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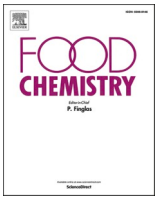

Development of a new eco-friendly ultrasound-assisted extraction method to quantify tryptophan in wild mushrooms and determination of its beneficial properties

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ABSTRACT

Bioactive compounds in food offer health benefits by influencing cellular and physiological functions. Tryptophan, an essential amino acid and precursor to neurotransmitters like melatonin and serotonin, regulates mood and circadian rhythms. However, its quantification in mushrooms is scarce studied. An eco-friendly ultrasound-assisted extraction (UAE) method has been optimized to quantify tryptophan using ultra high-performance liquid chromatography with a diode array and fluorescence detector (UHPLC-DAD-Fl). Tryptophan levels were determined in 26 wild mushroom samples of the genus *Lactarius* and *Boletus*. The concentrations ranged from 0.042 mg/g to 0.742 mg/g. The extracts' health benefits were assessed for antioxidant capacity using DPPH (17.7 % - 71.6 %) and ABTS (7.2 % - 24.9 %) methods, while acetylcholinesterase (AChE) inhibitory activity ranged from 13.1 % to 49.8 %. Promising results were obtained. Hierarchical cluster analysis demonstrated a correlation between tryptophan concentration, mushroom species, location, and extract properties, highlighting tryptophan's crucial role in these mushrooms' health benefits.

1. Introduction

Recently, there has been a growing interest in bioactive compounds, due to the incipient tendency of both the population and the pharmaceutical and food industry to consume and develop functional foods or food supplements. Thus, mushrooms are postulated as an interesting food due to the presence of bioactive compounds.

The consumption of mushrooms has increased in countries where they were not as integrated in the diet, as is the case of Spain (Ostos et al., 2015; Peintner et al., 2013), due to their numerous therapeutic properties, as the regulation of various biological processes or the reduction of the probability of suffering from diseases such as cancer or Alzheimer's disease (Kolniak-Ostek et al., 2022; Rai et al., 2021; Rizzo et al., 2021). Many studies have revealed that mushrooms can accumulate essential metals in high levels from the fruiting soil, which are indispensable for the proper functioning of the human body (Berthélémy, 2008; Kosanić et al., 2016; Rudawska & Leski, 2005). However, research related to the nutritional profile have shown that the

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AChE, Acetylcholinesterase; ANOVA, Analysis of variance; AOAC, Association of Official Agricultural Chemists; ATI, Acetylthiocholine iodide; BBD, Box–Behnken design; CV, Coefficient of variation; DNA, Desoxyribonucleic acid; DPPH, 2,2-difenil-1-picrilhidrazil; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); EtOH, Ethanol; FAMA, Federation of Andalusian Mycological Associations; HCA, Hierarchical cluster analysis; $K_2S_2O_8$, Potassium persulfate; LOD, Limit of detection; LOQ, Limit of quantification; MAE, Microwave-assisted extraction; PE, Polyethylene; ROS, Reactive oxygen species; SFE, Supercritical fluids extraction; UAE, Ultrasound-assisted extraction; UHPLC-DAD-Fl, Ultra high-performance liquid chromatography coupled to a diode array and fluorescence detector..

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numerous beneficial properties of mushrooms are also associated with the presence of different bioactive compounds. These are responsible for anti-inflammatory, anticarcinogenic or antibacterial properties, as well as for lowering blood pressure or increasing the activity of the immune system (Alkin et al., 2021; Dedousi et al., 2021).

Bioactive compounds are food components that, after ingestion, influence cellular and physiological activities, and provide a beneficial effect on health. Within this broad group, some amino acids stand out. These are obtained as final products of various biosynthetic routes, which constitute the secondary metabolism of mushrooms, not being essential for their growth, but playing important roles as defence agents or pathogen repellent (Çayan et al., 2020).

Amino acids are primary metabolites for humans because they are the building blocks, or monomers, that make up proteins. Amino acids also play an essential role in metabolism, neurotransmission, and the metabolism of proteins. Within this group, tryptophan is one of the most important amino acids because it is a precursor of numerous neurotransmitters, as is the case of melatonin and serotonin, which play an important role regulating the circadian rhythms or the states of mind and emotions, respectively, but it also can be metabolised into 5-hydroxyl-L-tryptophan, which is used as drug in order to treat different neurodegenerative diseases. Specifically, tryptophan is an exogen amino acid, so it is therefore vitally important to incorporate foods rich in this bioactive compound in the diet (Muszyńska et al., 2016; Podkowa et al., 2021).

In addition, amino acids and its derivatives in general, and tryptophan in particular, feature a high antioxidant capacity, eliminating reactive oxygen species (ROS) (Çayan et al., 2020; Islam et al., 2016; Kaewnarin et al., 2016; Nowacka et al., 2014). These ROS are molecules or free radicals that have oxygen in their composition, such as superoxide, hydroxyl or hydrogen peroxide radicals, produced in cell metabolism. However, if its concentration is high, they can cause irreparable damage to desoxyribonucleic acid (DNA), leading to cardiovascular or neurodegenerative diseases (Alvarez-Parrilla et al., 2007; Kaewnarin et al., 2016).

There are numerous methodologies based on the extraction of bioactive compounds, both conventional and advanced. Within the conventional methods, most are characterized for using large volumes of solvents, which is a major environmental problem, long operating times, working at high temperatures, which is not usually appropriate for extracting thermolabile compounds, and manual procedures, such as solid-liquid extractions, macerations or Soxhlet-type extractions (Alara et al., 2021).

On the other hand, due to the low yields and environmental problems presented by conventional methods, in recent years extraction methodologies based on more sophisticated techniques have been developed, such as extraction with supercritical fluids (SFE), microwave-assisted extraction (MAE) or UAE (Alara et al., 2021; Joradon et al., 2024; Nzekoue et al., 2022; Zahoor et al., 2023). These new technologies are known as green, clean or cold and, in general, present a series of advantages over conventional methods, such as some lower sample/solvent ratios, less extraction times or higher yields.

Among the sophisticated methods, ultrasound-assisted extraction is one of the techniques that is becoming more relevant with respect to the extraction of bioactive compounds in food samples, due to its high efficiency, low energy consumption and gentle heating of the sample (Wang et al., 2023). Ultrasound-assisted extraction is based on cavitation phenomena produced by ultrasonic waves, which facilitates the accessibility of the solvent to the interior of the cells, due to cell disruption, and the transfer of matter, increasing its extraction efficiency (Hazmi et al., 2023; Song et al., 2023; Wang et al., 2023).

