# PULSED ELECTRIC FIELD EXTRACTS OBTAINED FROM EDIBLE MUSHROOMS: A DETAILED ICP-MS ANALYSIS OF THEIR MINERAL AND HEAVY METAL CONTENTS AND THEIR CYTOTOXIC EFFECT ON CACO-2 CELLS

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**Pulsed electric fields extracts obtained from edible mushrooms: A detailed ICP-MS analysis of their mineral and heavy metal contents and their cytotoxic effect on CACO-2 Cells**

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**Abstract**

Mushrooms contain beneficial micro and macronutrients, as well as biologically active compounds, but they can pose a food safety risk by absorbing heavy metals from the environment. Therefore, pulsed electric fields (PEF) technology is of interest as it enables selective extraction of high-value compounds, offering an advantage over conventional methods. In this study, PEF extractions were performed under optimal conditions (2.5 kV/cm, 50 kJ/kg) from *A. bisporus, L. edodes, A. brunnescens, P. ostreatus* and *L. deliciosus*, and compared to aqueous maceration at 20°C. The total carbohydrate content was evaluated by spectrophotometric methods, and the mineral (Ca, Mg, Fe, Zn, P, K, Cu, Se) and heavy metal (As, Cd, Hg, Pb) content was determined by inductively coupled plasma mass spectrometry (ICP-MS). In addition, the content of the mycotoxins AFB1, AFB2, AFG1, AFG2, OTA, ENNA, EN-NA1, ENNB, ENNB1 was previously determined by UHPLC-MS/MS. Results showed that PEF extracts achieved higher carbohydrate yields, with increases of up to 1327.02% compared to conventional methods, with *L. edodes* and *P. ostreatus* standing out for their content. Different mineral and heavy metals profiles were observed, with *L. edodes* showing higher levels of As and Cd and *A. bisporus* of Pb. No mycotoxins were found in the original matrices. Furthermore, PEF extracts (0.78-25% *v/v*) demonstrated enhanced cytotoxic effects on human colorectal adenocarcinoma cells (CACO-2), suggesting that bioactive compounds, rather than heavy metals, were responsible for reducing cell viability. These findings highlight the PEF technology potential for extracting beneficial compounds from mushrooms, with minimal contamination from heavy metals.

**Keywords:** cytotoxicity, mushroom, bioactivity, heavy metals, food contaminant, cell viability

## 1. Introduction

The fruiting bodies of mushrooms have been valued for decades not only for their culinary value but also for their medicinal properties. Among them, *Agaricus bisporus*, *Agaricus brunnescens*, *Lentinula edodes* and *Pleurotus ostreatus* are the most consumed and studied mushrooms, as they are an excellent source of bioactive compounds, minerals, vitamins, beta-glucans, and proteins of high biological value (Bringye et al. 2021). In this context, China is the largest producer of mushrooms worldwide with 40,000,000 tons in 2020, followed by Japan, the USA and the Netherlands, the latter with 260,000 tons (FAO, 2022) . In addition, Europe is characterized by a great diversity of mushrooms on the market, with 60 cultivable mushrooms out of 268 (Bringye et al. 2021; De Cianni et al. 2023). According to the Food and Agriculture Organization of the United Nations (FAO, 2020), the promotion of indoor cultivation of *Pleurotus spp.* allows the diversification of livelihoods and strengthens the resilience of farmers in countries such as Uganda, as they can be grown in a short time and at low cost, being a sustainable alternative for certain regions with unfavorable climatic conditions. For these reasons, it is currently a growing market, diversifying its value on a nutritional and therapeutic level.

When it comes to nutrition, the mineral content of mushrooms is of crucial importance, as they are rich in potassium, phosphorus, magnesium, and some species are characterized by their zinc, copper, selenium, and iron content, so that nutrient deficiencies in the diet can be compensated (Anusha et al. 2023; López et al. 2022a). However, mushrooms can also bioaccumulate heavy metals such as lead, cadmium, and mercury, which can present a health risk if consumed in large quantities and whose accumulation may depend on the species or soil conditions during cultivation (Liu et al. 2022; Nambafu et al. 2022; Razanov et al. 2022). Therefore, an analysis of the profile of minerals and heavy metals in the most commonly consumed species should be carried out in order to find a balance between nutritional value and food safety.

In addition to their mineral content and contaminants such as heavy metals, mushrooms and extracts with different solvents have shown remarkable biological activity in the context of oncological research. Recent studies have indicated that mushrooms can exert cytotoxic effects on various tumor cell lines, suggesting their potential use as therapeutic agents and adjuvants in oncological therapies (Dincer et al. 2023; Park 2022). The evaluation of cell viability after exposure to the extracts allows a better understanding of their mechanisms and efficacy in inhibiting tumor growth. In this sense, several studies have suggested that their high content of polysaccharides and especially beta-glucans is responsible for this antitumor activity through the activation of cellular responses such as the expression of cytokines and nitric oxide, as well as the induction of apoptosis and the suppression of metastasis (Dong et al. 2023; Razin et al. 2023).

Against this background, the search for extraction methods and technologies that maximize the content of bioactive compounds and reduce contaminants such as heavy metals from matrices like mushrooms is of particular importance. Among them, pulsed electric fields extraction (PEF) has emerged as a promising technology that allows high intensity electric fields to be generated over short periods of time to produce electroporation of the cell membrane, enabling the release of high-value compounds into the external environment (Calleja-Gómez et al. 2023). In contrast, the application of PEF has proven to be effective in reducing the concentration of contaminants such as mycotoxins and pesticides in the final product, the attenuation and removal of which depends on factors such as the strength, duration, and frequency of the

electric field (Gavahian et al. 2020; Sebastià et al. 2023). In addition, this technology has proven to be effective in extracting macro- and micronutrients from plant (Zaky et al. 2024), animal (Martí-Quijal et al. 2023; Xu et al. 2023), and agricultural by-products (Calleja-Gómez et al. 2024). Therefore, its application in matrices with high nutritional value but with bioaccumulation of contaminants is of particular interest for the formulation of adjuvants and concentrates whose composition may be related to a reduction in the viability of cells in tumor lines.

For the aforementioned reasons, the aim of this study is to compare the content of carbohydrates, minerals, and heavy metals in the cultivated mushrooms *A. bisporus*, *A. brunnescens*, *L. edodes*, *P. ostreatus* and the wild mushroom *L. deliciosus* as well as in their extracts obtained by conventional and pulsed electric fields (PEF)- assisted extraction. In addition, the effects of these extracts on the cell viability of CACO-2 human colorectal adenocarcinoma cells will be evaluated to determine the efficacy of PEF in improving biological activity and reducing contaminants.

## 2. Materials and Methods

### 2.1 . Sample preparation

The fresh mushrooms *Agaricus bisporus* (Button mushroom), *Agaricus brunnescens* (Portobello), *Lentinula edodes* (Shiitake) and *Pleurotus ostreatus* (Oyster mushroom) were acquired in a local supermarket in Valencia (Comunitat Valenciana, Spain) and came from the same area. On the other hand, the wild mushroom *Lactarius deliciosus*, was collected in the area of Villafranca del Cid (Comunitat Valenciana Spain), whose coordinates are 40°25'16.4 "N 0°16'37.8 "W. The mushrooms were kept in the dark at 4 °C until use the day after purchase. For the extractions, the pileus of fruiting body of each mushroom was cut into thin slices with a knife (20 g for each extraction) and used immediately after cutting. The stems of each mushroom were discarded.

