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Ultrasound intensification of Ferrochelatase extraction from pork liver as a strategy to improve ZINC-protoporphyrin formation

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ABSTRACT

The enzyme Ferrochelatase (FeCH), which is naturally present in pork liver, catalyses the formation of Zinc-protoporphyrin (ZnPP), a natural pigment responsible for the typical color of dry-cured Italian Parma ham. The aim of this study was to evaluate the feasibility of using high power ultrasound in continuous and pulsed modes to intensify the extraction of the enzyme FeCH from pork liver. US application during FeCH extraction led to an improved enzymatic activity and further increase in the formation of ZnPP. The optimal condition tested was that of 1 min in continuous US application, in which time the enzymatic activity increased by 33.3 % compared to conventional extraction (30 min). Pulsed US application required 5 min treatments to observe a significant intensification effect. Therefore, ultrasound is a potentially feasible technique as it increases the catalytic activity of FeCH and saves time compared to the conventional extraction method.

1. Introduction

Nowadays, the pork industry is facing relevant challenges. Firstly, one of the most significant ones is linked to the large environmental load of animal protein production, which leads to the search for new protein sources with which to complement or replace meat products in the diet [1]. Secondly, the large amount of co-products and by-products generated in the pork industry are of low commercial value and represent a high impact in terms of waste treatment. However, some of these products, such as pork liver, possess high potential from a nutritional and technological point of view [2]. Finally, another relevant issue that affects the cured meat industry is the use of nitrifying agents (E249, E250, E251 and E252), which have a threefold purpose: to achieve the characteristic color in dry-cured meat products due to the formation of nitrosomyoglobin, to inhibit pathogen microorganisms, especially Clostridium botulinum, and to enhance the flavour [3]. Nitrites are responsible for all of these functions, while nitrates can be a source of nitrite through the action of nitrate reductase. However, the use of these chemicals may be controversial because nitrites are precursors of the formation of methemoglobin and nitrosamines, recognized toxic substances [4]. In Italian Parma ham manufacturing, the use of nitrates and nitrites is not allowed and microbial safety is ensured by an extended, 12-month minimum, manufacturing process. Thereby, the typical color of Parma ham is formed without adding nitrates and nitrites due to the formation of Zinc-protoporphyrin IX (ZnPP) which has a characteristic reddish color [5]. There is evidence of ZnPP formation under anaerobic conditions and in the presence of endogenous microorganisms and meat enzymes in pork loin [6]. Laursen et al. [7] and Moller et al. [8] also reported the presence of ZnPP in dry-cured Iberian and Parma ham. Wakamatsu et al. [9] reported that the formation of ZnPP did not take place via the substitution of zinc in the heme group, but via the insertion of this atom into independently-formed Protoporphyrin IX. Therefore, it has to be considered that the formation of ZnPP is strongly influenced by the endogenous formation of Protoporphyrin IX. Benedini et al. [10] found that fresh meat contains an enzyme, Ferroquelatase (FeCH), which promotes the formation of ZnPP in the presence of zinc and Protoporphyrin IX substrates at an optimum pH of 8. FeCH is a protein located in the mitochondria, associated with the inner mitochondrial membrane in meat, which is highly resistant since it remains unaltered during dry-cured ham manufacturing. It has an optimum pH and temperature of 7.5–8 and 37–40°C, respectively.

Pork liver is a co-product of the meat industry with a low market value, whose use has been limited to liver paste products and feed due to a lack of knowledge about its nutritional and technological functionality [11]. The liver, in addition to being a good source of protein, has a large number of enzymes and other valuable compounds that could be

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exploited industrially. Liver protein has special uses as a foaming agent and as a functional ingredient for the supply of nutrients in food [12]. Due to the high concentration of FeCH in the pork liver, its extracts could be used to catalyse the formation of ZnPP from hemoglobin or, when added to meat products, as a way to promote the formation and stability of redness. Previous studies about FeCH extraction had a marked analytical and biochemistry character since they were designed to separate and purify the enzyme for analysing its activity. Thus, Taketani and Tokunaga [13,14] addressed the extraction of FeCH in rat and bovine liver by differential centrifugation from the purified mitochondrial fraction and the homogenized liver, respectively. High FeCH concentrations in the extracts would facilitate not only the further purification steps, if necessary, but also its direct and effective application for in-vivo and in-vitro ZnPP formation. For this purpose, efficient extraction processes must be developed to optimize the release of the enzyme from the internal cellular structures of the liver without being damaged. To our knowledge, this is the first study dealing with the intensification of the extraction process for industrial purposes.

