Pulsed electric fields effects on proteins: extraction, structural modification and enhancing enzymatic activity

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Abstract

Pulsed electric fields (PEF) is an innovative physical method for food processing characterized by low energy consumption and short processing time. This technology represents a sustainable procedure to extend food shelf-life, enhance mass transfer, or modify food structure. The main mechanism of action of PEF for food processing is the increment of the permeability of the cell membranes by electroporation. However, it has also been shown that PEF may modify the technological and functional properties of proteins. Generating a high-intensity electric field necessitates the flow of an electric current that may have side effects such as electrochemical reactions and temperature increments due to the Joule effect that may affect food components such as proteins. This paper presents a critical review of the knowledge on the extraction of proteins assisted by PEF and the impact of these treatments on protein composition, structure, and functionality. The required research for understanding what happens to a protein when it is under the action of a high-intensity electric field and to know if the mechanism of action of PEF on proteins is different from thermal or electrochemical effects is underlying.

Keywords: Pulsed electric fields, Protein extraction, Protein modification, Enzymatic activity

23 **Introduction**

24 Proteins are remarkably complex long-chain molecules consisting of amino acid
25 residues linked by peptide bonds, likely constituting the most versatile compound found
26 in food. From a nutritional standpoint, proteins are macronutrients essential in relatively
27 substantial quantities to support bodily growth and repair, furnish energy, and supply
28 crucial amino acids that must be obtained from the diet since the human body cannot
29 produce them[1]. Proteins are present in a diverse array of traditional foods,
30 encompassing animal protein sources like meat, dairy, fish, and eggs, as well as plant
31 protein sources such as beans, lentils, and nuts. Beyond their nutritional contributions,
32 proteins serve as fundamental ingredients in the food industry, leveraging specific
33 functional properties that facilitate processing and form the foundation for product
34 performance [2]. The primary functional properties of proteins include gelation,
35 emulsification, foaming, water binding, coagulation, and flavor-binding capabilities.
36 These attributes, influencing protein behavior during food processing, arise from
37 complex interactions among protein composition, structural conformation,
38 physiochemical properties, and other components in the food matrix [3].

39 Enzymes, which catalyze chemical reactions, are proteins synthesized by all living
40 organisms. Frequently, endogenous enzymes within foods accelerate chemical
41 transformations leading to alterations in flavor, color, and texture, thereby diminishing
42 the overall edibility of the food [4]. Nevertheless, these enzymes also serve a
43 technological role by expediting various reactions in the processing of food
44 commodities or ingredients [5].

45 The configuration of protein structures hinges on the sequence of amino acids and the
46 chemical bonds within both the polypeptide backbone and the side chains of amino

47 acids. From a structural perspective, proteins exhibit four levels of organization:
48 primary, secondary, tertiary, and quaternary. The structural arrangement of a protein
49 plays a pivotal role in its functionality, as it determines the protein's ability to interact
50 with other molecules. Any alteration in the shape of the protein at any structural level is
51 termed denaturation. Denatured enzymes may lose their functionality, while,
52 conversely, the functional properties of proteins may necessitate the maintenance of
53 their structure or a previous denaturation [6].

54 Food processing significantly influences the physical, chemical, and functional
55 characteristics of proteins. Throughout this process, the intrinsic interactions among
56 amino acids may be altered, resulting in protein denaturation. Additionally, amino acids
57 may engage in reactions with other components present in the food matrix. These
58 reactions can lead to diminished bioavailability of amino acids, potentially reducing
59 digestibility [7]. However, they also contribute to color and flavor development in various
60 foods, exemplified by processes such as the Maillard reaction [8]. Furthermore,
61 endogenous enzymes in raw materials or commercial enzymes used during processing
62 may induce proteolytic events, breaking down proteins into peptides and amino acids
63 [9,10].

64 In recent years, innovative food processing technologies such as pulsed electric fields
65 (PEF) have emerged with the aim of efficiently manufacturing and preserving food,
66 thereby reducing energy costs and enhancing the sustainability of the food sector [11]. The
67 main goal of employing PEF in food processing is to induce electroporation in
68 microbial cells as well as cells from vegetable and animal tissues. This aims to achieve
69 microbial inactivation, enhance mass transfer, or improve process efficiency by
70 inducing alterations in the textural and rheological properties of the products [12].

71 PEF treatment is typically applied throughout two metallic electrodes that are in direct
72 contact with the liquid food product or with the liquid in which solid products are
73 submerged. To generate the necessary electric field for inducing cell electroporation, a
74 voltage of thousands of volts must be applied between the electrodes [13]. This process
75 involves the passage of electrical current through the product being treated. The current
76 that flows in the electrodes consists of free electrons and in the material placed between
77 the electrodes of charged particles such as ions [14].

78 The impact of different stresses such as heating acidification, pressure etc., imposed
79 during food processing on protein composition, structure, and functionality has been
80 extensively explored and is currently well-understood [15–17]. However, it is necessary a
81 more comprehensive understanding of the effect of PEF on the functional and biological
82 properties of proteins.