### 2.2 . Pulsed Electric Fields (PEF) pretreatment

The PEF extractions were performed in a 900 mL rectangular cell of the PEF-Cellcrack III (German Institute of Food Technologies (DIL)) equipment (ELEA, Quakenbrück, Germany) at the Faculty of Pharmacy and Food Sciences of the Universitat de València (Burjassot, Valencia, Spain). The distance between the two parallel rectangular electrodes was 10 cm. The ratio between sample (fresh mushroom) and solvent (distilled water) was 1:10 and the conductivity was adjusted between 600-800 µS/cm with NaCl using the ProfiLine Cond 3310 conductometer (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). Subsequently, the treatment cell was introduced into the equipment and the treatment was carried out at the optimum conditions determined by Calleja-Gómez et al. (2022): 2.5 kV/cm and 50 kJ/kg. The frequency was 2.00 Hz and the pulse duration 100 ms.

### 2.3 . Supplementary aqueous extraction

After PEF pretreatment, the extracts were stirred for 6 hours at 400 rpm according to the optimal conditions mentioned above (Calleja-Gómez et al. 2022). After 6h, the extracts were centrifuged at 4000 rpm for 10 min and filtered under vacuum to remove solid residues. Finally, the extracts were stored in the freezer at -20 °C until they were used for chemical analyses and *in vitro* assays.

2.4 Conventional extraction conditions

In order to compare the impact of PEF on the recovery of bioactive compounds, a conventional extraction based on aqueous maceration was performed. Each mushroom sample was placed in an Erlenmeyer flask together with 200 mL of distilled water and stirred for 6h, according to the optimal supplementary extraction time for PEF, but without pretreatment by this technology. In this sense, conventional extracts obtained by aqueous agitation for 6h at room temperature were compared with those obtained by PEF under optimal conditions (2.5 kV/cm, 150 kJ/kg and 6h total time). Conventional extracts were performed in triplicate for each matrix.

2.5 . Chemical Analysis

2.5.1 Determination of mycotoxins in source matrices

The analysis utilized an in-house validated QuEChERS method for extracting mycotoxins based on the study published by Pallarés et al. (2022). A 2 g portion of powdered mushroom sample was placed in a 50 ml Falcon tube, followed by the addition of 10 ml of 2% formic acid in water. The mixture was agitated for 30 minutes using a KS 260 IKA shaker (Staufen, Germany). Next, 10 ml of acetonitrile (ACN) were added, and the tube was shaken for an additional 30 minutes. Afterward, 4 g of $MgSO_4$ and 1 g of NaCl were added, and the mixture was vortexed for 30 seconds. The tubes were centrifuged at 2268 g for 10 minutes in an Eppendorf Centrifuge 5810R (Madrid, Spain). Following centrifugation, 2 ml of the supernatant were transferred to a 15 ml Falcon tube, to which 0.3 g of $MgSO_4$ and 0.1 g of octadecyl C18 were added, vortexed for 30 seconds, and centrifuged again at 2268 g for 10 minutes. The final supernatant was filtered through a 13 mm/0.22 µm nylon filter (Membrane Solutions, TX, USA) and the resulting solution was injected into the UHPLC-MS/MS system for analysis.

The quantification of mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ENNA, EN-NA1, ENNB, ENNB1) was carried out using UHPLC-MS/MS on a Sciex TRIPLE QUAD 6500+ system equipped with electrospray ionization (ESI). This was coupled with an Agilent 1260 HPLC UHPLC system, consisting of a degasser, quaternary pump, and column oven, along with an Eksigent ULC 100 HTC-xt autosampler. A BEH® C18 column (1.7 µm; 100 Å, 50 x 2.1 mm, Waters) was used for separation. The mobile phases consisted of (A) 5 mM ammonium formate with 0.1% formic acid in water, and (B) methanol with 0.1% formic acid. The linear gradient program was as follows: 0 min (95% A), 2 min (95% A), 13 min (0% A), 15.1 min (5% A), and 18 min (5% A), with a 5 µL injection volume and a column temperature maintained at 30 °C. The mass spectrometer operated in positive ionization mode using Selected Reaction Monitoring (SRM), with a Turbo Spray IonDrive ionization source. The operating conditions were: curtain gas (CUR) at 30 PSI, ion spray voltage (IS) at 4.4 kV, a temperature of 300 °C, and ion source gases (GS1 and GS2) at 55 PSI. Mycotoxin standards (aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), and enniatin B1 (ENNB1)) were purchased from Sigma (St. Louis, MO, USA). The UHPLC-MS/MS method performance parameters are shown in Table S1.

2.5.2 Total carbohydrate content

The phenol-sulfuric method described by Dubois et al. (1956) was used to determine the total carbohydrate content. This method is based on the acid catalysis of carbohydrates, which yield a colored product after condensation with phenol. 1 mL of the conventional and PEF extracts were taken in 1/10 dilution together with 2.5 mL of $H_2SO_4$ and 0.5 mL of a 5% phenol solution. The resulting solution was incubated at room temperature for 30 minutes and the absorbance was measured at 490 nm. A solution of D-glucose at different concentrations (10-100 mg/L) was used as a standard. The results were expressed in mg glucose/g dry weight (mg glu/g DW).

#### 2.5.3 Mineral and heavy metal content determination

The minerals Ca, Mg, Fe, Zn, P, Se, Cu and K as well as the heavy metals As, Cd, Hg and Pb were determined by inductively coupled mass spectrometry (ICP-MS) after prior digestion of the samples. For the digestion of the solid samples of mushrooms (*A. bisporus, L. edodes, A. brunnescens, P. ostreatus* and *L. deliciosus*), 10 mg of the sample was taken and digested with 1 mL of 69% $HNO_3$ together with 250 µL of $H_2O_2$ in an Ethos Easy high-pressure microwave oven (Milestone Srl, Sorisole (BG), Italy) at a maximum temperature of 180 °C. Subsequently, it was then brought to a final volume of 10 mL (minerals) or 5 mL (heavy metals) with MiliQ water. For the digestion of minerals, an aliquot of 100 µL is taken and diluted again in 10 mL MiliQ water. For the digestion of liquid extracts, 1 mL of the sample (minerals) or 0.5 mL (heavy metals) was taken and the procedure described before was followed, reaching a final volume of 5 mL, and taking an aliquot of 100 µL, which is dissolved with MiliQ water to 10 mL for minerals.

Multielement and heavy metal determination was performed using the Agilent 7900 ICP-MS equipment (Agilent Technologies, Santa Clara, CA, USA) with Micromist concentric nebulizer, Scott type spray chamber, platinum interface cones, off-axis double lens system, hyperbolic quadrupole as mass filter and an octopolar collision/reaction cell with He and $H_2$ gas, respectively. Certified Mg, Ca, P, Fe and Zn standards of 10000 µg/mL (HPS, ZeptoMetrix, North Charleston, USA) and internal standard dilutions of Sc and Ge of 20 µg/g (ISC Science, Gijón, Spain) were used for mineral analysis (Table S2). For the heavy metals, As, Cd, Hg and Pb standards of 10000 µg/mL and an internal standard solution of Ge, Rh and Ir of 20 µg/g were used.

### 2.6 . Cytotoxicity Assessment

#### 2.6.1 Cell culture

CACO-2 human colorectal adenocarcinoma cells were cultured in DMEM medium (1x) + GlutaMAX™-I with 4.5 g/L D-glucose and pyruvate. The medium was supplemented with 10% Fetal Bovine Serum (FBS), 1% Non-Essential Amino Acids Solution (MEM-NEAA, 100x), 1% HEPES buffer solution (1M), Fungizone (0.1%) and 1% Penicilin-Streptomycin (5000U/mL). All components were purchased from ThermoFisher Scientific, Waltham, MA, USA. Cells were incubated at 5% $CO_2$, 95% air atmosphere with constant humidity, pH 7.4 and 37°C. The medium was changed every 3 days.