Power ultrasound (US) is a technology that is frequently used to intensify the extraction of natural products, and as a strategy to increase the process rate or to obtain higher yields [15]. In a liquid medium, the main effect linked to ultrasound application is the implosion of cavitational bubbles, which are formed due to the cycles of compression and rarefaction that, at a certain frequency, are provoked by high intensity ultrasound waves [16,17]. Cavitation is characterized by high local heat and mechanical energy release, leading to a temperature rise and great turbulence, which positively induce a more intense solvent penetration, structural alteration and improved mass and heat transfer [18,19]. As for the extraction of the molecules that are tightly attached to the solid matrix, ultrasound improves the cellular lysis that will release the compounds of interest [20]. Both the physical phenomena associated with ultrasound extraction, as well as its performance, are mostly dependent on the process conditions used. As for the optimization of ultrasonic systems, the frequency, intensity, treatment time, shape and size of the vibrating surface of the emitter and of the treatment chamber are all essential, due to their effect on the energy released into the medium. As to the medium-related parameters, the temperature, solvent properties (density, viscosity, air dissolved, etc.), solid/liquid ratio and the nature of the matrix being treated are also key factors in solid–liquid extraction [15]. Power ultrasound represents an alternative to conventional enzyme extraction methods, since it could improve the yield and rate of the process [21,22,23,24,5,26,27]. US technology increased the extraction yield of the pectinase enzyme from the guava shell (Psidium guajava) by 96.2%, also improving the enzymatic characteristics of the extracts [28]. Szabo et al. [29] showed that the application of US in the extraction of ligninolytic and hydrolytic enzymes increased enzymatic activity by 129–413%. However, when applied at high power and for prolonged exposure times, US is also able to cause the alteration of the enzyme structure that can lead to its deactivation [30]. In this regard, the effect of US on the protein structure of duck liver was also reported by Xu et al. [31]. So far, no literature has addressed the intensification of the enzyme extraction process in meat products using US or the extraction of FeCH from pork liver. Therefore, the objective of this study was to evaluate the feasibility of using US to improve the extraction of the FeCH enzyme from pork liver.

2. Materials and methods

2.1. Raw material and sample preparation

The raw material used was pork livers from the slaughterhouse “Carnes de Teruel S.A.” (D.O. Jamón de Teruel, Spain). The pork livers were transported at a temperature of under 4°C and processed in the lab in <2 h. The fresh livers were ground for homogenization (Blixer 2, Robot Coupe, Vincennes Cedex, France), packaged (30 g portions) in vacuum bags (200×300 PA/PE, Sacoliva, Castellar del Vallès, Barcelona) and stored at −20°Cuntil used.

2.2. Ferrochelatase extraction

The process of Ferrochelatase extraction carried out was based on the procedure described by Parolari et al. [32]. Firstly, 4 g of the milled thawed (5 h, 20°C) pork liver were homogenized (Homogenizer DI 25 Basic, IKA, Germany) with 100 mL of extraction buffer for 1 min at 4°C and 8000 rpm, avoiding foam formation, using a 200 mL glass beaker. The extraction buffer contained Tris-HCl 50 mM, Glycerol 20 % (w/v), KCl 0.8 % (w/v) and Triton X-100 1 % (w/v) (Sigma Aldrich), and was adjusted to pH = 8 with NaOH. Conventional extraction (CV) was carried out using a magnetic stirrer (Magnetic Stirrer Hot Plate SM3, STUART, UK), the liver/solvent mixture was placed into a 100 mL glass beaker and extraction was conducted for 30 min. Temperature was kept at 4 ± 2°C to minimize enzyme thermal deactivation during extraction.

As an alternative to the conventional method, once the sample was homogenized, the enzymatic extraction was also performed with ultrasound (US) assistance using a probe-like device (UP400S, HIELSCHER, Germany) supplying the maximum power available (400 W) at a frequency of 24 kHz and using a sonotrode of 2.2 cm in diameter. The liver/solvent mixture was placed into a 300 mL glass jacketed beaker, using a volume of 50 mL of liver/solvent mixture and the tip of the ultrasonic sonotrode was immersed for 1 cm. US was applied for different times (1, 2.5 and 5 min) in continuous (100 % frequency) and pulsed (50 % frequency) operation modes. Pulsed (50 % frequency) US application consisted of on and off pulses of 0.5 s. In this case, temperature control was more critical than in the conventional mode because the cavitation generated by ultrasound could lead to a fast temperature rise. To avoid reaching high temperatures at which the enzyme would be inactivated, a glycol solution (20 %) was pumped (SUK-0220, Shurho, Mexico) at −20°C through the walls of the jacketed-beaker containing the extraction solution. The temperature was controlled by using a K-type thermocouple wired to a process controller (on/off control) (E5CN-R2MT-500, Omron, Japan), which acted on the pump to recirculate the glycol-solution. The control system allowed to keep the temperature at 10 ± 2°C. For each extraction condition (CV and US continuous and pulsed), five replications were carried out.

In order to separate the enzyme fraction, the extraction solution was centrifuged for 10 min at 12500 rpm and 4°C (Medifriger BL-S, SELECTA, Spain) and the supernatant was filtered (Whatman 597, GE LIFE SCIENCE, USA) to be used as the FeCH enzyme extract.