Both conventional and advanced extraction methods require the use of different types of solvents to carry out the extraction, which will depend on the nature of the analyte to be determined. It is worth mentioning that, although solvents such as petroleum ether, methanol or acetone have been used for decades as extractants of bioactive compounds, they have posed a serious risk both to the environment and to the health of researchers due to their high toxicity (Alara et al., 2021; Jin et al., 2023). Therefore, and related to the rise of green chemistry in recent years the use of solvents that are safer for health, environmentally friendly, and of high quality, such as ethanol, has been chosen (Barbosa et al., 2019; Bodoira et al., 2017; Plaskova & Mlcek, 2023; Tungmunnithum et al., 2019).

However, to the best of the authors knowledge, this is the first study related to the optimization of tryptophan extraction in mushrooms. Thus, this research aims: first, to obtain the optimal conditions for tryptophan UAE using a Box-Behnken design. Then, to apply this method for the quantification of tryptophan in several wild mushroom samples, and finally to evaluate the beneficial properties of the obtained extracts to human health.

2. Materials and methods

2.1. Solvents and chemicals

The reagent used to carry out the extraction (absolute ethanol) was purchased from Scharlab S.L. (Sentmenat, Spain). Tryptophan (reagent grade, $\geq$ 98 %) was purchased from Merck (Darmstadt, Germany). To measure the antioxidant capacity, the reagents were purchased from Panreac Quimica S.A. (Castellar del Valles, Spain): Methanol (HPLC grade), from Sigma-Aldrich (St. Louis, MO, USA): 2,2-difenil-1-picrilhidrazil (DPPH, 99.9 %), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 99 %), potassium persulfate ($K_2S_2O_8$, 99 %), and from Thermo Fischer Scientific (Walthman, MA, USA): Trolox (97 %). To quantify the AChE inhibition, the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA): 5,5-dithiobis(2-nitrobenzoic acid) (DTNB ($C_{14}H_8N_2O_8S_2$), 99 %), acetylthiocholine iodide (ATI ($C_7H_{16}INO_2$), 98 %), sodium dihydrogen phosphate (NaH_2PO_4, 99 %), disodium hydrogen phosphate (Na_2HPO_4, 99 %), and electric-eel AChE ($C_{31}H_{27}NO_4$, 98 %). All water-based solutions were prepared using ultrapure water obtained by passing twice-distilled water through a Milli-Q system (18 MΩ/cm, Millipore, Bedford, MA, USA).

2.2. Mushroom samples

A total of twenty-six samples belonging to *Lactarius* genus and *Boletales* order were analysed due to their gastronomic relevance and the fact that they are the most collected and consumed mushrooms in Spain (Fig. 1). Mushroom samples were collected from different locations in the South of Spain, sited in the provinces of Cadiz, Malaga, Granada, and Huelva, and in the North of Morocco, belonging to Bouhachem natural park (Supplementary Table S1). Their identification was based on their morphological characteristics and the habitat where they were collected. The identification was carried out by expert mycologists which are members of the Federation of Andalusian Mycological Associations (FAMA). With the purpose to obtain a representative pool of samples for each area, at least 10 specimens of each sample were collected. After collection, samples were washed using deionized water and then were lyophilized up to constant weight, using a freeze-dryer (BenchTop K, VirTis, Gardiner, NY, USA). Finally, the lyophilized samples were powdered using an agate pestle and mortar to homogenize them and stored in polyethylene (PE) bottles perfectly labelled according to the species and sampling area.

2.3. Tryptophan extraction

The extraction of the bioactive compounds present in the mushroom samples, were performed using an ultrasound assisted extraction equipment (Sonopuls HD 3200, BANDELIN electronic GmbH & Co. KG, Heinrichstrabe, Berlin, Germany), equipped with a 7 mm ultrasound probe (VS 70 T, BANDELIN electronic GmbH & Co. KG, Heinrichstrabe, Berlin, Germany), employing an ethanolic solution as extraction solvent.

Fig. 1. Mushrooms analysed: a) Lactarius deliciosus; b) Lactarius rugatus; c) Lactarius sanguifluus; d) Lactarius semisanguifluus; e) Lactarius vinosus; f) Boletus aereus; g) Boletus edulis; h) Suillus bellinii. Pictures: José G. López Castillo.

This equipment is coupled to a thermostatic bath (Frigiterm-10, J.P. Selecta, Abrera, Spain), which has a double-walled vessel where the sample will be introduced in order to control the extraction temperature. The equipment allows simultaneous control of different sonication parameters, such as amplitude and cycle, as well as operation time.

2.4. Tryptophan identification and determination by UHPLC-DAD-FL

For the identification and quantification of tryptophan, ultra-high-performance liquid chromatography (Acquity UPLC H-Class, Waters Corporation, Milford, MA, USA) coupled to diode array detector and a fluorescence detector (UHPLC-DAD-FL) was employed. Tryptophan was identified by comparison of retention time, UV–Vis spectrum and fluorescence spectrum with a commercial tryptophan standard. This chromatograph was composed of a quaternary pump, an autosampler, a diode array detector, a fluorescence detector and a reverse phase C-18 column (Acquity UPLC BEH, Waters Corporation, Milford, MA, USA), with a particle size of 1.7 μm, a length of 100 mm and a diameter of 2.1 mm. The chromatographic method employed a solvent mixture as mobile phase, consisting in water (2 % acetic acid) as solvent A, and acetonitrile acidified with 2 % acetic acid, as solvent B at a flow of 0.5 mL/min. Analyses were carried out using the following gradient elution (time, % solvent B): 0 min, 0; 1 min, 0, 3 min, 5 %; 4 min, 10 %; 4.5 min, 10 %; 5 min, 20 %; 7 min, 20 %; 8 min, 30 %; 9 min, 100 %. The column temperature was set at 47 °C. The retention time of tryptophan was 2.8 min.

Firstly, a calibration curve (fluorescence detection) was made employing the standard of tryptophan at different concentrations, between 0.1 and 100 mg L^{-1}. A calibration curve ($y = 19{,}429\times + 11{,}985$) was obtained, with a goodness of fit, i.e. a linear regression coefficient (R^2) equal to 0.9995. The excitation and emission wavelengths employed to determine tryptophan were 250 nm and 395 nm, respectively.

The limit of detection (LOD) and limit of quantification (LOQ) of tryptophan were calculated by 3 and 10 times the deviation of the signal from the blank, divided by the slope of the calibration curve, respectively. LOD and LOQ were 0.172 mg L^{-1} and 0.523 mg L^{-1} of tryptophan, respectively.