#### 2.6.2 Assessment of Cell Viability

To determine the cytotoxicity and cell viability of conventional (aqueous maceration) and PEF extracts of *A. bisporus, L. edodes, A. brunnescens, P. ostreatus* and *L. deliciosus*, the MTT assay was performed on

CACO-2 cells according to the method described by Martí-Quijal et al. (2023). This method is based on the formation of blue formazan crystals due to the reduction of the tetrazolium salt (MTT) by cell metabolism, which can be dissolved in an organic solvent and measured in a spectrophotometer. 25.000 cells/well were seeded in 96-well microplates and incubated for 24h until 80% confluence was reached. The medium was then removed, and the cells were exposed to various increasing concentrations (0.78 to 25%) of the conventional and PEF extracts for 24h. After the incubation period, the medium was removed together with the extract and replaced with 200 µL of medium together with 50 µL of MTT salt previously dissolved in 5 mL of PBS (5 mg/mL). Subsequently, a 3h incubation was performed in the dark at 37 °C and the crystals formed were dissolved with DMSO. A MultiSkanEX automatic plate reader (Labsystem, Helsinki, Finland) was used for measurement at 540 nm. Control groups were included in each experiment. The results were expressed as the relative percentage of cell viability compared to the control. The standard deviation is shown as an error bar for each concentration.

### 2.7 Statistical Analysis

The comparison between the carbohydrate, micronutrient, and heavy metal contents as well as the cytotoxicity results of the different mushrooms and extracts was performed by analysis of variance (ANOVA) assuming a significant difference of $p < 0.05$. All statistical treatment was performed with GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) considering n = 3 and assuming a significance level of 5%. Standard deviations are shown as error bars in the figures. A coefficient of variation <10% was assumed when analyzing the mineral and heavy metal content.

## 3 Results and discussion

### 3.1. Mycotoxins determination

None of the mycotoxins tested (AFB1, AFB2, AFG1, AFG2, OTA, ENNA, EN-NA1, ENNB, ENNB1) were detected in the samples of the fresh mushrooms *A. bisporus, L. edodes, A. brunnescens, P. ostreatus* and *L. deliciosus*.

### 3.2. Comparison of total carbohydrate content of different mushrooms and influence of the extraction methodology

The total carbohydrate content of the extracts of *A. brunnescens, L. edodes* and *P. ostreatus* mushrooms obtained by the conventional aqueous and PEF methods compared to that determined for *A. bisporus* in the earlier study carried out by our research group (Calleja-Gómez et al. 2022) is shown in Figure 1.

The total carbohydrate content varied across species, with values ranging from 8.14 ± 0.74 to 16.71 ± 0.72 mg glucose equivalents per gram dry weight (mg glu/g DW) for *A. bisporus* (Calleja-Gómez et al. 2022). For *L. edodes*, the carbohydrate content ranged from 7.66 ± 0.44 to 109.31 ± 8.63 mg glu/g DW, while for *A. brunnescens*, it was between 12.29 ± 0.64 and 19.12 ± 1.90 mg glu/g DW. *P. ostreatus* showed values between 64.30 ± 6.14 and 70.29 ± 3.29 mg glu/g DW, and *L. deliciosus* exhibited the lowest range, from 3.19 ± 0.08 to 6.33 ± 0.77 mg glu/g DW. These variations were observed when comparing conventional extraction methods with pulsed electric field (PEF) extraction techniques.

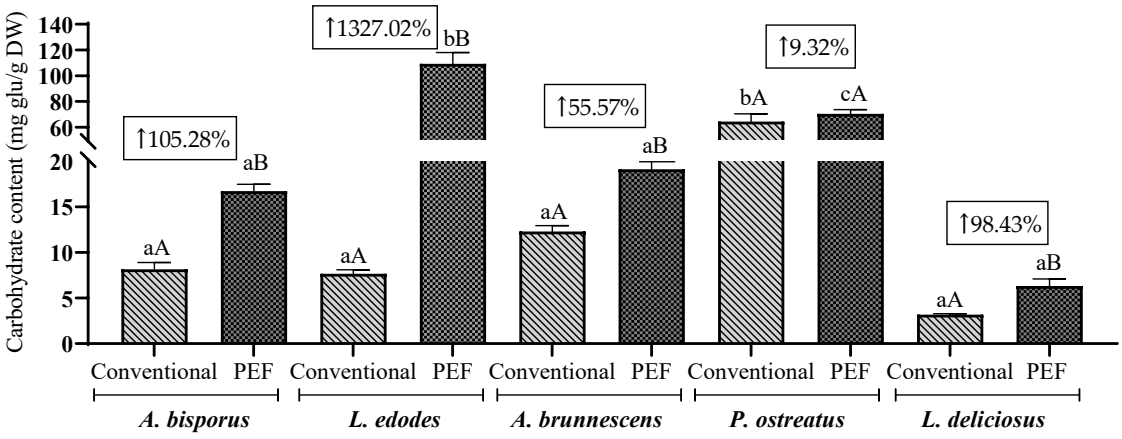



Figure 1. Total carbohydrate content (mg glucose/g dry weight) of the different extracts obtained by aqueous maceration and by PEF-assisted extraction from the cultivated mushrooms *A. bisporus* (data from Calleja-Gómez et al. 2022), *L. edodes, A brunnescens, P. ostreatus* and the wild mushroom *L. deliciosus*. The increases between the conventional method and PEF for the same mushroom are expressed as a percentage. Different capital letters indicate significant difference ($p$<0.05) in the extraction method for the same mushroom. Lower case letters indicate a significant difference ($p$<0.05) between the mushroom species for the same extraction method.

As can be observed, PEF-assisted extraction improved carbohydrate yield in all mushrooms, with the highest increase in *L. edodes* at 1327.02% and the lowest in *P. ostreatus* at 9.32%. In this sense, carbohydrate extraction by PEF showed significant differences ($p$<0.05) for *L. edodes, A. bisporus, A. brunnescens* and *L. deliciosus* and non-significant differences ($p$>0.05) compared to the conventional methodology for *P. ostreatus*.

Various authors have reported increased carbohydrate yield in different mushrooms by PEF. Sung et al. (2020) observed that PEF extracts from Chaga mushrooms at 0.5 kV/cm had higher carbohydrate and polyphenol content. Xue and Farid (2015) showed that after an intensive PEF treatment at 38.4 kV/cm on *A. bisporus*, a concentration of 7.9 mg/g of carbohydrates was achieved compared to 6.4 mg/g for a conventional extraction at 95 °C. Similarly, Li et al. (2013) observed that PEF treatment at 24 kV/cm on *Auricularia auricula* improved the recovery of polysaccharides with anticoagulant activity and achieved the best results compared to ultrasonic and microwave extraction. In addition, PEF technology has been used not only to obtain extracts, but also to stimulate the growth of various fungal species. In the study published by Zare et al. (2021), pulsed electric fields with a total energy of 62.3 kJ were applied to the culture of *P. ostreatus* for 30 days, resulting in an increase in production yield of up to 45.5%, suggesting that it is a useful technology that can be used before and after harvest for either cultivation or extraction, as it increases the nutritional quality of the fruiting body.

On the other hand, significant differences ($p$<0.05) were also observed among the different mushroom species when PEF was applied, with *L. edodes* and *P. ostreatus* standing out for their higher carbohydrate content, followed by *A. brunnescens* and *A. bisporus* with similar and lower contents, respectively. Finally, *L. deliciosus* had the lowest carbohydrate content in both the conventional and PEF extracts, with values below 10 mg glu/g DW.

In this sense, *P. ostreatus* stands out among the rest of the mushrooms for its high content of carbohydrates (Rodrigues Barbosa et al. 2020). It is also remarkable *L. edodes* high content of glucans such as lentinan, with different bonds responsible for the biological activity against infections and mutations, showing synergy with the antioxidant components of the mushroom (Singdevsachan et al. 2016; Zhu et al. 2015). Several studies have shown that the total carbohydrate content of *P. ostreatus* ranges from 46.10% to 66.54% DW (Irshad et al. 2023; Onuoha et al. 2021), while the total carbohydrate content of *L. edodes* ranges from 47.98% to 75.56% DW (C et al. 2023; Q. Li et al. 2021), being a food that stands out less for its quantity than for its carbohydrate quality (such as β-glucans). It should also be noted that the content may vary depending on the growing conditions and the part of the mushroom, with the stipe generally having a higher content (Rahmann 2023).