2.3. Zinc-protoporphyrin formation kinetics

Following the experimental procedure developed by Parolari et al. [3032], the enzymatic reaction of ZnPP formation catalyzed by the FeCH was conducted in microtubes incubated at 37 ± 0.5°C in a water bath. The reactants used were as follows: 250 μL of $ZnSO_4$ 400 μM in Tris-HCl buffer 360 mM, adjusted to pH = 8.0, 50 μL of protoporphyrin IX 0.25 mM in Tris-HCl buffer 360 mM, adjusted to pH = 7.0, 200 μL of ATP 25 mM in NaCl at 20 % (w/v), 35 μL of EDTA 50 mM and 300 μL of FeCH enzyme extract from pork liver. With these reagents, two different batches were prepared: samples and blanks. In the batch of blanks, 300 μL of the extraction buffer was added instead of an enzyme extract (no enzymatic reaction). The blank value was subtracted from that of each sample to correct any background fluorimetric signal from the reagents. Microtubes were incubated at different times (0, 15, 30, 45, 60, 90, 105 and 120 min) for the purposes of monitoring the reaction kinetics.

Zinc-protoporphyrin (ZnPP) was quantified as the product of the reaction due to its ability to emit fluorescence (unlike myoglobin and nitrosylmyoglobin) with excitation and emission peaks at around 420 nm and 590 nm, respectively [9]. Fluorescence measurements were taken with an 86-well plate fluorometer (Infinite 200 Microplate Reader, TECAN, Switzerland) adjusted to the previously mentioned range. Before ZnPP quantification, cold absolute ethanol (840 μL) was added to

the microtubes and then centrifuged for 30 min at 13200 rpm and 4°C (5415R, EPPENDORF, Germany).

In order to quantify the ZnPP concentration (μmol/L), a calibration curve was obtained. For that purpose, different dilutions (0 μmol/L – 18 μmol/L) were prepared from concentrated ZnPP (Sigma-Aldrich) using the extraction buffer as dilution medium. The calibration curve (r^2 = 0.995) is shown by Eq. (1).

$$\text{ZnPP} = \frac{\text{F - 1451}}{6502} \quad (1)$$

Where F is fluorescence (RFU) and ZnPP is the concentration of the product formed (μmol/L)

From the increase in the amount of ZnPP, the reaction rate was calculated as the product formation velocity (r) (μmol of ZnPP/L × min) [3133]. As shown in Eq. (2), the velocity at which ZnPP is formed can be defined as the amount of product formed per unit of time.

$$r = \frac{dP}{dt} \quad (2)$$

Where P is the ZnPP concentration (μmol/L) and *t* is the time (*min*).

With the aim of comparing the experimental results obtained with other studies, the reaction rate values can also be expressed as specific enzymatic activity (SEA, nmol of ZnPP/g dry matter × min) by considering the mass of liver used for each extraction.

2.4. Ultrasonic field characterization

In order to characterize the acoustic intensity applied to the solution, the calorimetric method was used [34]. For this purpose, the temperature was measured with a type K thermocouple located in the center of the extraction beaker and recorded with an Agilent 34970A Data Acquisition/Switch Unit (4970 A, Hewlett-Packard Española, S. A., Madrid, Spain). Temperature data were transferred to a computer using proprietary software (Agilent BenchLink Data Logger 3).

The calorimetric measurement was taken in continuous mode (100 % cycle) and in pulsed mode (50% cycle). The experiments involved the measurement of temperature every 0.2 s for the first 2 min of US application. Equation (3) was used to determine the ultrasonic power.

$$P = M\, C_p \frac{dT}{dt} \quad (3)$$

Where P (W) is the ultrasonic power, M (kg) the mass of the solution, Cp (J/Kg °C) the heat capacity (C_p of water was considered) and dT/dt the rate of temperature increase. The ultrasonic power was measured 5 times for every US mode tested.

2.5. Statistical analysis

The influence of the extraction conditions was statistically evaluated by analysis of variance (multifactorial ANOVA) and the differences between the averages were compared by the LSD (Least Significant Difference) intervals. In every case, a significance level of 95 % ($p < 0.05$) was considered. The response, or dependent, variable studied was ZnPP concentration, while the factors, or independent variables, were the reaction time and the extraction mode (conventional, continuous-ultrasound and pulsed-ultrasound). The analysis was performed by using Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA).