83 This paper seeks to provide a critical review of the recent research conducted on the
84 effect of PEF in protein processing including protein extraction, its impact on
85 physicochemical and functional properties and its effect on enzyme activity. Those
86 aspects need to be more deeply investigated to understand what happens to a protein
87 when it is under the action of a high-intensity electric field are highlighted.

88 Fundamentals of pulsed electric fields processing

89 PEF technology constitutes a physical treatment method involving the intermittent
90 application of high-voltage (kV) pulses of a duration ranging from microseconds to
91 milliseconds to a product positioned between two electrodes. The applied voltage
92 generates an electric field, the intensity of which is contingent upon the gap between the
93 electrodes [11].

94 The key process parameters defining the efficiency of a PEF treatment include the
95 electric field strength, the processing time (which relies on the number of pulses
96 delivered and the pulse width) and the pulse frequency, denoting the number of pulses
97 administered per second (**Figure 1**). Another key parameter is the total specific energy
98 (kJ/kg) dispensed in the treatment chamber to generate the electric field which is
99 determined by the applied voltage, processing time, and the electrical resistance of the
100 treatment chamber that depends on its dimension and conductivity of the product.
101 Despite being a non-thermal technology, PEF treatment is associated with an increase in
102 the sample's temperature due to the conduction of an electric current through the food
103 material with conductive properties (Joule effect) [18].

104 The main effect of the application of an external electric field to a biological material is
105 the electroporation phenomenon. Electroporation consists of the formation of local
106 defects or pores in the lipid bilayer of the cytoplasmic membrane enveloping the
107 cytoplasm of eukaryotic or prokaryotic cells. Consequently, the cytoplasmic membrane
108 becomes permeable to molecules that would otherwise be unable to traverse it in its
109 intact state [19]. Depending on the intensity of the treatment applied (electric field
110 strength, processing time, specific energy) and cell characteristics (size, shape,
111 orientation in the electric field), the electroporation of the lipid bilayer can be either
112 reversible or irreversible [13]. It is reversible if the bilayer returns spontaneously to its pre-
113 breakdown state by recovering membrane integrity. If structural changes in the lipid
114 bilayer due to PEF treatment are permanent, electroporation is irreversible.

115 For electroporation to occur in a cell subjected to an external electric field, the
116 transmembrane potential must surpass a specific threshold, resulting from the build-up
117 of oppositely charged ions on either side of the non-conductive membrane. The
118 transmembrane potential induced by an external electric field varies based on factors

119 such as cell size and the intensity of the applied electric field. As a result, the critical
120 electric field strength necessary to induce electroporation in larger cells, such as those
121 found in plant or animal tissues (0.5–1 kV/cm), is lower than that required for microbial
122 cells (10–15 kV/cm) [20,21].

123 The electroporation of microbial membranes results in the loss of their selective
124 permeability, leading to microbial death at temperatures lower than those utilized in
125 traditional thermal processing [22–24]. Additionally, electroporation of the cytoplasmic
126 membrane proves to be an effective method for enhancing mass transfer phenomena
127 across cell membranes. This process finds applications in various food industry
128 operations, aiming to obtain specific intracellular compounds of interest such as
129 proteins or to remove water from foods through drying [25,26]. Alterations in the textural
130 and rheological properties of products, where the structure is largely dependent on cell
131 integrity may be achieved by irreversible electroporation of cells of food material. The
132 modification of textural properties in plant and animal tissues serves as the basis for
133 diverse PEF applications, including reducing the energy required for cutting, facilitating
134 the peeling of fruits and vegetables, and tenderizing meat [27–29].

135 The primary objective of processing through PEF is to generate an electric field of
136 enough electric field to cause cell electroporation to achieve microbial inactivation,
137 enhance mass transfer or modify the food structure. However, generating a high-
138 intensity electric field necessitates the flow of electric current from one electrode to
139 another through the treated product. This current flow may lead to undesirable side
140 effects such as electrochemical reactions, electrophoresis-based phenomena, and an
141 increase in the sample's temperature [30]. These side effects may result in electrode
142 fouling due to the formation of a particle film on the electrode surface, migration of
143 electrode material, and modification of components, that may impact the nutritional,

144 organoleptic, or functional properties of the processed product [31,32]. On the other hand, 145 the lack of homogeneity in the distribution of the electric field in the treatment zone 146 may cause over or under-processing or dielectric breakdowns [33]. Consequently, any 147 study aiming to understand the effect of PEF on protein processing requires a correct 148 definition of the processing conditions (electric field strength, pulse width and 149 frequency, total specific energy, etc), to reduce the unwanted effects caused by the 150 current flow and an appropriate design of the treatment chamber [34]. These issues are 151 essential to prevent methodological artefacts and to minimize the unwanted effects that 152 may occur during the application of PEF.