For all the above reasons, the application of technologies such as PEF that allow better extraction of carbohydrates from mushrooms with a higher carbohydrate content is interesting, especially in mushrooms such as *P. ostreatus* and *L. edodes* with a higher content of biologically active components such as β-glucans, in order to formulate extracts that have a high concentration of bioactive components.

### 3.3. Mineral content

The contents of Ca, Mg, Fe, Zn, K, Cu, P and Se of the different fresh mushrooms and their extracts obtained by aqueous maceration (conventional method) and PEF measured by ICP-MS are listed in Table 1. Mineral data (Ca, Fe, Zn, Se, P, Mg) for *A. bisporus* was obtained from the previous study published by Calleja-Gómez et al. (2022). The results were expressed in mg or µg/kg and mg or µg/150g (recommended serving size (Valero et al. 2018)) to determine the percentage of the Nutritional Reference Intake (NRI) covered by a portion.

Differences in mineral content were observed between the different mushroom species, but with a generally high content of P, K, Fe, Mg and Se, being P and K the most important minerals. In this sense, *A. brunnescens, P. ostreatus* and *L. edodes* were the mushrooms with the highest Mg and Fe content, with 258, 326 and 745 mg/kg Mg and 63.50, 13.59 and 19.30 mg/kg Fe, respectively. In addition, *P. ostreatus* and *L. edodes* also stood out for their Zn content, with 15.02 mg/kg covering 20.48% of the RNI for *P. ostreatus* and 20.34 mg/kg covering 27.74% of the RNI for *L. edodes*.

In addition, *A. bisporus* and *A. brunnescens* were characterized by a high P, K and Se content, which reached 131.36% of the NRI for *A. brunnescens*. Together with these mushrooms, a high P content was also observed in *L. edodes*, reaching 63.34% of the NRI. Finally, it can be concluded from the observed results that the mushrooms do not have a high Ca content, since they all contribute <1% of the NRI, with the exception of *L. edodes* and *A. brunnescens*, whose maximum contribution is 3.6%.

Table 1. Ca, Mg, Fe, Zn, P, K, Cu and Se content of fresh mushrooms *A. bisporus, L. edodes, A. brunnescens, P. ostreatus*, conventional (Conv) and pulsed electric fields (PEF) extracts together with *L. deliciosus* extracts. Mineral data (Ca, Fe, Zn, Se, P, Mg) for *A. bisporus* was obtained from the previous study published by Calleja-Gómez et al. (2022).

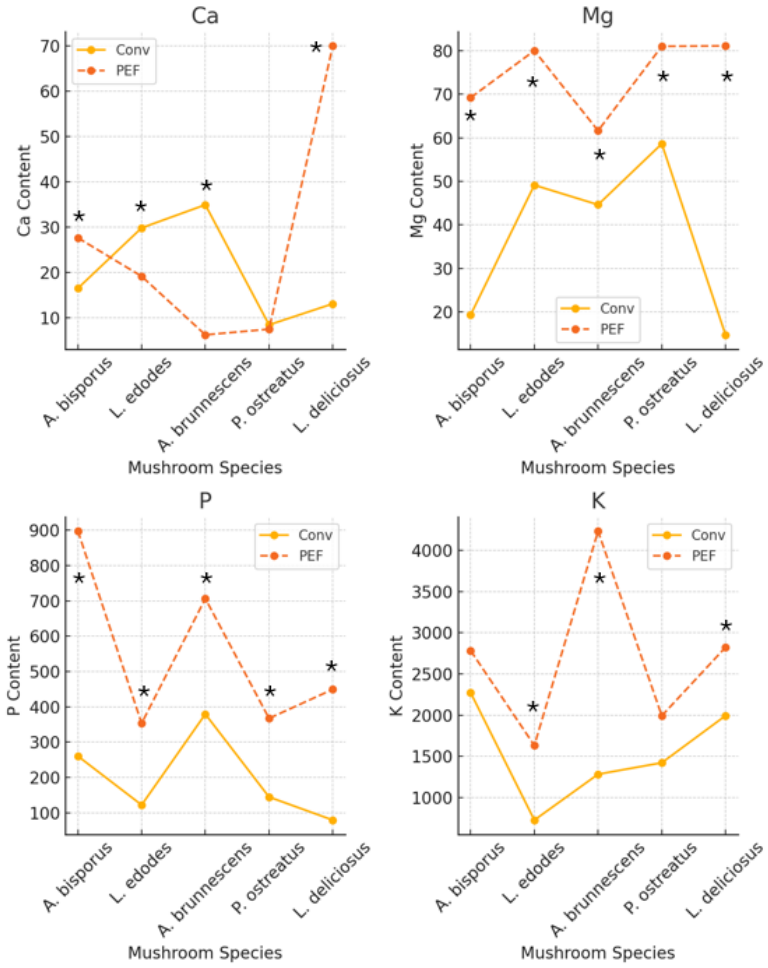
| | | *A. bisporus* | | | *L. edodes* | | | *A. brunnescens* | | | *P. ostreatus* | | | *L. deliciosus* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | FM | Conv | PEF | FM | Conv | PEF | FM | Conv | PEF | FM | Conv | PEF | Conv | PEF |
| Ca | mg/kg | 30.20 | 16.47 | 27.61 | 226.20 | 29.81 | 19.12 | 77.10 | 34.92 | 6.18 | 21.06 | 8.39 | 7.44 | 13.04 | 70.04 |
| | mg/150g | 4.53 | 2.47 | 4.14 | 33.93 | 4.47 | 2.87 | 11.57 | 5.24 | 0.93 | 3.16 | 1.26 | 1.12 | 1.96 | 10.51 |
| | % NRI | <1 | <1 | <1 | 3.57 | <1 | <1 | 1.22 | <1 | <1 | <1 | <1 | <1 | <1 | 1.11 |
| Mg | mg/kg | 141 | 19.33 | 69.23 | 745 | 49.13 | 80.03 | 258 | 44.63 | 61.63 | 326 | 58.63 | 81.03 | 14.73 | 81.13 |
| | mg/150g | 21.15 | 2.90 | 10.38 | 111.75 | 7.37 | 12.00 | 38.7 | 6.69 | 9.24 | 48.90 | 8.79 | 12.15 | 2.21 | 12.17 |
| | % NRI | 6.04 | <1 | 2.97 | 31.93 | 2.11 | 3.43 | 11.06 | 1.91 | 2.64 | 13.97 | 2.51 | 3.47 | 0.63 | 3.48 |
| Fe | mg/kg | 8.60 | 0.58 | 1.78 | 19.30 | 0.86 | 1.20 | 63.50 | 0.70 | 0.65 | 13.59 | 1.75 | 3.67 | 0.21 | 3.79 |
| | mg/150g | 1.29 | 0.09 | 0.27 | 2.90 | 0.13 | 0.18 | 9.53 | 0.10 | 0.10 | 2.04 | 0.26 | 0.55 | 0.03 | 0.57 |
| | % NRI | 14.18 | 0.95 | 2.93 | 31.81 | 1.41 | 1.97 | 104.67 | 1.15 | 1.08 | 22.40 | 2.88 | 6.04 | <1 | 6.25 |
| Zn | mg/kg | 9.90 | 2.17 | 3.11 | 20.34 | 3.97 | 3.61 | 10.38 | 2.93 | 2.10 | 15.02 | 4.53 | 3.21 | 0.59 | 2.47 |
| | mg/150g | 1.49 | 0.33 | 0.47 | 3.05 | 0.60 | 0.54 | 1.557 | 0.44 | 0.31 | 2.25 | 0.68 | 0.48 | 0.09 | 0.37 |
| | % NRI | 13.50 | 2.96 | 4.24 | 27.74 | 5.42 | 4.92 | 14.15 | 3.99 | 2.86 | 20.48 | 6.18 | 4.37 | 0.80 | 3.37 |
| P | mg/kg | 1461 | 260.45 | 897.45 | 2956 | 122.35 | 353.45 | 2130 | 379.45 | 706.42 | 1319 | 144.85 | 367.41 | 79.55 | 449.45 |
| | mg/150g | 219.15 | 39.07 | 134.62 | 443.40 | 18.35 | 53.02 | 319.50 | 56.92 | 105.97 | 197.85 | 21.73 | 55.12 | 11.93 | 67.42 |
| | % NRI | 31.31 | 5.58 | 19.23 | 63.34 | 2.62 | 7.57 | 45.64 | 8.13 | 15.14 | 28.26 | 3.10 | 7.87 | 1.70 | 9.63 |
| K | mg/kg | 4000 | 2272.10 | 2782.10 | 2700 | 727.10 | 1632.10 | 4290 | 1283.10 | 4232.10 | 2980 | 1422.10 | 1992.10 | 1993 | 2822.1 |
| | mg/150g | 600 | 340.82 | 417.32 | 405 | 109.07 | 244.82 | 643.50 | 192.47 | 634.82 | 447 | 213.32 | 298.82 | 299 | 423.32 |
| | % NRI | 17.14 | 9.74 | 11.92 | 11.57 | 3.12 | 6.99 | 18.39 | 5.50 | 18.14 | 12.77 | 6.09 | 8.54 | 8.55 | 12.09 |
| Cu | mg/kg | 1.71 | 0.33 | 0.43 | 0.57 | 0.09 | 0.2 | 3.46 | 0.60 | 1.74 | 0.65 | 0.33 | 0.32 | 0.36 | 0.44 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/150g | 0.26 | 0.05 | 0.06 | 0.08 | 0.01 | 0.03 | 0.52 | 0.09 | 0.26 | 0.10 | 0.05 | 0.05 | 0.05 | 0.07 |
| | % NRI | 19.73 | 3.81 | 4.92 | 6.52 | 1.08 | 2.35 | 39.92 | 6.97 | 20.06 | 7.52 | 3.77 | 3.66 | 4.11 | 5.06 |
| Se | µg/kg | 243 | 60 | 150 | 83.4 | <60 | <60 | 613 | 104 | 76.10 | 81 | <60 | 63.50 | 50 | 46 |
| | µg/150g | 36.45 | <9 | 22.50 | 12.51 | <9 | <9 | 91.95 | 15.60 | 11.42 | 12.15 | <9 | 9.53 | 7.50 | 6.90 |
| | % NRI | 52.07 | ±12.86 | 32.14 | 17.87 | ±12.86 | ±12.86 | 131.36 | 22.29 | 16.31 | 17.36 | ±12.86 | 13.61 | 10.71 | 9.86 |