3. Results and discussion

3.1. Conventional FeCH extraction

Regardless of the procedure used for the FeCH extraction (conventional, CV or ultrasound-assisted, US), the ZnPP formation kinetics showed the same pattern (Figs. 1 and 2). A linear, steady phase was preceded by an initial, burst phase. This initial phase is shown by a y-intercept of the linear relationship that is significantly ($p < 0.05$) different from zero (Fig. 1). The burst phase indicates an initial stage in the enzyme reaction at a very high rate, which is related to the first turnover of the active sites [35]. Afterwards, the enzyme reaction enters the steady state phase in which a constant reaction rate is manifested, coinciding with the slope of the linear relationship (Fig. 1). Thereby, and according to the Michaelis–Menten model, the slope of the linear fit for the steady state phase is proportional to the active enzyme concentration (E) and the product release rate constant (K_2), while the y-intercept is only proportional to the active enzyme concentration [36]. The extension of the initial burst phase ranged between 0 and 15 min, but it cannot be precisely assessed from the present experiments since the initial sampling time was 15 min. According to previous literature, the burst phase only generally covers the first seconds of the enzymatic reaction [37]; for this reason, its experimental assessment is, in many cases, extremely complex, and is not this study's aim. To our knowledge, previous literature has not identified the burst phase in ZnPP formation catalyzed by FeCH, since reaction rates have been computed at a single specific time, when the reaction is stopped, without analyzing the kinetic evolution. Thus, [14] reported that the Zn metal bound to protoporphyrin in the presence of bovine liver FeCH followed a Michaelis-Menten model illustrating the influence of the limiting substrate on the reaction rate, although no data is provided about the formation of ZnPP kinetics (ZnPP calculated at 30 min reaction).

If what is considered is only the reaction rate at the steady phase (Fig. 1), the specific enzymatic activity (SEA) was 7.6 nmol of ZnPP/g dry matter × min and the product formed at 120 min of 0.684 μmol/L ZnPP. However, if the burst phase is not considered and the ZnPP formed at 45 min is used to calculate the SEA [38], a value of 18.2 nmol of ZnPP/g dry matter × min is obtained. De Maere et al. [38] reported a higher value of SEA for pork liver (123.81 nmol of ZnPP/g dry matter × min) and a value of 31.51 nmol of ZnPP/g dry matter × min for pork shoulder. Parolari et al. [32] showed enzymatic activities ranging from 4.42 to 0.88 nmol of ZnPP/g dry matter × min throughout the different stages of Parma ham processing (from green to dry-cured hams). On the other hand, in bovine liver, Taketani and Tokunaga [14] obtained a SEA

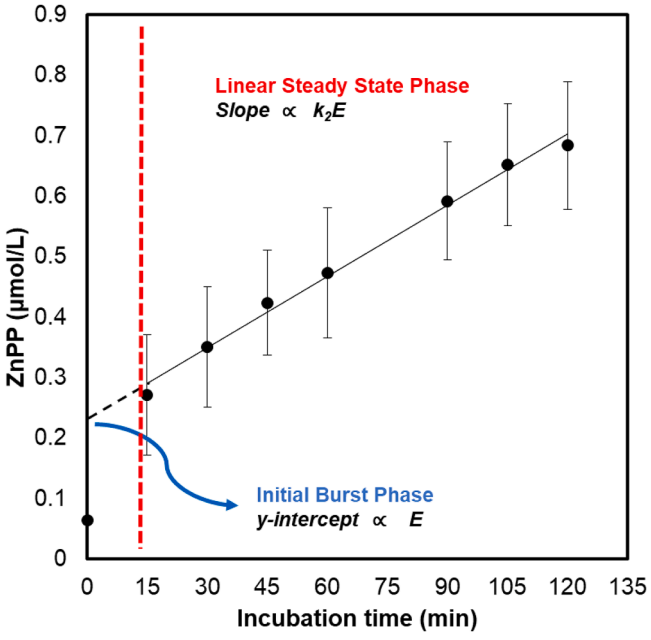

Fig. 1. Kinetics of ZnPP formation using a FeCH extract from pork liver obtained by conventional extraction (CV, 30 min magnetic stirring). Average values ± standard deviation are shown for each experimental time (t).

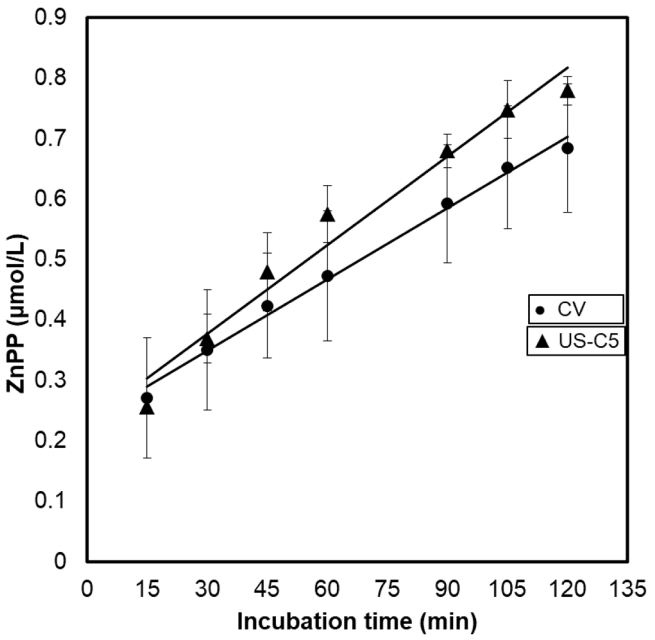

Fig. 2. Kinetics of ZnPP formation using a FeCH extract from pork liver obtained by conventional (CV, 30 min magnetic stirring) and continuous ultrasound extraction for 5 min (US-C5). Average values ± standard deviation are shown for each experimental time (t).