153 Extraction of proteins assisted by PEF

154 The escalating demand for total dietary protein, driven by the ongoing global population 155 growth, necessitates the exploration of sustainable and nutritious protein sources. This 156 exploration extends to alternative sources such as microorganisms, insects, seaweed, 157 microalgae, and by-products generated during food processing [35]. Typically, these non-158 traditional protein sources require prior protein extraction to enhance digestibility and 159 bioavailability.

160 The efficiency of protein extraction is augmented through cell disruption methods. 161 Mechanical grinding, osmotic shock, high-pressure homogenization, ultrasound, and 162 enzymatic hydrolysis represent various physical and chemical techniques employed for 163 this purpose [36]. While these methods effectively release proteins from cellular 164 compartments by inducing complete cell disruption, they often result in non-selective 165 extraction of compounds and micronization of cellular debris. This undesired outcome 166 impedes the attainment of a pure protein-rich extract. In contrast, PEF increases the 167 permeability of the cytoplasmic membrane through electroporation, facilitating protein

168 extraction without disintegrating the cell structure at a minimum processing time and
169 energy consumption when compared to other techniques [37].

170 Variables such as the strength of the electric field and the duration of treatments
171 significantly influence the extraction process [38–40]. Furthermore, extraction conditions
172 following PEF treatment, such as pH, temperature, or extraction time, have been found
173 to impact protein extraction efficiency [41–43]. Due to the huge number of factors
174 influencing extraction assisted by PEF, optimizing protein extraction conditions should
175 involve the use of response surface methodology, allowing for the simultaneous
176 optimization of individual factors along with their possible interactions, rather than the
177 more time-consuming one-factor-at-a-time approach typically used [44].

178 The interest in the application of PEF to improve the extraction of proteins from
179 microbial cells, especially yeast and microalgae, is growing (**Table 1**). The cytoplasm
180 of microorganisms contains proteins of varying molecular weights and structural
181 conformations. Consequently, the size of pores generated by PEF treatment
182 significantly influences the extraction process. Using various, fluorescent-labeled
183 dextran molecules with different molecular weights, it has been observed that the radius
184 of membrane pores may range from 0.77 to 5.11 nm, depending on the electric field and
185 the duration of the pulses [45]. This finding, coupled with the fact that PEF-induced pores
186 allow access to the cytoplasm without disintegrating the cell wall, offers the possibility
187 of sequentially extracting peptides and proteins based on their molecular weight. During
188 the initial moments after the application of the treatment, amino acids and small
189 peptides are released from the cytoplasm, while macromolecules like proteins and
190 nucleic acids are retained inside the cytoplasm [46]. Extending the incubation time is
191 required for efficient protein release. Consequently, an electroporated cytoplasmic
192 membrane could be used as an ultrafiltration unit for separating compounds of different

193 molecular weights. Combining treatments that cause membrane pores of different sizes
194 with extended incubation times could permit obtaining protein fractions of varying
195 molecular weights [47].

196 Yeast has become one of the most utilized hosts to produce recombinant proteins for
197 industrial and pharmaceutical use. Recently, the applicability of PEF for improving the
198 extraction of recombinant proteins accumulated in the cell cytoplasm from
199 *Saccharomyces cerevisiae*, *Pichia pastoris*, or *Hansenula polymorpha* has been
200 evaluated [25,48,49]. On the other hand, the efficiency of PEF has also been demonstrated in
201 cases where recombinant proteins are secreted by the yeast into the growth media.
202 Electroporation of the yeast biomass once the production process has finished allows
203 the extraction of proteins that remain in the cytoplasm, improving the yield of the
204 process [50]. Large intracellular proteins may require subsequent incubation with lytic
205 enzymes to increase cell wall porosity for efficient recovery [25].

206 The possibility of inducing reversible electroporation by applying PEF treatments of
207 moderate intensity opens the doors to designing more eco-friendly biotechnological
208 processes. This approach requires adjusting the intensity of the PEF treatment to
209 reversibly increase the permeability of the cytoplasmic membrane, facilitating the
210 release of proteins from the cytoplasm without killing the cells. This approach has been
211 demonstrated for the biocompatible extraction of proteins from the microalgae
212 *Haematococcus pluvialis* and *Chlorella vulgaris* [40,51].

213 PEF technology also emerges as an innovative tool capable of embodying the principles
214 of a circular economy by facilitating the extraction of proteins from by-products
215 generated in the food industry that represents a significantly underutilized source of
216 protein. Larger animal and plant cells require lower electric field strength for
217 electroporation compared to microbial cells. PEF, coupled with aqueous extraction

218 enabled obtaining high-purity protein extracts from complex animal and vegetable by-
219 products matrices [27,37]. The energy efficiency and scalability of PEF in reclaiming
220 proteins from chicken waste has been highlighted [52]. Compared to traditional methods,
221 PEF consumed significantly less energy, positioning it as a more sustainable and
222 environmentally friendly alternative. The scalability of PEF enhances its potential for
223 widespread adoption in industrial protein extraction processes. Mirroring its success in
224 meat processing, PEF's versatility is further demonstrated in the valorization of fish
225 processing by-products such as fishbone or viscera isolating collagen and proteins with
226 improved emulsifying properties compared to conventional enzymatic methods [53]. In
227 emerging protein sources such as insects, PEF enables enhanced cell permeabilization
228 and biomass disintegration, facilitating the production of high-protein, dry, defatted
229 insect-based food while maintaining protein integrity [54].