3 NRI: Nutritional Reference Intake. FM: Fresh Matter.

Regarding the methodology used, a decrease in mineral content of more than 50% compared to the original matrix was generally observed. However, there was a trend towards higher recovery by PEF compared to conventional extraction. This general trend is clearly visualized in Figure 2, which shows the behavior of the minerals towards both extraction methodologies across the various mushroom species.

As can be seen, an increase in the amount of minerals, especially Ca, was observed in the extracts of *L. deliciosus*, with the exception of Se, which was extracted in similar amounts. Thus, PEF generally increased Mg and Fe extraction compared to the conventional method, with a notable enhancement in *P. ostreatus* and *L. deliciosus*, while the trend is less pronounced for Zn content. As for P and K content, PEF drastically increased the content in all species, especially in *Agaricus spp*. and *L. deliciosus*. These results suggest that the higher extraction of minerals by PEF is related to the phenomenon of electroporation of the membranes, which allows an efflux of minerals into the extraction medium compared to aqueous maceration.



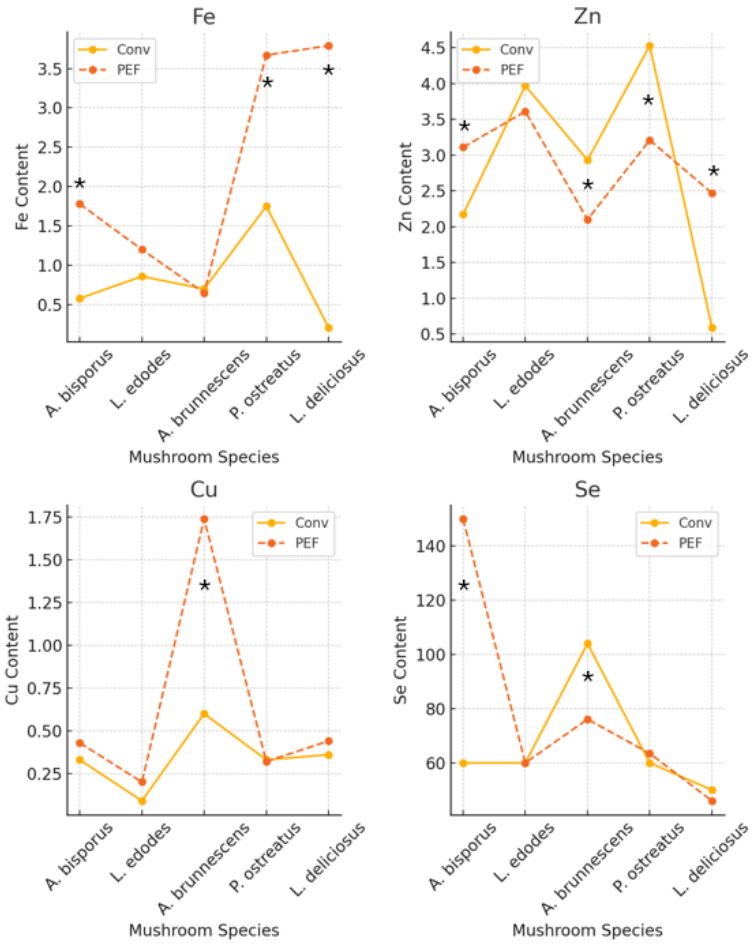



Figure 2. Overview of the recovery trend of Ca, Mg, Fe, Zn, P, K, Cu and Se minerals based on the extraction methodology across *A. bisporus* (Calleja-Gómez et al. 2022), *A. brunnescens, P. ostreatus, L. edodes* and *L. deliciosus* mushrooms. The results were expressed in mg/kg for all minerals except for Se, which was expressed in µg/kg. * $p < 0.05$ between extraction methodologies.

In general, several authors have found that mushrooms are a source of minerals, especially K and P, followed by Mg, Fe and Zn (Anusha et al. 2023; Effiong et al. 2023). However, great variability is observed in the species analysed in different studies, due to interspecies variability and environmental conditions, which have a greater influence on wild species. In this sense, in the study published by López et al. 2022, in which several wild *Lactarius* species were studied, it was observed that the most abundant minerals were K (13845 ± 1.00 mg/Kg for *L. deliciosus*) and P (mean value of 3909 mg/Kg), while a low Ca content was observed. However, they pointed out that the consumption of mushrooms should be considered due to the presence of heavy metals such as Cr or As. On the other hand, Mleczek et al. 2022 observed that anthropogenic pollution affects the mineral profile of cultivated mushrooms, with an increase in mineral elements (Ca, Hg, Pb, Fe, Mn, among others) in urban areas compared to rural areas.

Finally, due to the low Ca content and the influence of growing conditions, it has been observed in recent years that the enrichment of growing soils with $CaCO_3$, $CaCl_2$ or $Ca(NO_3)_2$ makes it possible to obtain mushrooms with a higher content of this mineral, which in turn increases the content of valuable compounds such as phenolics, other minerals and polysaccharides (Oyetayo 2023; Tang et al. 2023).

Therefore, the in-depth study of the mineral profile as well as its variability is interesting not only for its own nutritional value, but also for its influence on other bioactive compounds.