of 18 nmol of ZnPP/h × mg of liver, a value much higher than that obtained in the present study (0.315 nmol of ZnPP/h × mg of liver, for a moisture of 71 gH_2O/100 g liver). This could be due to the fact that the reaction for the ZnPP formation was carried out using purified FeCH, obtained by solubilization, ammonium sulfate fractionation and blue Sepharose CL-6B chromatography while in the present study, an unpurified extract was used. The direct comparison of the values reported in this study and the ones found by De Maere et al. [38] is complex, due to there being different factors, such as differences in the content of endogenous metals, which could affect the reaction rate. In this sense, divalent ions from the endogenous metals (Fe^{2+}, Co^{2+}, Ni^{2+}…) which are present in the mitochondrial membranes can modify the enzymatic activity, since they can serve as substrates, competing against and limiting the formation of ZnPP [39]. In previous studies, ferrous ion (Fe^{2+}) was already reported to lead to a competitive inhibition of the Zinc-chelatase activity and a decrease in the ZnPP formation catalyzed by FeCH from *Saccharomyces cerevisiae* [40]. In this sense, Camadro et al. [39] postulated that the FeCH capacity to synthesize ZnPP in human liver may be affected by the mitochondrial Fe^{2+} reserve, which leads to the formation of Ferro-protoporphyrin instead of ZnPP and confirmed that Protoporphyrin IX consumption differed from the ZnPP formation. The influence of Fe^{2+} was also evidenced by Nunez et al. [41], who showed that when 2,2′-bipyridine was used to reduce endogenous Fe^{2+} without affecting Zn^{2+}, FeCH activity, in terms of ZnPP production, was increased [42,43]. Finally, sample heterogeneity should also account for the differences in enzymatic activity; thus, Benedini et al. [10] reported that the variation of FeCH concentration in pork loins reached 66 %, which was linked to different factors, such as the breed, feeding or slaughter technique.

3.2. Comparison between conventional and ultrasonically-assisted FeCH extraction

Fig. 2 compares the reaction kinetics of ZnPP formation using FeCH extracts obtained by two modes of extraction: conventional (CV, 30 min, agitation) and continuous, ultrasound-assisted for 5 min (US-C5). Ultrasound assistance during FeCH extraction did not alter the linear pattern found in the reaction kinetics. The CV and US-C5 linear fits presented similar y-intercepts but the use of ultrasound significantly ($p < 0.05$) increased the slope, from 0.0039 to 0.0049 µmol of ZnPP/L × min (Table 1). These facts confirm that the same amount of enzyme was extracted in both CV and US-C5 procedures, but ultrasound significantly ($p < 0.05$) improved the performance of the enzyme. Thereby, the reaction rate was 25.7 % higher for the FeCH extract obtained with ultrasound assistance (Table 1), which amounts to a difference of 13.9 % (0.096 µmol/L ZnPP) in the final ZnPP concentration at 120 min (Table 1) compared to the conventional method. Therefore, the experimental results highlighted that the enzyme extracted with ultrasound behaved differently to the one obtained by the conventional stirring procedure. This implies that ultrasound is inducing some conformational modification in the enzyme, improving its performance. No previous studies have addressed the influence of ultrasound on the extraction of FeCH but there is a wide number of applications for other enzymes. Thus, Li et al. [44] studied the effects of the US-assisted extraction of pectinase, endoglucanase and xylanase collected from *Aspergillus japonicus* through solid state fermentation and obtained a maximum enzymatic activity increase of 1.2, 1.48 and 1.3, respectively. Szabo et al. [29] applied US to extract various enzymes from *Trichoderma virens and* the enzymatic activity increased between 1.2 and 4.13 times compared to the conventional extraction mode, depending on the sonication parameters applied. Both previous studies presented similar values for the ultrasonic enhancement of the enzymatic activity to that found in the present study (1.31 increase). Ultrasound has also been applied to improve the extraction of enzymes from microbial cultures [45]. In this sense, Avhad et al. [46] used a 3-phase ultrasonically-assisted extraction partitioning to obtain fibrinolytic enzyme from *Bacillus sphaericus,* achieving 7 times more purity and activity than conventional extraction. Pakhale et al. [47] reported that the application of ultrasound shortened the extraction time of serratiopeptidasa from *Serratia marcescens* to 5 min compared to the 60 min of conventional extraction and increased the enzyme activity. Therefore, previous literature supports the results achieved in this study, since ultrasound may dramatically speed-up the extraction process of the enzyme FeCH and additionally, improve its activity.