2.5. Antioxidant capacity

According to the literature, the most commonly used methods to determine the antioxidant capacity of food samples are DPPH and ABTS (Alkan et al., 2020; González-Centeno, 2013; Re et al., 1999; Xu et al., 2019). Therefore, in this article, both methods were used to determine the antioxidant capacity of the different mushroom extracts obtained.

2.5.1. DPPH assay

For the DPPH method, 100 mL of a 0.06 mmol L^{-1} solution of DPPH in methanol was prepared. Subsequently, 15 μL of each extract was deposited in each position of a multiwell plate and 165 μL of the DPPH solution was added. Finally, the absorbance of each sample was measured at 515 nm using a microplates' spectrophotometer (SpectraMax 190, Molecular Devices, San José, CA, USA).

In order to quantify the antioxidant capacity of each of the samples, it was necessary to perform a trolox calibration curve, under the same operating conditions described above, in a range of concentrations from 0 to 100 mg L^{-1}. This calibration curve ($y = 0.7377\times + 15.371$) presented a goodness of fit of 0.9999, both calculated using Microsoft Office Excel 2021 (Microsoft Corporation). In addition, a blank was made with methanol and another with the extraction solvent employed. The antioxidant capacity of the samples was determined by calculating the percentage inhibition of DPPH using the expression shown in Eq. 1, where *A* represents the absorbance.

$$\%Inhibition = \frac{A_{blank} - A_{sample}}{A_{blank}} x100 \quad (1)$$

2.5.2. ABTS assay

For the ABTS method, an aqueous solution of 7 mmol L^{-1} ABTS and another of 2.45 mmol L^{-1} $K_2S_2O_8$ were prepared. Equal volumes of both solutions were mixed, and the mixture was left to react for 16 h under dark conditions. Subsequently, and immediately prior to analysis, 8 mL of the above mixture was taken and made up to a final volume of 100 mL, diluted with a 25 % aqueous solution of ethanol.

To perform the analysis, 190 μL of ABTS is placed in each well of the multi-plate and incubated at 25 °C for 10 min and absorbance measured at 734 nm. Then, 10 μL of each sample is added to each corresponding well, the reaction takes place, and an absorbance measurement is made after 30 min, using a microplates' spectrophotometer (SpectraMax 190, Molecular Devices, San José, CA, USA).

To quantify the antioxidant capacity of each of the samples, it was necessary to perform a trolox calibration curve, under the same operating conditions described above, in a range of concentrations from 0 to 100 mg L^{-1}. This calibration curve ($y = 0.3197\times - 0.9622$) had a goodness of fit of 0.9918, calculated using Microsoft Office Excel 2021. The antioxidant capacity of the samples was determined by calculating the percentage inhibition of ABTS using the expression shown in Eq. 1, where *A* represents the absorbance.

2.6. AChE assay

To determine the AChE inhibitory activity of the mushroom samples analysed, a bioassay based on the Ellman method was used (Ellman et al., 1961). For this purpose, 500 mL of a 200 mmol L^{-1} monobasic sodium phosphate solution (A) and the same volume of a 200 mmol L^{-1} dibasic sodium phosphate solution (B) were prepared. In order to obtain a phosphate buffer, 13.25 mL of A and 236.75 mL of B were mixed and brought to a final volume of 500 mL by making up to the mark with Milli-Q water and adjusting the pH of the solution to a value of 8, using either solution A or B.

Once the phosphate buffer was obtained, 50 mL of a 3 mmol L^{-1} solution of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and 25 mL of a 15 mmol L^{-1} solution of acetylthiocholine iodide (ATI) were prepared. To determine the inhibitory activity of each sample, 125 μL of DTNB, 25 μL of ATI, 50 μL of buffer and 25 μL of extract of each sample were added to each well of a microplate. In addition, a blank was made by adding the same amounts of each reagent, but 25 μL of buffer instead of sample. Then, 25 μL of AChE was added to each well, and the absorbance was measured at 405 nm and repeated 5 min later, employing a microplates' spectrophotometers (BioTek Synergy HTX, Agilent Technologies, Santa Clara, CA, USA). The percentage inhibition of the enzyme AChE was calculated using the expression shown in Eq. 2, where A represents absorbance.

$$\%Inhibition = \frac{\Delta A_{buffer\ blank} - \Delta A_{sample}}{\Delta A_{buffer\ blank}} x100 \quad (2)$$

2.7. Box–Behnken design (BBD)

To determine the variables with the greatest influence on the extraction of bioactive compounds, as well as the optimal extraction conditions, a response surface Box-Behnken design was carried out. The Box-Behnken design employs statistical tools, based on a central point, for the development of a quadratic fitting model.

This design predicts the value of the dependent variables studied, without the need to apply a full three-level factorial design. Therefore, in this way, the number of experiments is reduced, avoiding the realization of these under extreme conditions, due to the fact that, by not presenting axial points, a more spherical arrangement of the design points is obtained (Giri & Mishra, 2023; Meshram et al., 2023).

The independent variables can be set to three possible values, coded as −1, 0 and + 1, depending on whether they are the lower, middle or upper value, respectively. In this case, the independent variables studied were: (1) percentage of ethanol in the extraction solvent, (2) extraction temperature, (3) amplitude, (4) ultrasound cycle, and (5) sample/solvent ratio. In addition, a dependent variable was studied: (6) tryptophan concentration. The different minimum, mean and maximum values of each of the independent variables are shown in Table 1.

Thus, after combining the five independent variables, a Box-Behnken design composed of 46 experiments, detailed in Supplementary Table S2, was obtained. The Box-Behnken design was carried out employing the sample #26: *S. bellinii* (Dehesa de las Yeguas, Cadiz).

After performing the 46 experiments, the response surface method was used, from which a mathematical model of a second-degree equation is obtained, as shown in Eq. 3.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (3)$$

This equation shows the response provided by the system as function of the independent variables and the interactions existing between them, being *y* the response variable (concentration of tryptophan in the samples), *βi* the coefficient for each main effect, *βii* the coefficient of the quadratic factors representing the curvature of the surface, *βij* the coefficient of the interactions between *i* and *j*, *X* the factors studied and *ε* the residual value or random error (Haque et al., 2023).

2.8. Statistical analysis

To carry out the statistical study and process the results obtained after the performance of the Box-Behnken design, Statgraphics Centurion software (Version XVIII, StatPoint Technologies Inc., Warrenton, VA, USA) was employed. On the other hand, to perform the rest of the graphs presented in this work Microsoft Office Excel 2021 (Microsoft Corporation, Redmond, WA, USA), was used.

Table 1
Lower, middle and upper values of each independent variable of the BBD.