### 3.4. Heavy metal content

The contents of As, Cd, Hg and Pb (µg/kg) for each fresh mushroom, conventional and PEF extracts are shown in Table 2, also expressed by the content of each heavy metal per mushroom ration (150g) and the percentage it represents according to the Tolerable Weekly Intake (TWI) for a 70 kg person (Walpole et al. 2012).

Among the mushroom species, different profiles were observed for each heavy metal, with *L. edodes* standing out as the mushroom with the highest As and Cd content, with 11.87% and 31.11% TWI in a single portion respectively, while it does not exceed 1% TWI in the other mushrooms. In terms of Pb content, *A. bisporus* had the highest content with 24.75 µg/150g, followed by *A. brunnescens* and *L. edodes* with 10.35 and 9.27 µg/150g, respectively. The mushroom with the lowest total heavy metal content was *P. ostreatus*, which together with its high carbohydrate content makes it a valuable source of nutrients. In contrast, *L. edodes* had a high total heavy metal content, except for Hg, whose content was also low in the other mushrooms. For this reason, despite its high content of carbohydrates and bioactive compounds, a different method to extract compounds of interest without a high heavy metal content should be considered at the food safety level and especially in the development of extracts and food supplements from this mushroom.

Table 2. As, Cd, Hg and Pb content of fresh mushrooms *A. bisporus, L. edodes, A. brunnescens, P. ostreatus*, conventional (Conv) and pulsed electric fields (PEF) extracts together with *L. deliciosus* extracts.

| | | *A. bisporus* | | | *L. edodes* | | | *A. brunnescens* | | | *P. ostreatus* | | | *L. deliciosus* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | FM | Conv | PEF | FM | Conv | PEF | FM | Conv | PEF | FM | Conv | PEF | Conv | PEF |
| As | µg/kg | 15.20 | 6.7 | 15.10 | 831 | 74.00 | 80.00 | 27.10 | 12.20 | 11.60 | 22.60 | 9.80 | 7.30 | 56.00 | 45.5 |
| | µg/150g | 2.28 | 1.01 | 2.27 | 124.65 | 11.10 | 12.00 | 4.07 | 1.83 | 1.74 | 3.39 | 1.47 | 1.10 | 8.40 | 6.83 |
| | % TWI | 0.22 | 0.10 | 0.22 | 11.87 | 1.06 | 1.14 | 0.39 | 0.17 | 0.17 | 0.32 | 0.14 | 0.10 | 0.80 | 0.65 |
| Cd | µg/kg | 27.00 | 1.00 | 2.30 | 363 | 14.00 | 27.00 | 30.00 | 2.00 | 1.50 | 19.50 | 4.50 | 5.16 | 3.64 | 3.34 |
| | µg/150g | 4.05 | 0.15 | 0.35 | 54.45 | 2.10 | 4.05 | 4.50 | 0.30 | 0.23 | 2.93 | 0.68 | 0.77 | 0.55 | 0.50 |
| | % TWI | 2.31 | 0.09 | 0.20 | 31.11 | 1.20 | 2.31 | 2.57 | 0.17 | 0.13 | 1.67 | 0.39 | 0.44 | 0.31 | 0.29 |
| Hg | µg/kg | 4.50 | 2.80 | 4.40 | 6.00 | 3.70 | 2.50 | 2.20 | 2.51 | 3.22 | 2.50 | 3.10 | 2.50 | 4.00 | 3.40 |
| | µg/150g | 0.68 | 0.42 | 0.66 | 0.90 | 0.56 | 0.38 | 0.33 | 0.38 | 0.48 | 0.38 | 0.47 | 0.38 | 0.60 | 0.51 |
| | % TWI | 0.24 | 0.15 | 0.24 | 0.32 | 0.20 | 0.13 | 0.12 | 0.13 | 0.17 | 0.13 | 0.17 | 0.13 | 0.21 | 0.18 |
| Pb | µg/kg | 165 | 6.20 | 11.20 | 61.80 | 11.30 | 13.60 | 69.00 | 60.20 | 6.62 | 15.90 | 10.50 | 11.20 | 0.61 | 2.50 |
| | µg/150g | 24.75 | 0.93 | 1.68 | 9.27 | 1.70 | 2.04 | 10.35 | 9.03 | 0.99 | 2.39 | 1.58 | 1.68 | 0.09 | 0.37 |
| | % TWI | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

TWI: Tolerable Weekly Intake. FM: Fresh Matter

For this reason, the heavy metal content in the extracts obtained by PEF and conventional extraction was evaluated. A general trend was observed in all of them, leading to a strong decrease in content compared to the original matrix, without the TWI exceeding 3% in both types of extracts. Thus, the general trend of heavy metal extraction according to extraction technology is shown in Figure 3.

When comparing the different methods, a variability in the recovery of heavy metals was observed. In general, PEF extraction increased the content, but with small differences compared to conventional extraction. In addition, conventional methodology increased the recovery in certain cases, as in the conventional extract of *L. deliciosus* for all heavy metals except Pb and Cd. Moreover, regarding Hg, PEF extracts had lower values than conventional extraction for *L. edodes, P. ostreatus* and *L. deliciosus*. These results suggest that PEF would be an advantageous alternative to recover valuable compounds from mushrooms, reducing the levels of heavy metals compared to the original matrix, so that the addition of these components from the extracts would allow the formulation of nutraceuticals or the fortification of other food matrices.

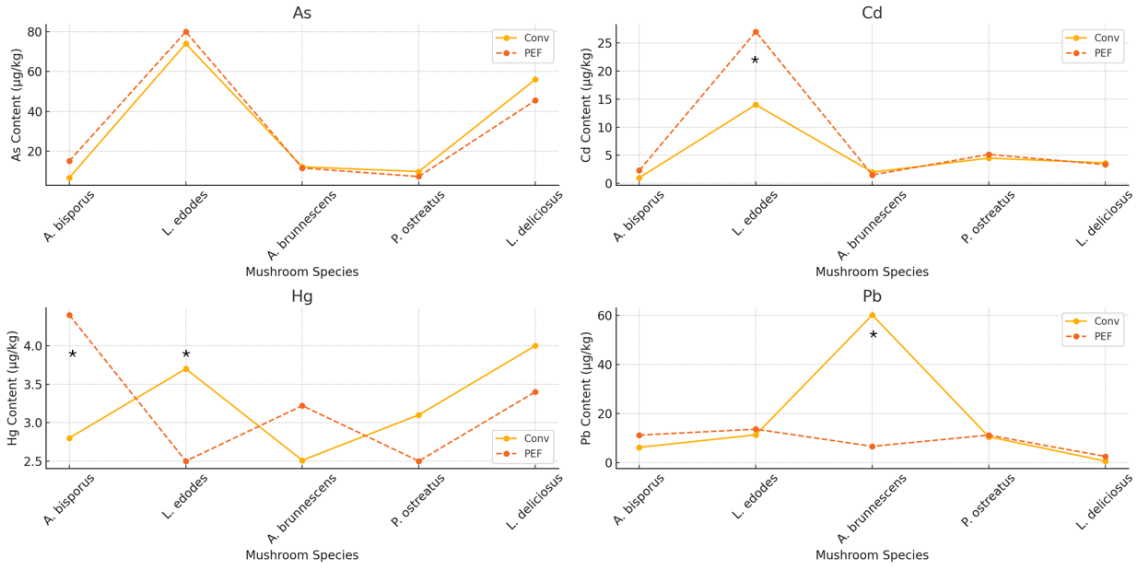



Figure 3. Overview of the recovery trend of As, Cd, Hg and Pb based on the extraction methodology across *A. bisporus, A. brunnescens, P. ostreatus, L. edodes* and *L. deliciosus* mushrooms. The results were expressed in µg/kg. * $p < 0.05$ between extraction methodologies.