3.3. Influence of ultrasonic application time.

In order to evaluate the influence of US application time on FeCH extraction, times shorter than 5 min (1 and 2.5 min) were analyzed (Fig. 3). Thus, the best performance was achieved by applying US for 1 min (US-C1). The maximum product formed was 0.852 µmol/L ZnPP after 120 min (Table 1) and the reaction rate was 0.0052 µmol of ZnPP/L × min (Table 1), which led to an increase of 24.6 % in the maximum product formed and 33.3 % in the reaction rate, compared to the values obtained using the conventional extraction mode (Table 1). Therefore, US extraction for 1 min involved a noticeable shortening in the process

Table 1
Linear fit for steady state phase of ZnPP formation kinetics: slope (b), y-intercept (a) correlation coefficient (r) and µmol ZnPP/L at 120 min.

	a (μM *ZnPP*)	b $\left(\frac{\mu mol\ ZnPP}{L \times min}\right)$	r	ZnPP* $\left(\frac{\mu mol}{L}\right)$
CV	0.231 ± 0.094 $_{AB}$	0.0039 ± 0.0001 $_{Y}$	0.995	0.684
US-C1	0.262 ± 0.083 $_{A}$	0.0052 ± 0.0013 $_{Z}$	0.991	0.852
US-C2.5	0.180 ± 0.072 $_{BCE}$	0.0051 ± 0.0008 $_{Z}$	0.993	0.786
US-C5	0.229 ± 0.110 $_{AB}$	0.0049 ± 0.0015 $_{Z}$	0.975	0.779
US-P1	0.155 ± 0.021$_{C}$	0.0034 ± 0.0004 $_{X}$	0.991	0.565
US-P2.5	0.160 ± 0.036$_{C}$	0.0035 ± 0.0003 $_{X}$	0.992	0.561
US-P5	0.226 ± 0.095 $_{AB}$	0.0043 ± 0.0007 $_{Y}$	0.984	0.717

For slope and y-intercept average values ± LSD intervals are given.
(A, B, C) and (X, Y, Z) show homogeneous groups established from LSD intervals ($p < 0.05$) for a and b, respectively.
* Final ZnPP concentration at 120 min.

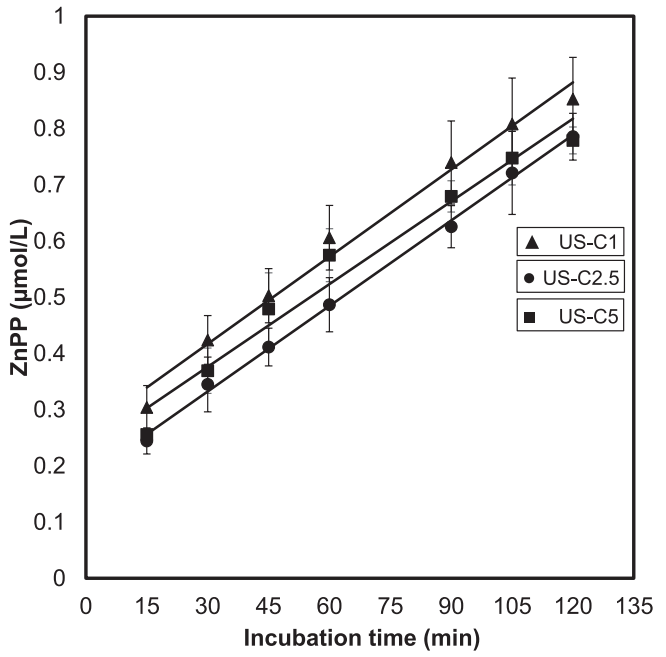

Fig. 3. Kinetics of ZnPP formation using a FeCH extract from pork liver obtained by continuous ultrasound extraction for 1 min (US-C1), 2.5 min (US-C2.5) and 5 min (US-C5). Average values ± standard deviation are shown for each experimental time (t).