Independent variable	−1	0	+1
Ethanol (%)	0	30	60
Temperature (°C)	10	35	60
Amplitude (%)	30	50	70
Ultrasound cycle (s^{-1})	0.2	0.6	1.0
Ratio (g sample in 20 mL solvent)	0.1	0.3	0.5

3. Results and discussion

3.1. Study of the extraction solvent range

In order to establish the optimum range for the percentage of ethanol in the extraction solvent, 6 trials were carried out in duplicate, varying the percentage of ethanol (0, 20, 40, 60, 60, 80 and 100 %), while the other variables were set at the following values: temperature (30 °C), extraction time (5 min), amplitude (50 %), cycle (0.5 s^{-1}), sample amount (0.5 g), and solvent volume (20 mL). After performing the corresponding assays, the extracts were centrifuged for 10 min at 4.000 r min^{-1} (1702 g) and the supernatant was brought to a volume of 25 mL with the solvent used.

Therefore, in view of the results obtained, it can be seen that the most effective range for the percentage of ethanol were from 0 % to 60 %, decreasing considerably from 80 % onwards, as it can be observed in Supplementary Fig. S1. A statistical analysis was carried out to study whether there were significant differences between the concentration obtained as a function of the percentage of ethanol. Thus, the optimal range of the percentage of ethanol in the extraction solvent selected to carry out the Box-Behnken design would be between 0 and 60 %.

3.2. Study of the extraction temperature

To establish the optimum extraction temperature range, 7 trials were carried out in duplicate, varying the temperature (10, 20, 30, 40, 50, 50, 60, and 70 °C), while the other variables were set at the following values: percentage of ethanol (30 %), extraction time (5 min), amplitude (50 %), cycle (0.5 s^{-1}), sample amount (0.5 g) and solvent volume (20 mL).

As in the previous study, the extracts were centrifuged for 10 min at 4000 r min^{-1} (1702 g) and the supernatant was brought to a volume of 25 mL with the solvent used.

A statistical analysis was carried out to study whether there were significant differences between the concentration obtained depending on the extraction temperature. Thus, in view of the results obtained (Supplementary Fig. S2), it is observed that, the most effective range of temperature for the extraction of tryptophan were from 10 °C to 60 °C. When working at 70 °C, a decrease in extraction efficiency is observed.

Therefore, the optimal range of extraction temperature selected for the Box-Behnken design would be between 10 and 60 °C.

3.3. Box-Behnken design and extraction optimization

Once the corresponding studies had been carried out to evaluate the optimum range of solvent concentration and extraction temperature, the lower, middle and upper levels were established for each of the five studied variables, being: percentage of ethanol (0 %, 30 % and 60 %), temperature (10 °C, 35 °C and 60 °C), amplitude (30 %, 50 % and 70 %), cycle (0.2 s^{-1}, 0.6 s^{-1} and 1.0 s^{-1}) and sample/solvent ratio (0.1 g/20 mL, 0.3 g/20 mL and 0.5 g/20 mL).

Subsequently, the different levels of the variables were combined and the 46 experiments of the Box-Behnken design were obtained. After carrying out these experiments, the concentration of tryptophan present in each of the extracts were determined and the effect of each of the variables, both individually and the possible interactions between them, on response variable was studied. For this purpose, an analysis of variance (ANOVA) was performed, and those parameters with a *p*-value of less than 0.05, for a confidence level of 95 %, are considered to have a significant influence on the response variable. Thus, in Table 2, it can be seen which parameters have a significant influence for tryptophan, as well as the estimated coefficient of the second-order polynomial equation for each of them.

Table 2
ANOVA results for the response variable, estimated coefficients of the second order polynomial equation and *p*-values for a confidence level of 95 %.

Parameter	Estimated coefficient tryptophan	*p*-value
%EtOH	0.0549313	0.0204
Temperature	−0.0138937	0.5367
Amplitude	−0.00619375	0.7824
Cycle	0.00376875	0.8664
Ratio	−0.100925	0.0001
%EtOH-%EtOH	0.0644188	0.0419
%EtOH-Temperature	0.009125	0.8387
%EtOH-Amplitude	0.009525	0.8317
%EtOH-Cycle	0.0093	0.8356
%EtOH-Ratio	−0.110675	0.0196
Temperature-Temperature	−0.0152479	0.6161
Temperature-Amplitude	−0.023825	0.5960
Temperature-Cycle	−0.023925	0.5944
Temperature-Ratio	0.00075	0.9866
Amplitude-Amplitude	−0.00213125	0.9440
Amplitude-Cycle	−0.007275	0.8711
Amplitude-Ratio	0.0328	0.4666
Cycle-Cycle	−0.0100479	0.7407
Cycle-Ratio	−0.002525	0.9551
Ratio-Ratio	0.0618937	0.0499

Therefore, the second-order equation showing the response provided by the system as a function of the independent variables and the interactions existing between them, can be reduced to those terms in which the significantly influencing parameters appear. Thus, the reduced mathematical expression shown in Eq. 4 is obtained.

$$Tryptophan\ Concentration\ (mg/g) = 0.0549313 \cdot \%EtOH - 0.100925 \cdot Ratio + 0.0644188 \cdot \%EtOH^2 - 0.110675 \cdot \%EtOH \cdot Ratio + 0.0618937 \cdot Ratio^2 \quad (4)$$

It shows that, for tryptophan extraction the most influential parameters are the percentage of ethanol and its quadratic interaction, the ratio and its quadratic interaction, and the interaction between the percentage of ethanol and the ratio.

These results can be expressed graphically using the Pareto's chart (Fig. 2). In this way, the importance of each of the independent variables and the interactions between them on the response variable can be observed. Therefore, those parameters that present a standardized error, with a value greater than 2.17, will have a significant effect on the response variable.

3.4. Optimized conditions

Once the statistical analysis was carried out, the optimal extractions conditions for tryptophan were obtained, which were as follows: extraction solvent with 60 % EtOH, extraction temperature of 25 °C, ultrasound amplitude of 30 %, cycle of 1.0 s^{-1} and 0.1 g/20 mL as the optimum solvent-solid ratio.