230 PEF represents a useful tool as a pre-treatment to improve the extraction of proteins
231 from alternative sources and by-products of the food industry. The scalability of PEF
232 enhances its potential for widespread adoption in industrial protein extraction processes.
233 However further studies on the optimization of extraction conditions to design efficient
234 and reproducible extraction processes for industrial applications accompanied by the
235 evaluation of the impact of extraction on subsequent purification operations are
236 required.

237 Effect of PEF on structure and functional properties of proteins

238 The exploration of PEF for altering protein structure and, consequently, functional
239 properties has attracted considerable attention. Investigations encompass both animal-
240 plant and plant-based proteins, with studies aimed at comprehending the mechanisms
241 underlying the effects of electric fields on proteins isolated from diverse food sources.

242 Whey proteins, caseins, ovalbumin, and soybean protein isolates have frequently served
243 as models for assessing the impact of PEF on protein structure and functionality (**Table**
244 **2**). As shown in the table, different studies have found that PEF may modify the
245 physicochemical and functional properties of proteins by improving solubility, water-
246 holding capacity, emulsifying, foaming, and gelation properties. Fundamental studies
247 conducted with sophisticated techniques such as Raman, FTIR, or fluorescent
248 spectroscopy conclude that PEF may alter the secondary and tertiary structure of
249 proteins by reducing the α-helix content and loss in β-sheets [55–57]. Polarization of
250 protein molecules, exposure of hydrophobic amino acids to solvents, and the absorption
251 of energy by polar groups of proteins that may generate free radicals capable of
252 affecting interactions among protein molecules have been hypothesized as the primary
253 effects of PEF on proteins [29,58].

254 The aforementioned PEF effects on proteins that may cause modifications in surface
255 hydrophobicity, protein denaturation, and protein aggregation affecting functional
256 properties are similar to those caused by thermal or chemical effects [29,59]. Generally, the
257 modification of the physicochemical and functional properties of proteins requires the
258 application of very intense PEF with an electric field strength and duration much longer
259 than those required for electroporation of cell membranes (**Table 2**). Under these
260 intense treatments, the spatial and temporal distribution of the electric field strength and
261 temperature inside the treatment chamber, especially in continuous flow treatment, may
262 make it difficult to discern the effect of the electric field from other unwanted side
263 effects such as temperature increments and electrochemical reactions.

264 The potential of PEF to alter the secondary and tertiary structure of proteins has led to
265 investigations into the modification of allergenic properties in food proteins. Results on
266 this matter suggest that PEF treatments do not significantly reduce the allergenicity of

267 plant or animal proteins [60,61]. Studies have shown that PEF treatment did not induce
268 structural modifications in allergenic proteins found in peanuts and apples [62]. While the
269 primary allergenic protein in peaches was denatured by a combination of PEF treatment
270 (25 kV/cm) and moderate temperatures (50ºC), in vitro allergenicity remained
271 unaffected [60]. Conversely, it has been observed that the immunoreactivity of egg
272 proteins was only minimally impacted by the application of very intense electric fields
273 (35 kV/cm)[63].

274 Currently, results presented in the literature on protein modification by PEF are
275 challenging to compare and sometimes contradictory. Different experimental
276 approaches used by various authors and the lack of a correct report of treatment
277 conditions make it difficult to replicate experiments in other laboratories. Basic studies
278 aiming to understand the mechanism of action of PEF on food proteins should be
279 conducted with protein isolates using a batch parallel treatment chamber that permits a
280 uniform distribution of the electric field and strictly controlled treatment conditions [64].
281 The extent of electrochemical reactions and temperature increments occurring during
282 PEF treatment depends on the total amount of electrical charge passing through the
283 electrodes during the pulse treatment. In this regard, different strategies, such as keeping
284 the applied voltage, pulse width, and frequency as the minimum value required for
285 generating a sufficient electric field, as well as using an electrode material that
286 minimizes electrochemical reactions, may be effective in significantly reducing the
287 number of electrochemical reactions and temperature increments [34]. The integration of
288 molecular dynamics simulation of the effect of the electric field on protein structure,
289 with numerical simulation techniques that permit the evaluation of the distribution of
290 temperature and electric field strength in the treatment chamber and the occurrence of

electrochemical reactions, could also result in a very useful approach to understanding what happens to a protein when it is under the action of a high-intensity electric field [65].

Therefore, before exploiting the potential of PEF as a tool for improving protein functionality more basic research is needed to understand the exact mechanisms by which the application of an external electric field affects food proteins.

Inactivating, boosting and triggering enzymatic activity by PEF

It is widely acknowledged that endogenous enzymes can expedite undesirable reactions in foods, leading to spoilage [66]. However, the remarkable catalytic efficiency of these ubiquitous biomolecules has been harnessed to enhance processing in the food and biotechnology industries for centuries [67]. While the potential of PEF technology to decrease or impair enzymatic activity has been extensively explored, its ability to positively modify enzymatic activity has received comparatively less attention.