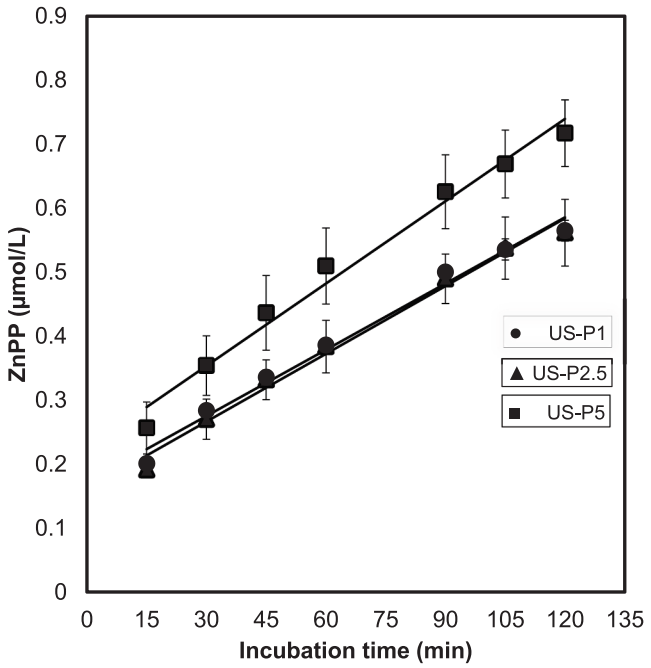

Fig. 4. Kinetics of ZnPP formation using a FeCH extract from pork liver obtained by pulsed ultrasound extraction for 1 min (US-P1), 2.5 min (US-P2.5) and 5 min (US-P5). Average values ± standard deviation are shown for each experimental time (t).

time compared to conventional stirring for 30 min, which is a relevant finding from an industrial point of view.

No significant ($p > 0.05$) differences were found for the y-intercepts or the slopes identified in the linear fits for the US experiments at the different times (Table 1). This suggests that the same amount of enzyme was extracted and it acted in a very similar way. However, that the best performance was achieved with an extraction time of 1 min points to the fact that a prolonged exposure to the ultrasonic energy could lead to the degradation of the enzyme. Ultrasound waves released into the liquid medium at high intensity could cause a disruption to or modification in the structure of the enzymes due to the mechanical and thermal stress produced by the cavitation [48,49].The collapse of the cavitation bubbles generates very high localized temperatures and pressure shock waves [50], which may alter the structure of the enzymes [51]. Previous literature has not addressed a minimum temperature for the enzyme deactivation, but [52] reported that the optimum temperatures for mouse and rat liver mitochondria FeCH were 45 and 50°C, respectively. Thus, there is evidence of protein denaturation at temperatures of over 50°C. Moreover, in barley leaves, FeCH activity was completely destroyed after a treatment of 1 min at 100°C[53].

3.4. Comparison between pulsed and continuous ultrasonic extraction

Pulsed ultrasound application (50 % frequency) (US-P) was tested (Fig. 4) and compared to continuous (US-C) extraction at different times (1, 2.5 and 5 min). Fig. 4A shows the product formation kinetics with enzyme extracts obtained with US-P application at different times. Among the conditions tested, the best performance was achieved with pulsed US application for 5 min. Thus, US-P5 conditions resulted in a maximum product formation of 0.717 (µmol/L ZnPP) (Table 1) due to a more pronounced reaction rate than in the CV experiments, which was evidenced in an increase of 10.3% in the slope of the linear fits (Table 1), while y-intercepts remained similar. However, when US-P was applied for shorter times (1 and 2.5 min), the enzymatic activity for ZnPP formation evolved in a similar way to that in the conventional extracts of FeCH. This indicates that a very small amount of energy was introduced into the medium and did not cause any relevant modifications in the enzyme.

In general terms, pulsed US application was less efficient than continuous, as shown in Fig. 5, in which average product formation kinetics for both application modes are shown for comparison purposes. Thus, pulsed US application involved a decrease in the maximum product formed (0.609 µmol/L ZnPP) compared to continuous US application (0.804 µmol/L ZnPP), as well as in the reaction rate (from

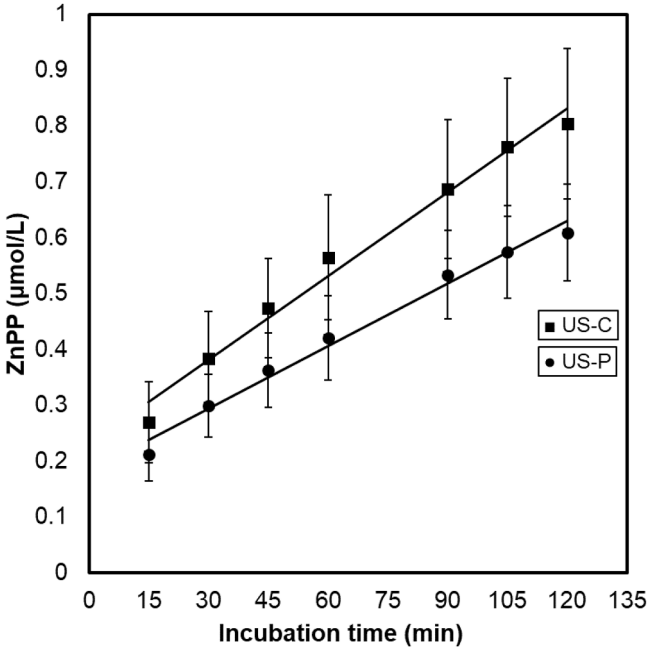

Fig. 5. Kinetics of ZnPP formation using a FeCH extract from pork liver obtained by continuous ultrasound extraction (US-C) and pulsed ultrasound extraction (US-P). Average values for the different US application times (1, 2.5 and 5 min) ± standard deviation are shown for each experimental time (t).