3.5. Extraction time optimization

Once the optimum extraction conditions were established, a study to determine the optimum extraction time was carried out. In this way, six experiments were carried out, in triplicate, where, keeping the optimum values of the independent variables constant, the extraction time was varied. Thus, the six trials were carried out applying the optimum conditions for 2, 5, 10, 15, 20 and 30 min and, subsequently, the

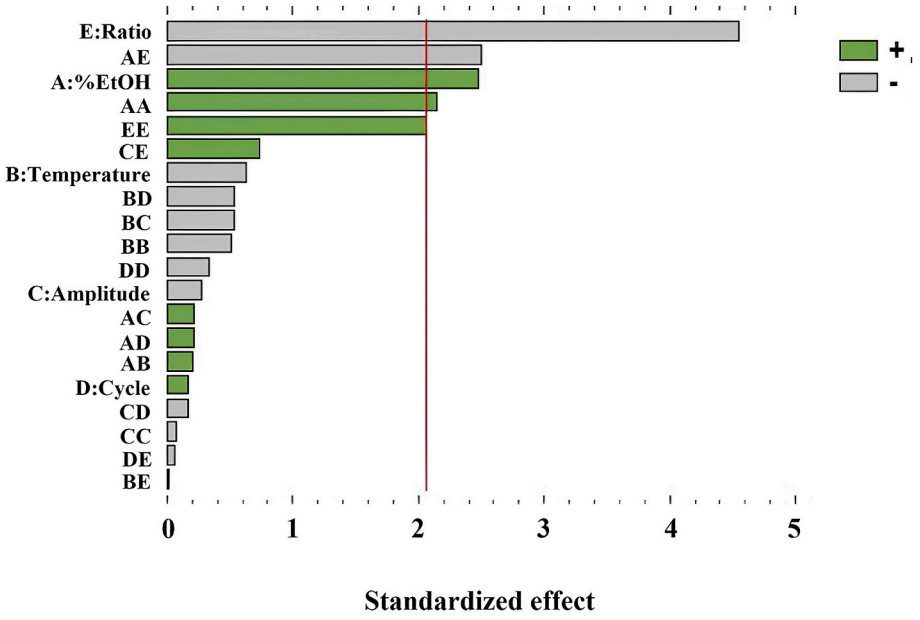

Fig. 2. Pareto charts for the standardized effects for tryptophan.

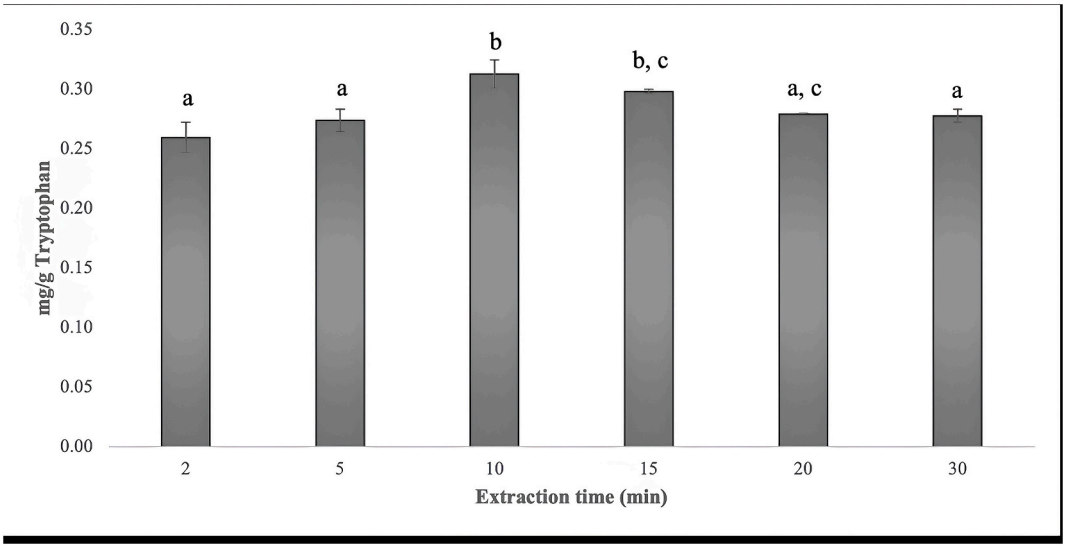

Fig. 3. Tryptophan concentration plotted versus extraction time ($n = 2$).

concentration of tryptophan in each of the extracts was determined. The concentrations of tryptophan versus time are plotted in Fig. 3. In this figure, it can be observed that the highest concentration of tryptophan is obtained after prolonging the extraction for 10 min. Therefore, as significant differences were observed between the extraction times, it was concluded that the optimum extraction time was 10 min.

3.6. Analytical characteristics of the UAE method

To conclude the optimization of the extraction method, the last step consisted of a study of the repeatability and intermediate precision. In order to evaluate the repeatability, nine extractions under the optimal conditions along the same day were carried out. Whereas the intermediate precision was evaluated by carrying out nine extractions, under the optimal conditions, during three consecutive days, performing a total of 27 extractions. Repeatability and intermediate precision results were found to be 3.02 % and 4.81 %, respectively. As it can be observed, the values of the coefficients of variation (CV) obtained were below a 5 %. Thus, it can be considered that the method is precise since it presents a high repeatability and intermediate precision, being both repeatability and intermediate precision within the acceptable limits (±10 %) defined by the Association of Official Agricultural Chemists (AOAC) (AOAC AOAC, 1998).

3.7. Tryptophan's stability under optimal extraction conditions

After establishing the optimal extraction conditions, the stability of tryptophan by conducting a degradation kinetic study was evaluated. A 100 mg L^{-1} solution of commercial tryptophan was subjected to the optimal extraction conditions with variations in treatment time (0, 5, 10, 20, and 30 min), and tryptophan concentration was measured after each time point to assess stability, as illustrated in Supplementary Fig. S3. A one-way ANOVA statistical analysis showed no significant differences in tryptophan concentrations throughout these tests (p-value >0.05). Therefore, based on the results, it can be concluded that using UAE does not significantly degrade tryptophan under the optimal conditions,

making it a viable method for extracting tryptophan from wild mushrooms while maintaining its stability.

3.8. Extraction procedure of mushroom samples

After optimal extraction conditions were obtained, the tryptophan extraction procedure was as follows: 0.1 g of lyophilized mushroom samples was weight into a Falcon tube. Then, 20 mL of 60 % ethanol were added. The tryptophan was extracted by applying a temperature of 25 °C, an amplitude of 30 % and a cycle of ultrasounds of 1.0 s^{-1}, during 10 min. After that, samples were centrifuged at 4000 r min^{-1} (1702 g) during 10 min and supernatant were transferred into a 25 mL flask and filled with the 60 % ethanol solution.

3.9. Application to real samples

To quantify the concentration of the bioactive compounds, it was necessary to construct a calibration curve for tryptophan ($y = 19{,}429\times + 11{,}985$), obtaining a high correlation coefficient ($R^2 = 0.9995$). After the application of the method to real samples ($n = 3$), the concentration of tryptophan found was ranged from 0.042 mg/g to 0.742 mg/g (dry weight), being the mean value 0.411 mg/g (dry weight). In Fig. 4, it is showed a bar chart where the concentration of tryptophan of each sample is represented.