Several studies have demonstrated the efficacy of PEF in inactivating various enzymes that may cause undesirable changes in food attributes such as loss of texture, color, or flavor. **Table 3** presents examples of recent PEF applications for enzyme inactivation in both model systems and foods. It is shown different sensitivities of enzymes to PEF, along with processing conditions such as electric field strength, frequency, pulse width, treatment time, specific energy, temperature, and characteristics of the media in which enzymes are suspended, significantly impact enzyme inactivation. However, as it happens with the effect of PEF on protein functionality, the mechanism of enzyme inactivation by PEF is not yet clearly understood. Destruction of enzymatic activity by PEF has been linked to structural modification and protein unfolding due to changes in the secondary and tertiary structure rather than the primary structure [29]. Similar to the effect of PEF on protein structure in general, the application of very intense PEF

315 treatments is required to significantly impair enzymatic activity by PEF. Although
316 thermal effects can strongly influence the impact of PEF treatment on enzyme
317 inactivation, most studies in the literature do not distinguish between electric field and
318 thermal effects. Some authors have reported that the inevitable increment of temperature
319 that occurs during the application of the intense PEF treatments or the combined effect
320 of the temperature and the electric field are the major reason for enzymatic inactivation
321 [33].

322 PEF technology has been found not only to decrease enzymatic activity but also to
323 enhance it by either boosting or triggering the enzymatic activity. The boosting of
324 enzymatic activity through PEF is associated with structural modifications of the
325 enzyme. However, triggering occurs due to cell electroporation, causing the enzyme to
326 uncouple from cell organelles, facilitating contact with the substrate.

327 The impact of PEF on enzymatic activity can yield opposing outcomes, either
328 stimulating or inhibiting enzymatic activity, depending largely on the electric field
329 intensity. A gradual increase in enzyme activity of α-amylase, β-glucosidase, alcalase,
330 or pectinase with electric field intensity up to 12-15 kV/cm, beyond which the activity
331 declined [68–71]. In contrast, papain activity was inhibited at a relatively lower electric
332 field intensity of 10 kV/cm [72]. This illustrates that the enzyme type and specific
333 conditions of the PEF treatment can substantially influence the enzyme's response.
334 While the precise mechanisms behind the PEF-induced increase in enzymatic activity
335 remain elusive, the mentioned studies suggest that these changes likely result from
336 modifications in the secondary and tertiary structures of the enzymes. These
337 modifications can lead to the creation of additional active sites, alterations in the
338 enzyme's overall globular structure, or modifications of existing active sites. Such

339 changes can also lower the free energy of activation in enzymatic reactions by fostering
340 proper substrate-enzyme orientation [73].

341 The potential of PEF to trigger endogenous enzymatic activity in various cell types has
342 been demonstrated in several studies. Electroporation of the cytoplasmic membrane of
343 *S. cerevisiae* by PEF not only facilitated the release of intracellular compounds but also
344 triggered yeast autolysis, representing the self-degradation of cellular constituents by its
345 own enzymes [74,75]. This effect is attributed to the release of hydrolytic enzymes such as
346 proteases and β-glucanase from plasmolyzed vacuoles after electroporation. The influx
347 of water, a consequence of uncontrolled molecular transport through the electroporated
348 cytoplasmic membrane of the yeast, decreases the osmotic pressure of the cytoplasm,
349 causing vacuole plasmolysis and enzyme release (**Figure 2**).

350 The rapid liberation of mannoproteins from the cell wall of yeast treated by PEF was
351 correlated with the increment of β-glucanase and protease activity in the supernatant
352 containing the electroporated cells (**Figure 2A**) [76]. The potential of PEF to reduce the
353 duration of the "ageing on the lees" step by triggering *S. cerevisiae* autolysis has been
354 demonstrated in both white and red wine [76,77]. On the other hand, the fact that the
355 amount of total amino acids released from yeast biomass treated by PEF along the
356 incubation time was higher than the total content of amino acids determined before the
357 application of the treatment has been associated with the hydrolysis of proteins by
358 endogenous proteases released from plasmolyzed vacuoles (**Figure 2A**) [74,75]. Promoting
359 proteolysis by endogenous proteases during extraction by incubating cells under optimal
360 protease conditions could permit obtaining protein-hydrolyzed compounds like essential
361 amino acids or peptides that possess bio-functional properties such as antihypertensive,
362 antioxidant, and antimicrobial effects [56,78].