In this sense, the effectiveness of PEF extraction lies in its ability to induce electroporation in mushroom cells, creating temporary or permanent pores in the cell membranes. This significantly improves mass transfer and the release of heavy metals into the extracellular medium (Raso and Heinz, 2006). However, the observed differences can be attributed to several factors. Among them, the composition of the fungal matrix could have a significant influence due to the amount of quinine and polysaccharides, affecting the ease of pore creation during pretreatment (Vorobiev and Lebovka, 2008). These matrix differences were demonstrated in the previous study by Calleja-Gómez et al. 2023, in which the differences in the initial matrix of *P. ostreatus*, *A. brunnescens* and *L. edodes* were investigated by scanning electron microscopy. In addition, the type of heavy metal is also a factor to consider, as some such as Cd and Pb bind strongly to

intracellular proteins, making their extraction difficult, so the chemical affinity of the metals studied with the intracellular components must be taken into account in order to select the extraction conditions (Raso and Heinz, 2006).

Additionally, these results are consistent with those reported by other authors. In the study published by Dowlati et al. 2021, which reviewed 59 articles published between 1970 and 2020 on the content of heavy metals in different mushrooms, the authors observed that mushrooms had higher amounts of Fe, Zn and Cu than Pb (2.4844 mg/kg) and Cd (1.3925 mg/kg) amounts. Furthermore, it indicated that the bioaccumulation of metals such as Pb, Cu, Fe and Cd occurs mainly in the caps of the mushrooms, the part selected for the present study.

Furthermore, the amount of heavy metals in wild mushrooms is particularly striking, even in the PEF extract of *L. deliciosus*. According to Pajak et al. 2020, the Pb and Cd concentrations of several evaluated mushrooms obtained in the wild environment of Northern Europe significantly exceeded the food safety limits by 30-fold. Similarly, Ronda et al. 2022 found that the consumption of certain wild mushroom species exceeded the limits for Cd, Cu and the radioisotope Cs-137 and thus posed a risk to food safety.

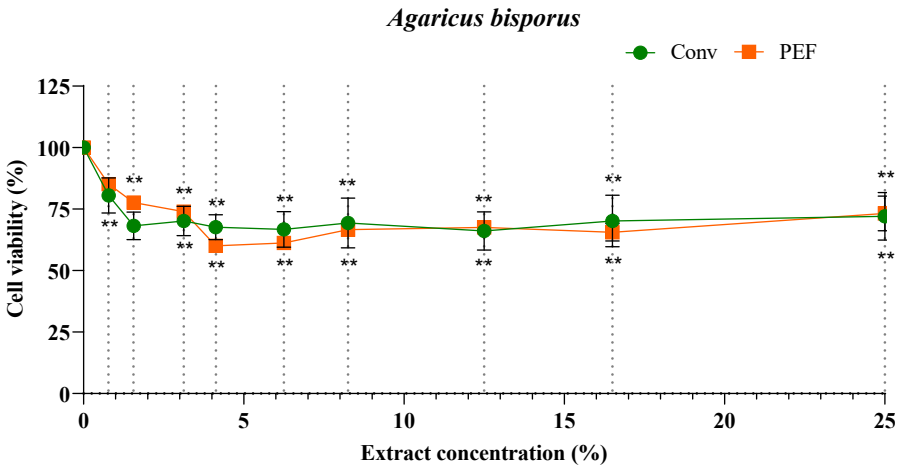
In this sense, the environmental conditions for mushroom cultivation should be considered, as a high concentration of heavy metals has been reported worldwide (Selvi et al. 2019), with metals such as Cu, Cr, Pb, Zn, Ni, Hg and Cd being the most prevalent in the environment and posing a risk to the population. Moreover, they are not only found in cultivated soils, but also in water, rocks and vapors (European Environment Agency 2022; United States Envirnomental Protection Agency n.d). Therefore, quality control in the cultivation of edible mushrooms, which can accumulate very high concentrations of heavy metals, would be necessary to minimize the risks and increase food safety.

### 3.5. Cytotoxicity of extracts obtained by conventional extraction and PEF on CACO-2 cells

Although the primary focus of this work is the chemical characterisation of the different extracts obtained using conventional extraction or PEF from five different mushroom varieties, we considered it worthwhile to conduct preliminary tests on human intestinal cell cultures, specifically the CACO-2 cell line, derived from colon cancer.

The cell viability (%) of human colorectal adenocarcinoma cells (CACO-2) by MTT assay after exposure to increasing concentrations of extracts of the different mushrooms obtained by aqueous maceration and PEF for 24 hours is shown in Figure 4.

**(a)**

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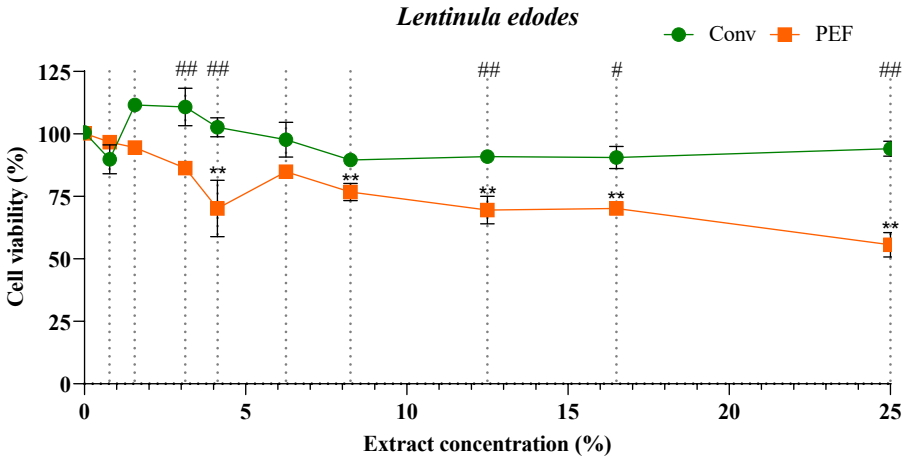
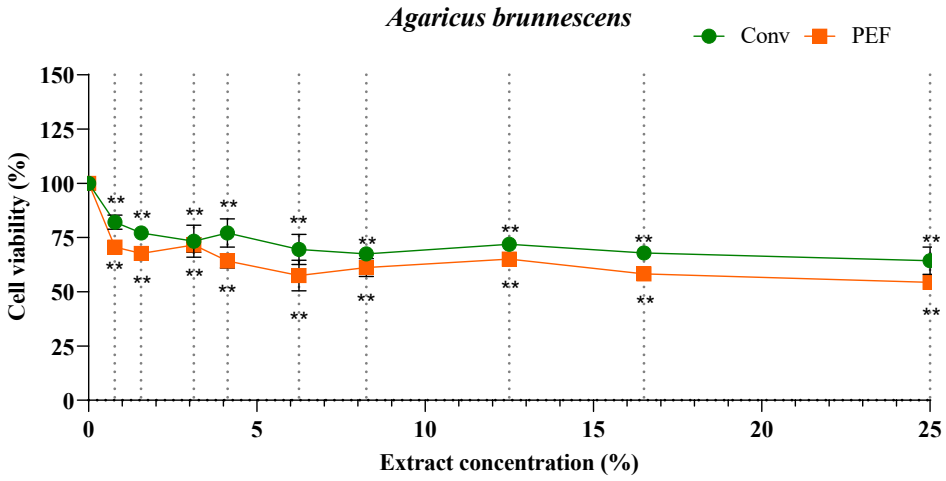
117 **(b)**