It can be observed that samples #4: *L. deliciosus* (Bouhachem, Chauen), #11: *L. deliciosus* (Pinar San Walabonso, Huelva) and #18: *L. semisanguifluus* (Puerto de la Mora, Granada) were the samples with highest concentration, while samples #13: *L. rugatus* (Cortes de la Frontera, Malaga) and #16: *L. sanguifluus* (Cortes de la Frontera, Malaga) had the lowest concentration. In Table 3, the concentration of tryptophan of each sample is shown.

A comparison of the tryptophan results obtained in this research with the scarce articles found in the literature showed higher values in the mushrooms studied in this work. It can be observed that, the mean value determined in mushrooms belonging to Andalusia and Morocco (0.411 mg/g) is a little high to the concentration of tryptophan reported by Kivrak et al. (Kivrak et al., 2014) in *Calvatia gigantea* collected in Turkey (0.301 mg/g).

However, comparing with the results of Muszynska et al. (Muszyńska et al., 2007, 2011, 2013, 2015) who determined tryptophan in both, commercial, as *Agaricus bisporus*, and wild mushroom collected in the south of Polish, specifically, *Cantharellus cibarius, Boletus badius* and *Lactarius deterrimus*, the concentration of tryptophan was ranged between 0.0001 and 0.0083 mg/g, being much lower that the values determined in this research.

3.10. Determination of the antioxidant capacity: DPPH and ABTS method

Based on the results obtained by the DPPH method, the inhibition percentage is ranged between 17.7 and 71.6 %, with a mean value of 31.5 %. Sample #25: *B. edulis* (Bouhachem, Chauen) was the mushroom with the higher percentage. However, sample #2: *L. deliciosus* (Puerto Real, Cadiz) showed the lower inhibition percentage. Expressing the results as mg of Trolox equivalents per gram of sample, the antioxidant capacity was ranged between 1.44 and 19.02 mg/g, being the mean value 2.56 mg/g.

On the other hand, the results obtained by the ABTS method show that sample #25: *B. edulis* (Bouhachem, Chauen) was the sample with the higher inhibition percentage, reaching 24.9 % and sample #2: *L. deliciosus* (Puerto Real, Cadiz) and #17: *L. sanguifluus* (Sierra Alfaguara, Granada) had the lowest percentages (7.2 % in both cases). The mean value of the inhibition percentages determined in the samples was 11.3 %. Expressing the results as mg of Trolox equivalents per gram of sample, the antioxidant capacity was ranged from 6.34 mg/g to 20.14 mg/g, with a mean value of 9.95 mg/g.

After carrying out a literature search on antioxidant activity in mushrooms, it was observed that authors such as Bach et al. (Bach et al., 2019) performed spectrophotometric tests such as DPPH and ABTS on different species of commercial mushrooms from Brazil of high gastronomic value. This study showed that the samples analysed had an antioxidant capacity, measured in mg of trolox per gram of sample, between 3.28 mg/g and 12.67 mg/g, according to the DPPH assay. Whereas, after applying the ABTS method, the antioxidant capacity determined ranged from 17.79 mg/g to 66.18 mg/g. Therefore, it can be seen that the mushroom samples analysed by Bach et al. have an antioxidant capacity, determined by the DPPH method, similar to that quantified by Bach et al. in samples of mushrooms from southern Andalusia and northern Morocco. However, the ABTS test carried out on the Brazilian commercial samples gave higher values.

On the other hand, Alkan et al. (Alkan et al., 2020) determined the antioxidant capacity of different mushroom samples, including species of the genus *Lactarius*, such as L. *deliciosus*. Thus, the values obtained in the studies of Alkan et al. show a range of mg trolox per gram of sample, ranging from 5.66 to 41.89 mg/g for the DPPH test and 17.53–84.33 mg/g for the ABTS test, with the sample of L. *deliciosus* having the lowest values for both methods. In view of these results, it can be seen that, in general, the samples analysed by Alkan et al. have a higher antioxidant capacity. However, the values obtained for the species L. *deliciosus* species coincide with those obtained in the samples from southern Andalusia and northern Morocco.

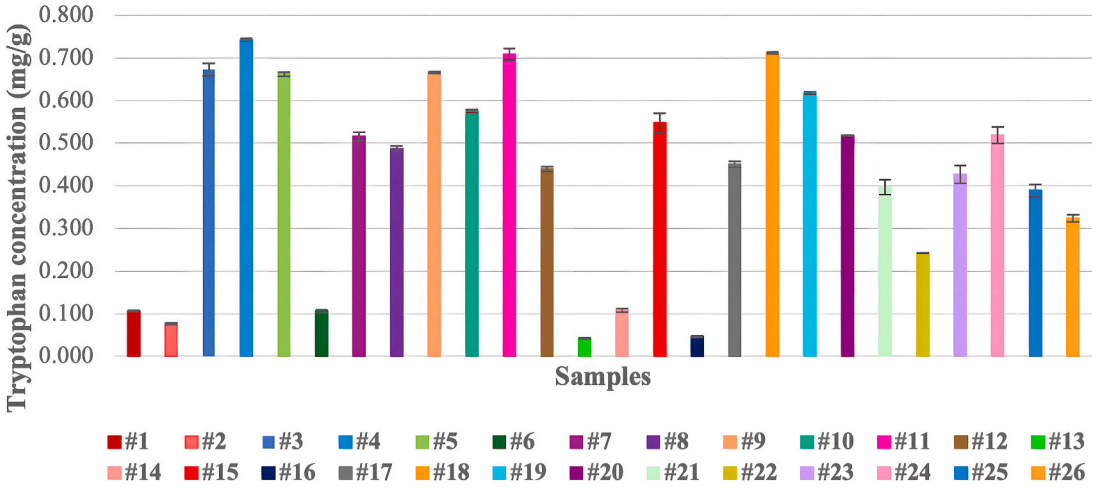

Fig. 4. Bar chart of the concentration of tryptophan mg/g sample ($n = 2$).

Table 3
Mean values of tryptophan concentration (mg/g), percentage inhibition obtained in DPPH and ABTS assays (%) and percentage inhibition of acetylcholinesterase (%), with coefficient of variation values in brackets (%).