363 The extraction of carotenoids from PEF-treated fresh biomass of yeasts and the
364 microalgae using ethanol as a green solvent, was also correlated with triggering esterase
365 activity after electroporation [79–81]. Ethanol was ineffective for extracting carotenoids
366 when the electroporated biomass was suspended in this solvent just after the treatment,
367 as the interaction between ethanol and carotenoids was too weak to disrupt the linkage
368 of carotenoids with the cell lipids. However, ethanol was effective after incubation of
369 the PEF-treated biomass in an aqueous medium for several hours. This effect was
370 correlated with the triggering of esterase activity in the electroporated cells, which
371 hydrolyzed the association of carotenoids with lipids (**Figure 2B**). In this way, the
372 ethanol-carotenoids complex could diffuse across the electroporated cell membrane
373 driven by a concentration gradient. Esterase activity triggered by PEF also resulted in a
374 positive improvement in the extraction of carotenoids from the dry biomass of
375 electroporated cells yeast using as a solvent ethanol or eutectic mixtures [82]. The
376 successful enhancement of the lipid bioaccessibility of *C. vulgaris* biomass by PEF also
377 required incubating the biomass suspension after PEF treatment. Proteome analysis
378 identified four endogenous cell wall-degrading enzymes that may be involved in cell
379 wall lytic activity during incubation after PEF [83].

380 The triggering of the activity of endogenous enzymes enables the extraction of cellular
381 compounds without the need for commercial enzymes and with a minimal
382 environmental footprint for extracting lipophilic compounds, such as carotenoids, from
383 both fresh and dried biomass using eco-friendly solvents like ethanol or eutectic
384 solvents. Consequently, in addition to improving mass transfer through the cytoplasmic
385 membrane, electroporation presents a promising tool to modulate the activity of
386 endogenous enzymes, with wide-ranging applications in the food and biotechnological
387 industries.

388 ## Conclusions

389 PEF represent a valuable tool for the food and biotechnological industry for enhancing
390 the recovery of valuable proteins, improving protein functionality, and inactivating,
391 boosting, or triggering enzymes. These effects may contribute to enhancing food shelf
392 life and quality, as well as improving the sustainability of the food sector by reducing
393 energy costs and contributing to the reuse of byproducts generated during food
394 processing.

395 The mechanisms of action of PEF in improving protein extraction and triggering the
396 enzymatic activity of endogenous enzymes located in the vacuoles are associated with
397 the well-understood electroporation phenomenon. However, broadening its scope as a
398 procedure for modification of the structures and technological functionalities of proteins
399 requires more in-depth studies on the mechanisms involved under appropriately
400 controlled treatment conditions that permit to discriminate of the effect of electric fields
401 from other unwanted side effects that may occur during PEF processing.

402 ## Data availability

403 No data were used for the research described in the article.

404 ## Author Contributions

405 **J. Marín-Sánchez:** Conceptualization, Writing - Original Draft, Visualization. **A.**
406 **Berzosa:** Supervision. **I. Álvarez:** Supervision. **C. Sánchez-Gimeno:**
407 Conceptualization, Writing - Review & Editing, Visualization, Supervision. **J. Raso:**
408 Conceptualization, Writing - Review & Editing, Visualization, Supervision.

Declaration of Competing Interest

The authors affirm that the research was carried out without any affiliations or financial ties that might be perceived as potential conflicts of interest.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGTP 3.5 in order to improve readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Table 1. Recent studies on the application of Pulsed Electric Fields (PEF) for improving protein extraction from microorganisms.

Microorganism	Treatment conditions	Protein extraction after PEF treatment	Reference
Nannochloropsis sp.	20 kV/cm, 4 ms (53.1 kJ/kg)	6% of total proteins (no incubation period).	[84]
Haematococcus pluvialis	3 kV/cm (8 kJ/kg)	8-fold increase in protein extraction after 24 hours of incubation compared to untreated cells.	[85]
Chlorella vulgaris	17.1 kV/cm (111 kJ/kg)	5% of total proteins (no incubation period).	[86]
Chlamydomonas reinhardtii	7.5 kV/cm, 0.5 ms (180 kJ/kg)	23% of total proteins (1 hour of incubation).	[87]
Chlorella vulgaris	20 kV/cm (100 kJ/kg)	5.2% of total proteins (1 hour of incubation).	[88]
Chlorella vulgaris	20 kV/cm, 100 µs (7.76 kJ/kg)	30% of total proteins (24 hours of incubation).	[51]
Arhtrospira platensis	40 kV/cm (122 kJ/kg)	100% of total proteins (6 hours of incubation).	[89]
Chlorella vulgaris	20 kV/cm, 47 µs (150 kJ/kg)	50% of total proteins (24 hours of incubation).	[47]
Arhtrospira platensis	20 kV/cm (100 kJ/kg)	17% of total proteins (3 hours of incubation).	[90]
Saccharomyces cerevisiae	3.3 kV/cm, 12 ms (120 kJ/L)	90% of total proteins (16 hours of incubation).	[46]
Arthrospira maxima	25 kV/cm (100 kJ/kg)	72% of total proteins (2 hours of incubation).	[91]
Haematococcus pluvialis	1 kV/cm, 20 ms (200 kJ/L)	46% of total proteins (45 min of incubation).	[40]
Saccharomyces cerevisiae	20 kV/cm, 240 µs	70% of total proteins (24 hours of incubation).	[48]
Saccharomyces cerevisiae	15 kV/cm, 150 µs (87.7 kJ/kg)	66% of total proteins (24 hours of incubation).	[75]
Hansenula polymorpha	5.85 kV/cm, 21 ms (158.8 kJ/L)	30% of total proteins (2 hours of incubation).	[25]

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Table 2. Recent studies on the effect of Pulsed Electric Fields (PEF) on protein structure and functional properties modification.