0.0050 to 0.0037 μmol of ZnPP/L × min) (Fig. 5). This is explained by a noticeable reduction in the average power released into the medium and, consequently, in the total energy supplied, which produces less intense effects, such as cavitation and stirring. In this sense, calorimetry tests revealed that pulsed ultrasound application (50% frequency) led to a 65.8% reduction in the power applied (24.6 W in US-P vs 71.9 W in US-C). Therefore, a 50% reduction in the application time involved a proportionally larger reduction in the energy released, since cavitation cycles are interrupted and a high portion of the energy is misspend. The observed differences between US-C and US-P application were dependent on the treatment time (Fig. 6A–C). The longer US was applied during FeCH extraction (Fig. 6C), the smaller the differences between the pulsed and continuous modes. Thus, for an extraction time of 1 min, the average concentration of ZnPP was 33.8 % higher in continuous mode than in pulsed, whereas after 5 min, the concentration was only 7.1 % higher for the continuous method. As is the case for many ultrasound-assisted processes, there is probably an energy threshold value that is necessary to observe the effect of US; thus, until this threshold is not exceeded, no differences could be observed between the US-C or US-P compared to CV. On the other hand, it was necessary to apply 5 min treatments in pulsed mode (7380 J, Table 2) in order to obtain energy values greater than those of 1 min in continuous mode (the optimum time for the continuous US treatment, 4314 J, Table 2), which would explain why the difference between FeCH extraction in continuous and pulsed modes for 5 min treatments was minimal. Moreover, in terms of energy efficiency, which was defined as the increase in the ZnPP concentration (compared to the conventional treatment) divided by the ultrasonic energy applied (Table 2), US-C1 was the treatment showing the highest energy efficiency (0.0389 μM ZnPP/kJ), being 9 times higher than the US-C5 one (0.0044 μM ZnPP/kJ). On the other hand, US-P1 and US-P2 did not improve the ZnPP formation, compared to the CV treatment. Finally, the US-P5 obtained an energy efficiency value similar to US-C5. It is important to remark that the energy efficiency is a relevant aspect to be considered for the use of US in industrial applications.

4. Conclusions

Power ultrasound (US) application has proven to be an effective method for the intensification of Ferrochelatase (FeCH) extraction from pork liver, shortening the process time and improving the further formation of Zinc-protoporphyrin (ZnPP). Thus, the application of US improved the rate of ZnPP formation by up to 33.3 % compared to conventional extraction. Ultrasound did not increase the amount of FeCH extracted but did improve its enzymatic activity. The best US application performance was achieved for 1 min of continuous treatment. It was evidenced that longer US application times of over 1 min could cause enzyme degradation. The pulsed US application system allowed the energy released into the medium to be modulated but, in general terms, led to a worsening of the extraction performance compared to continuous application. Further research should address the optimization of the ultrasonic treatment by exploring relevant process variables, such as the liver-solvent ratio, temperature, pH and power density. In addition, for future industrial applications, continuous US-assisted extraction has to be addressed for residence times of close to 1 min. [25,33].

CRediT authorship contribution statement

B. Abril: Investigation, Formal analysis, Data curation, Visualization, Writing - original draft, Writing - review & editing. **E.A. Sanchez-Torres:** Visualization. **R. Bou:** Conceptualization, Methodology. **J.V. Garcia-Perez:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **J. Benedito:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision, Project administration, Funding acquisition.

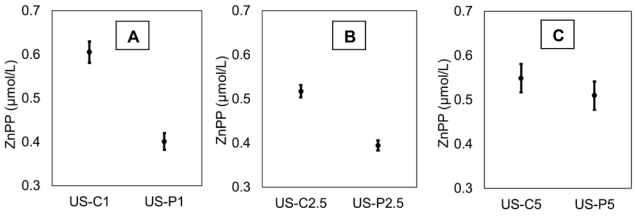

Fig. 6. (A) Analysis of average values of ZnPP concentration and LSD intervals from multifactorial ANOVA (A) by continuous ultrasound extraction for 1 min (US-C1) and pulsed ultrasound extraction for 1 min (US-P1). (B) by continuous ultrasound extraction for 2.5 min (US-C2.5) and pulsed ultrasound extraction for 2.5 min (US-P2.5). (C) by continuous ultrasound extraction for 5 min (US-C5) and pulsed ultrasound extraction for 5 min (US-P5).

Table 2
Energy applied and energy efficiency in ultrasound applications.

Time (min)	Energy applied (J)		Energy efficiency* (μM ZnPP/kJ)	
	US-C	US-P	US-C	US-P
1	4314	1476	0.0389	-
2.5	10,785	3690	0.0095	-
5	21,570	7380	0.0044	0.0045

- No increase compared to the conventional treatment.
* Calculated as the increase in the ZnPP concentration (compared to the conventional treatment) divided by the energy applied.

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.

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