Sample ID	Tryptophan	DPPH	ABTS	AChE
#1	0.107 ± 0.001 (0.9)	28.6 ± 11.6 (40.7)	9.4 ± 0.6 (6.4)	16.4 ± 0.3 (1.9)
#2	0.077 ± 0.002 (2.5)	17.7 ± 0.4 (2.5)	7.2 ± 0.8 (10.6)	13.1 ± 0.6 (4.5)
#3	0.673 ± 0.014 (2.1)	22.6 ± 3.3 (14.7)	10.8 ± 0.5 (4.8)	21.7 ± 0.5 (2.2)
#4	0.742 ± 0.003 (0.4)	23.2 ± 0.2 (0.9)	9.2 ± 0.8 (8.2)	17.6 ± 0.7 (4.2)
#5	0.662 ± 0.004 (0.7)	27.8 ± 1.0 (3.6)	11.0 ± 0.9 (8.3)	21.7 ± 0.4 (2.0)
#6	0.106 ± 0.003 (3.2)	23.0 ± 1.1 (4.6)	8.4 ± 0.3 (3.6)	19.3 ± 0.6 (3.0)
#7	0.516 ± 0.010 (2.0)	25.5 ± 2.7 (10.5)	8.0 ± 0.8 (10.0)	18.6 ± 0.4 (2.4)
#8	0.487 ± 0.006 (1.2)	25.2 ± 0.2 (0.6)	9.5 ± 0.6 (6.1)	20.0 ± 0.9 (4.4)
#9	0.665 ± 0.002 (0.4)	24.0 ± 1.2 (4.9)	12.4 ± 0.2 (1.3)	25.5 ± 0.1 (0.6)
#10	0.575 ± 0.003 (0.6)	23.9 ± 0.8 (3.2)	9.3 ± 0.1 (1.2)	22.7 ± 0.6 (2.6)
#11	0.709 ± 0.013 (1.8)	25.1 ± 0.2 (0.7)	12.6 ± 0.0 (0.1)	15.6 ± 2.6 (17.0)
#12	0.439 ± 0.005 (1.2)	24.4 ± 1.5 (6.1)	10.0 ± 0.7 (7.4)	49.8 ± 0.1 (0.3)
#13	0.042 ± 0.001 (2.7)	38.8 ± 1.3 (3.5)	12.4 ± 0.3 (2.6)	15.9 ± 0.1 (0.9)
#14	0.108 ± 0.004 (3.9)	44.3 ± 0.6 (1.4)	13.2 ± 0.6 (4.8)	17.7 ± 0.3 (1.7)
#15	0.547 ± 0.023 (4.2)	25.4 ± 1.0 (3.8)	8.9 ± 0.5 (5.5)	17.5 ± 0.3 (1.7)
#16	0.046 ± 0.002 (4.2)	24.1 ± 0.1 (0.4)	8.0 ± 0.2 (3.1)	21.4 ± 0.6 (2.7)
#17	0.450 ± 0.007 (1.5)	21.9 ± 2.2 (8.8)	7.2 ± 0.9 (12.2)	17.4 ± 0.7 (4.2)
#18	0.711 ± 0.002 (0.3)	25.4 ± 2.2 (8.8)	8.7 ± 0.9 (10.2)	15.7 ± 0.1 (0.9)
#19	0.618 ± 0.002 (0.4)	29.6 ± 2.8 (9.5)	9.4 ± 0.6 (6.5)	21.3 ± 0.4 (2.1)
#20	0.517 ± 0.001 (0.3)	26.6 ± 0.4 (1.4)	10.5 ± 0.3 (3.0)	22.9 ± 0.9 (3.9)
#21	0.397 ± 0.018 (4.4)	33.0 ± 0.5 (1.5)	11.9 ± 2.1 (17.7)	24.6 ± 0.7 (3.0)
#22	0.243 ± 0.001 (0.2)	29.7 ± 0.0 (0.1)	11.0 ± 2.2 (20.0)	15.3 ± 0.7 (4.8)
#23	0.427 ± 0.021 (4.9)	33.7 ± 0.2 (0.7)	14.3 ± 0.1 (1.0)	19.4 ± 0.4 (2.3)
#24	0.519 ± 0.019 (3.7)	65.8 ± 0.9 (1.4)	18.5 ± 0.0 (0.1)	23.9 ± 1.2 (4.9)
#25	0.389 ± 0.015 (3.8)	71.6 ± 4.0 (5.6)	24.9 ± 3.2 (12.8)	21.5 ± 1.0 (4.8)
#26	0.324 ± 0.009 (3.0)	57.4 ± 4.1 (7.2)	16.6 ± 0.3 (1.7)	21.5 ± 1.0 (4.8)

Xu et al. (Xu et al., 2019) focused on studying the composition and different biological activities of the species *Lactarius deliciosus*. Thus, it was observed that its antioxidant capacity presented values, referred to as mg of trolox per gram of sample, of 4.60 mg/g, according to the DPPH method, while for the ABTS method, the values obtained were 10.33 mg/g. In both cases, it can be seen that this antioxidant capacity is in agreement with the ranges obtained in the samples analysed in this work. Therefore, the mushroom samples analysed by Bach et al. have an antioxidant capacity, determined by the DPPH method, similar to that quantified in the samples of mushrooms from the south of Andalusia and the north of Morocco. However, the ABTS test carried out on the Brazilian commercial samples provided higher values than those determined in this research.

In Table 3, it is shown the percentage of antioxidant capacity determined by DPPH and ABTS method of each sample.

3.11. Determination of the inhibition of AChE enzyme

Tryptophan has numerous important roles into the organism, highlighting its activity against neurodegenerative diseases, as Parkinson's or Alzheimer. The latter is affected by the loss of cholinergic neurons, i.e. those neurons that are activated or secrete the neurotransmitter acetylcholine (Meden et al., 2019; Pohanka, 2014; Tallini et al., 2018). Thus, the loss of these neurons decreases the levels of this neurotransmitter, affecting areas of the brain related to memory or memory functions. To reduce the loss of function of cholinergic neurons, drugs are used that inhibit cholinesterase enzymes, specifically against AChE, which is mainly responsible for the loss of function of these neurons. Because tryptophan and various phenolic compounds contribute to the inhibition of AChE, they are postulated as candidates for such drugs (Meden et al., 2019).

For that reason, in this research, the inhibition of the mushroom extracts obtained against AChE was studied. Thus, the percentage of inhibition of that enzyme was ranged from 13.1 % to 49.8 %, with a mean value of 20.7 %, being sample #2: *L. deliciosus* (Puerto Real, Cadiz) which had the lowest activity and sample #12: *L. deliciosus* (Pinar Raboconejo, Huelva) the mushroom with the higher inhibition activity against AChE.

Several authors have carried out studies in which the inhibitory activity of different mushroom species against AChE has been determined. Thus, Prodanovic et al. (Prodanović et al., 2023) measured the activity of extracts of species belonging to the genus *Boletus* and *Agaricus*, which ranged from 11.49 to 17.46 %. Therefore, it is clear that the mushrooms of the genus *Lactarius* collected in southern Andalusia and northern Morocco, show percentages of inhibition against the enzyme AChE higher than those found in the species of the genus *Boletus* and *Agaricus* species.