Protein	Treatment conditions	Changes con structure	Influence on protein	Reference
Canola protein	35 kV/cm	Secondary and tertiary structures altered by changing α-helices and β-sheets, increasing amount of free sulfhydryl groups and surface hydrophobicity.	Significant increment in solubility, water-holding capacity, oil- holding capacity, emulsifying capacity, emulsion stability, foaming capacity, and foam stability.	[58]
Pine nut protein	5-20 kV/cm	Altered α-helix, β-sheet, β-turn, and random coils of peptides.	Increased antioxidant activity.	[92]
Ovoalbumin	16 kV/cm, 0-2260 μs	The combined effect of metal ions and PEF the surface hydrophobicity and surface tension increased with pulsed time firstly and then decreased.	Enhanced surface properties.	[93]
β-Lactoglobulin	20 kV/cm, 300 μs	Increased α-helix, decreased β-sheet and random coil elements.	Increased tryptic and chymotryptic hydrolysis of β-Lactoglobulin.	[94]
Pea, rice and gluten concentrates	1.65 kV/cm, 100-300 ms	PEF induced unfolding, intramolecular rearrangement, and aggregate formation, altering protein structure.	Increased water and oil holding capacity and solubility in gluten concentrate.	[95]
Ovomucin	10-40 kV/cm	Significant alterations in the primary, secondary, and tertiary structures of the protein.	Changes in the spatial conformation could reduce sensitization.	[96]
Wheat gluten	2.5-12.5 kV/cm, 2-9.0 s ($1.650x10^3$-$2.475x10^4$ kJ/kg)	As field strength increased, α-helix reduced, β-sheet increased, while random coils remained stable.	Significant effect on solubility, water-holding capacity, oil-holding capacity, foamability, foam stability, and emulsion stability.	[97]
Whey protein isolate	10 kV/cm	Surface hydrophobicity, exposed sulphydryl, and total sulphydryl decreased.	Improved succinylation.	[98]
Micellar casein isolate	16 kV/cm, 6-31 μs (24-100 kJ/L)	Induced reorganization in the configuration of the micelle.	Enhanced protein digestibility and peptide formation, potentially enhancing its nutritional value.	[99]

Table 3. Recent studies on enzyme inactivation in model systems and food by Pulsed Electric Fields (PEF).

Matrix	Enzyme	Treatment conditions	Observations	Reference
Sodium-caseinate hydrolysates	Commercial protease Protamex™	14-18.2 kV/cm, 1-90 s	The maximum reductions in enzyme activity were 66% and 72% at 14 and 18.2 kV/cm for 900 and 500 pulses, respectively.	[100]
Unpasteurized sake	α-amylase Acid carboxypeptidase	40 kV/cm, 1-9 ms	PEF caused slight inactivation of α-amylase. Acid carboxypeptidase was inactivated by PEF at 4ºC but activated at 25ºC.	[101]
Bovine milk	Alkaline phosphatase Xanthine oxidase Plasmin	25.7 kV/cm, 34 µs	Alkaline phosphatase activity decreased by 96-97%, xanthine oxidase activity by 30%, and plasmin activity by 7% after PEF treatment.	[102]
Carrot and apple mashes	Peroxidase (POD) Polyphenol oxidase (PPO)	0.8 kV/cm, 0.5 ms. Pretreatment at 20-80 ºC	PEF treatment at of 20 and 40 ºC had no significant impact on POD activity. PEF treatment at 80 ºC resulted in a 90% reduction in both POD and PPO activities compared to the control.	[103]
Phosphate buffer	Papain	10-13 kV/cm	Maximum inactivation (64%) was achieved using 13 kV/cm, 288 pulses, and a flow rate of 0.2 L/min.	[72]
Apple juice	Polyphenol oxidase (PPO) Peroxidase (POD) Pectin methylesterase (PME)	12.5-40 kV/cm, 100-400 µs	12.5 kV/cm (76.4 kJ/L) resulted in a 36% reduction in PPO activity and a 49% reduction in POD activity. 12.5 kV/cm (132.5 kJ/L) led to the inactivation of PPO, POD and PME by over 90%.	[104]

Figure legends

Figure 1. Main process parameters of PEF technology.

Figure 2. Schematic diagram illustrating the improvement of the extraction of cell compounds by triggering endogenous enzymatic activity through PEF treatment: (**A**) extraction of intracellular and cell wall compounds, (**B**) extraction of lipophilic compounds from fresh biomass using ethanol as a green solvent.