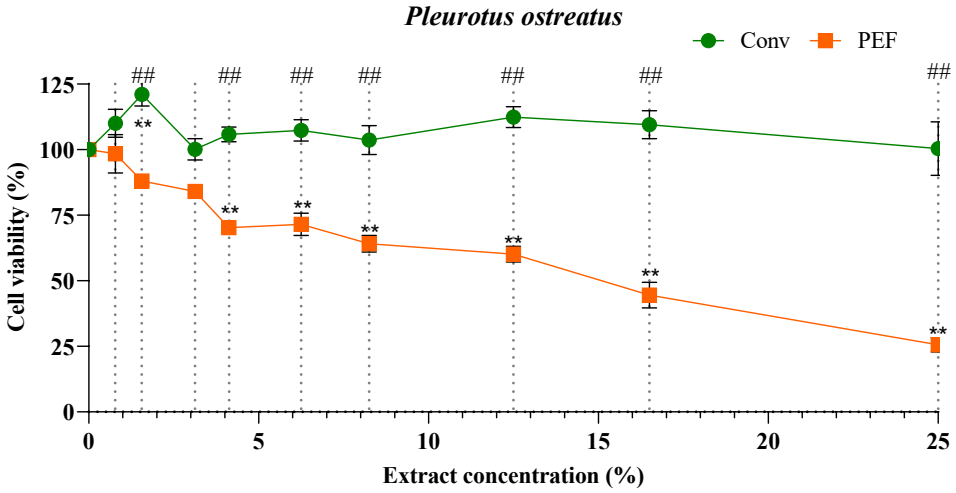

118

119 **(c)**




120

121 **(d)**




(e)

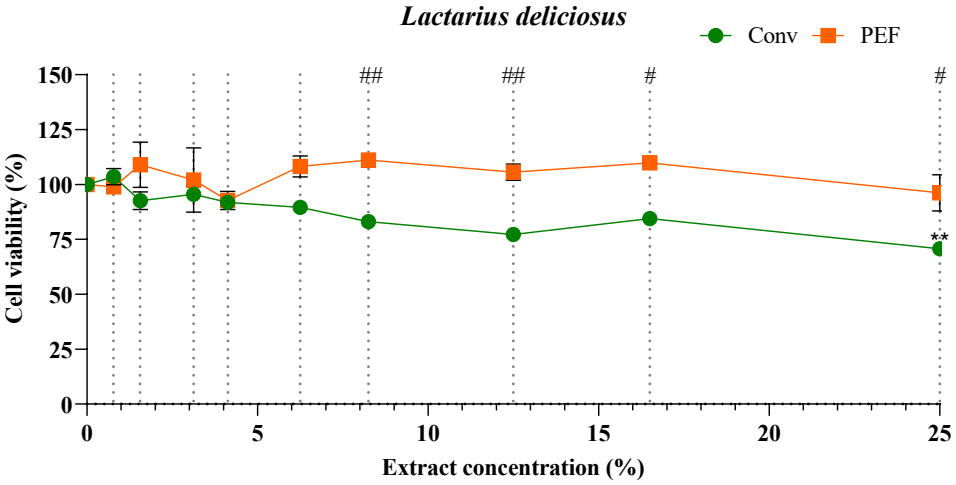



Figure 4. Effect of *A. bisporus* (**a**), *L. edodes* (**b**), *A. brunnescens* (**c**), *P. ostreatus* (**d**) and *L. deliciosus* (**e**) mushroom extracts obtained by aqueous maceration (Conv) and pulsed electric fields (PEF) on cell viability of CACO-2 cells after 24h exposure, at concentrations from 0.78 up to 25 % (v/v in medium). Cell viability was measured using the MTT assay. Results are expressed as mean ± SEM after three replicates per extract. ** = $p < 0.01$ vs. its respective cell control (0% extract); # = $p < 0.05$ between PEF and conventional extracts; ## = $p < 0.01$ between PEF and conventional extracts.

As observed, the extracts obtained from the mushrooms studied by PEF significantly decreased cell viability ($p < 0.01$), even at low concentrations with the exception of the wild mushroom *L. deliciosus* (Figure 2e). This observation was especially notable for *A. brunnescens* and *A. bisporus*, showing a decrease to 54.39 ± 2.06% and 73.26 ± 7.02% at 25% concentration, respectively, whose conventional extracts also showed the same trend. In addition, *P. ostreatus* also stood out, whose PEF extract decreased cell viability strongly as the concentration of the studied extract increased, reaching a cell viability lower than 50% at concentrations higher than 15%. However, the extract obtained by aqueous maceration did not present cytotoxicity in comparison with the control ($p > 0.01$), so significant differences were observed between

methodologies used ($p < 0.01$). In this sense, a slight increase in cell viability was observed in *P. ostreatus* and *L. edodes* at very low concentrations (< 5%) for the conventional extracts, with a significant difference ($p < 0.01$) in the case of *P. ostreatus* at a concentration of 1.56% with a cell viability of 121.10 ± 4.41% (Figure 2d).

Furthermore, a concentration-dependent trend of decrease in cell viability was observed for these cultivable mushrooms, with a clear difference in relation to the methodology used, being the extracts obtained by PEF more cytotoxic than the conventional ones without the influence of the solvent, which showed no significant difference ($p < 0.05$) with respect to the control. Thus, the results suggest that the electroporation produced by PEF and, therefore, the content of bioactive compounds belonging to the mushrooms and present in the extract, may play a fundamental role in the viability of CACO-2 cells.

Several authors have reported on the potential of mushrooms as antitumor agents that exhibit cytotoxicity against various tumor lines by activating different mechanisms. In this context, purified extracts of the mushroom *Pleurotus highking* have been shown to reduce the viability of breast cancer cells by inducing apoptosis by altering the balance of pro- and anti-apoptotic genes and suppressing Akt signaling and exhibiting antiproliferative and antimigrative effects (Haque et al. 2020; Haque and Islam 2019). In addition, extracts of *P. ostreatus* have also induced rapid apoptosis in prostate tumor cells with dose-dependent cytotoxicity (Gu and Sivam 2006). In the study published by Dulay et al. 2022, ethanolic extracts of *Oudemansiella canarii* and *Ganoderma lucidum* mushrooms inhibited cell proliferation between 44.2% and 72.5% of malignant hematologic cells by activating apoptotic markers.

Furthermore, the results of this study agree with those of Durgo et al. 2013, who suggested that different fungal constituents such as carbohydrates and phenolic compounds act synergistically and contribute to the cytotoxic effect observed in different tumor lines. Therefore, and according to the observations of this study, a stronger extraction of carbohydrates by PEF with a significant difference from conventional extraction, which is more evident in *L. edodes* and *P. ostreatus*, could explain the differences in cytotoxicity observed in each of these extracts. In addition, the higher carbohydrate extraction is accompanied by a higher recovery of total phenolic compounds and compounds with antioxidant capacity, which is consistent with a previous study by Calleja-Gómez et al. 2023.

However, as mentioned earlier, these are preliminary studies, and further research is needed to thoroughly investigate the mechanisms behind these differences, both in tumour and non-tumour cells, in order to better understand the observed effects of these extracts on Caco-2 cells and their potential physiological relevance.

## 4. Conclusions

After evaluating the recovery of carbohydrates and determining the mineral and heavy metal profile of *A. bisporus, A. brunnescens, L. edodes, P. ostreatus* and *L. deliciosus* mushrooms, it can be concluded that PEF extraction has an advantage over conventional methods, which do not recover a large proportion of the minerals present in the original matrix, but also do not extract the heavy metals bioaccumulated in the original matrix. Therefore, the cytotoxicity and reduced cell viability observed in the CACO-2 cells is hardly attributable to this heavy metal content due to the low presence of heavy metals in the aqueous

extracts obtained. However, the recovery of carbohydrates would show a correlation with the general trend observed, where the PEF extracts decreased cell viability to a greater extent. In this sense, further research is needed to evaluate the synergy of mushroom components such as carbohydrates and phenolic compounds in decreasing cell viability, as there is a lack of information on the combination of both effects produced by these compounds on cells. However, the application of technologies such as PEF that have been shown to increase the recovery of bioactive compounds from mushrooms would provide a double advantage by producing electroporation that allows this increased recovery without producing thermal degradation of thermosensitive compounds such as phenolic compounds.

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