On the other hand, Petrovic et al. (Petrović et al., 2023) determined the activity of both methanolic and acetonic extracts of the species *Laetiporus sulphureus* from Serbia. The bioactivity of this species, in both solvents, was in the range of 3.64–8.84 %, i.e. lower values than those determined in the mushroom samples analysed in this research.

Other authors, such as Orhan et al. (Orhan & Üstün, 2011) carried out ethanolic extractions on different mushroom species collected in various locations in Turkey, in order to determine the inhibition capacity against AChE. The results obtained show that the species of the genus *Polyporus*, the species *Cantharellus cibarius*, *Trametes versicolor* and *Lactarius deliciosus* show inhibition percentages between 6.81 and 37.61 %. Thus, it can be observed that these mushroom species show an activity against AChE that agrees with the results obtained by the species of the genus *Lactarius* analysed in this article. Furthermore, it is worth mentioning that the *Lactarius deliciosus* species analysed by Orhan et al. showed an inhibition percentage of 24.04 %, which is close to the average value obtained in samples from southern Andalusia and northern Morocco, which was 20.7 %.

Other authors, such as Çol Ayvaz et al. (Çol Ayvaz et al., 2019) and Cayan et al. (Çayan et al., 2021), also determined the activity against AChE of different species collected in different provinces of Turkey, after extraction with methanol. The percentage inhibition values obtained for the different species range from 3.91 to 46.74 %, being in the range determined for mushrooms from southern Andalusia and northern Morocco. However, among these species, the *Lactarius deliciosus* species was found to have an inhibition percentage of 11.74 %, a lower value than those obtained for the mushrooms of the genus *Lactarius* analysed in this research. In Table 3, it can be observed the percentage of inhibition of AChE of each sample.

3.12. Hierarchical cluster analysis (HCA)

Finally, to stablish possible clustering trend of mushroom samples regarding to the location, species, parameters analysed, and the concentration of tryptophan found in the extracts a hierarchical cluster

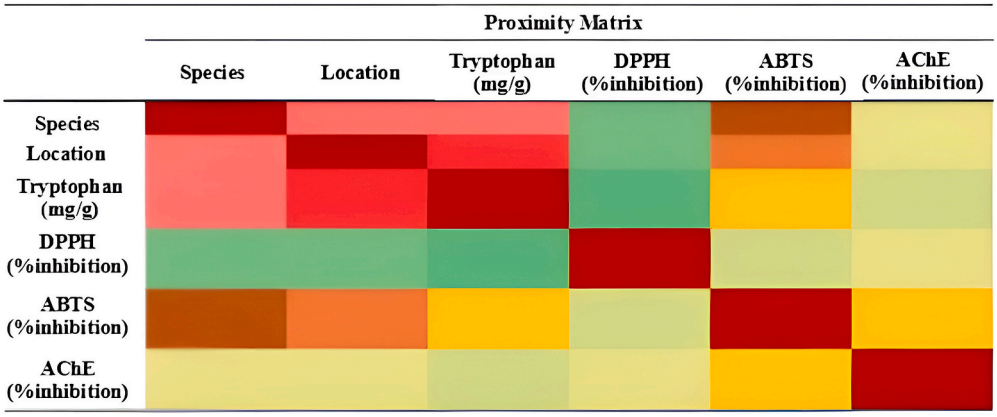

Fig. 5. Heatmap using Euclidean distance and Ward's method to identify clustering trend patterns among the concentration of tryptophan and the other bioactive compounds found in the extracts and the parameters analysed (antioxidant capacity and inhibition of acetylcholinesterase).

analysis (HCA) with Euclidean distance squared and Ward's method was carried out. That technique establishes recognition patterns in the dataset allowing the observance of groupings by species, geographic location, or both. The results obtained using HCA are represented in the heatmap shown in Fig. 5. In that heatmap, red colours are associated with a lower distance between the parameters, i.e. higher correlation, whereas green colours are related to a higher distance, i.e. lower correlation. According to the results, it can be seen that tryptophan's concentration is highly correlated with both extrinsic factors, such as location, and intrinsic factors, such as species. In addition, it can be observed that tryptophan's concentration not only correlates with the location and species but also, to a lesser extent, with the other properties determined in the extracts obtained (ABTS, AChE inhibition and DPPH).

These results are in concordance with the literature (Ji et al., 2019). Thus, it can be confirmed that the tryptophan, one of the major compounds in analysed samples, take an important role in the principal properties of the extracts obtained.

4. Conclusions

Based on the results, it can be enumerated a series of conclusions. Firstly, an eco-friendly ultrasound assisted extraction method for the quantification of tryptophan in wild mushrooms has been successfully developed, highlighting its high repeatability and intermediate precision. Thus, after the application of the developed method in mushrooms belonging to *Lactarius* genus and *Boletus* order, the concentration of tryptophan has been determined in a range from 0.042 mg/g to 0.742 mg/g, with a mean value of 0.411 mg/g. These results are in agreement with, and even exceed, those found in the bibliography, which highlights the importance of mushrooms collected in southern Andalusia and northern Morocco as a source of tryptophan. On the other hand, the antioxidant capacity of the extracts obtained was measured by the DPPH and ABTS method, showing concordance with the percentages reported by other authors. In addition, the inhibition of the AChE enzyme was ranged from 13.1 % to 49.8 %, being higher results than the found in other mushrooms, according to literature. A HCA was carried out, showing a strong correlation of tryptophan's concentration both with location and species and with the properties determined in mushrooms' extracts (antioxidant capacity and AChE inhibition). Finally, the analysis of the AChE inhibitory activity of the tested extracts has shown promising results. These could offer significant benefits as a dietary supplement for people with neurodegenerative diseases, such as Alzheimer's, or those at risk of developing them. However, although the results are encouraging, it is essential to carry out additional studies in vitro using cell culture models and in vivo using animal models to confirm the efficacy and safety of these extracts under complex physiological conditions.

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CRediT authorship contribution statement

Alejandro R. López: Writing – original draft, Investigation, Formal analysis. **Elena Ortega-Caneda:** Investigation, Formal analysis. **Estrella Espada-Bellido:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Nuria Chinchilla:** Methodology, Investigation, Formal analysis. **Miguel Palma:** Supervision, Resources. **María José Aliaño-González:** Methodology, Formal analysis. **Gerardo Fernández Barbero:** Writing – review & editing, Supervision, Resources, Investigation. **Ceferino Carrera:** Writing – review & editing, Supervision, Software, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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