Electric field strength (*E*)

$$E = \frac{V}{d}$$

V: voltage (kV)
d: distance between electrodes (cm)

Treatment time (t_i)

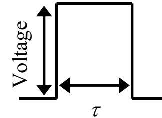
$$t_i = n \cdot \tau$$

n: number of pulses
τ: pulse width (μs)

Frequency (*f*)

f: number of pulses per second (Hz)

Square wave pulse



Specific energy (*W*)

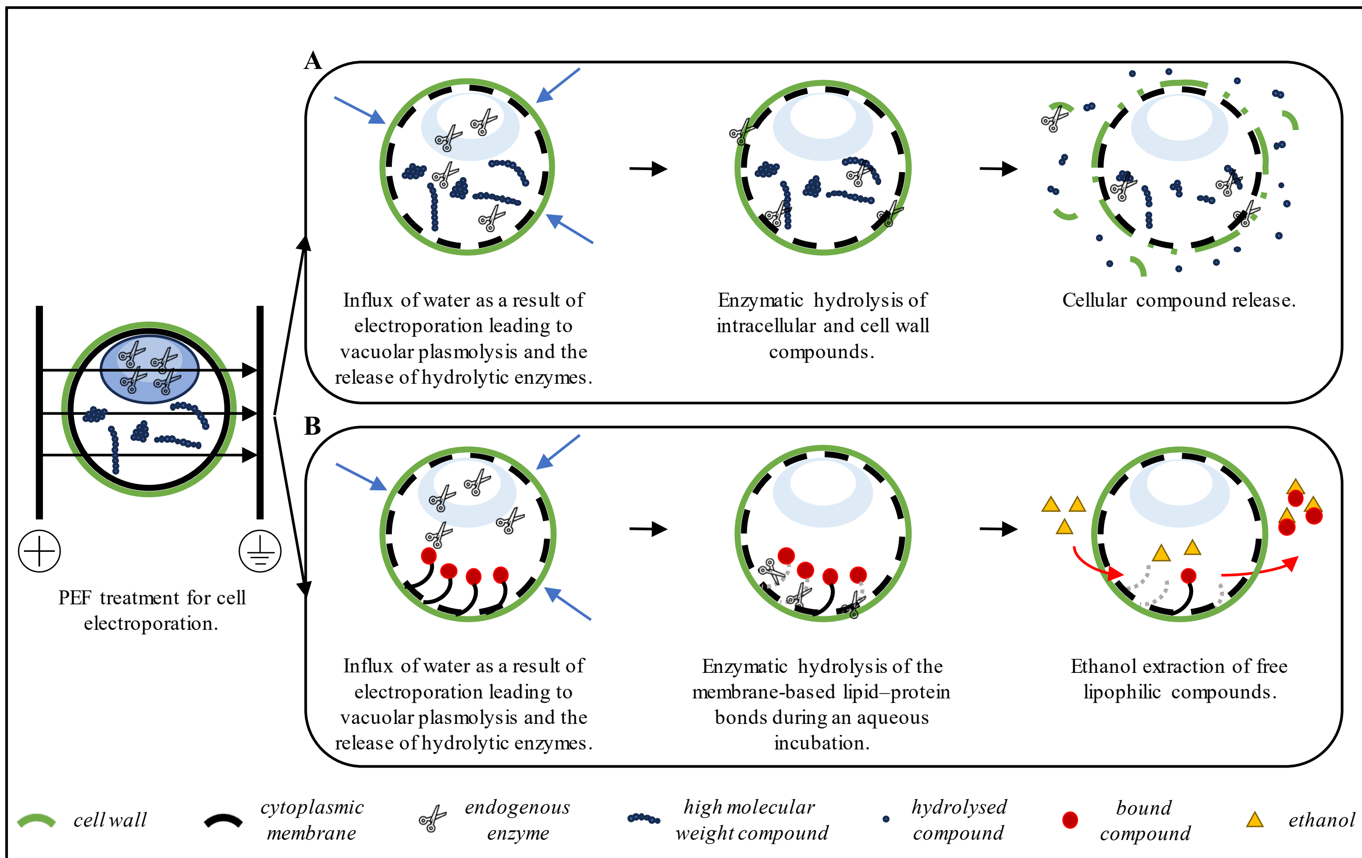
$$W = \frac{1}{m}\int_0^{\infty} \kappa \cdot E^2(t)\, dt = \frac{1}{m}\int_0^{\infty} \frac{V^2}{R}\, dt$$

m: mass (kg)
κ: electrical conductivity (mS cm^{-1})
V: voltage (V)
R: electrical resistance (Ω)

Temperature increment (ΔT)

$$\Delta T_{estimated} = \frac{W}{C_p}$$

C_p: specific heat (kJ kg^{-1} K^{-1})


A
Influx of water as a result of electroporation leading to vacuolar plasmolysis and the release of hydrolytic enzymes.
Enzymatic hydrolysis of intracellular and cell wall compounds.
Cellular compound release.
B
Influx of water as a result of electroporation leading to vacuolar plasmolysis and the release of hydrolytic enzymes.
Enzymatic hydrolysis of the membrane-based lipid–protein bonds during an aqueous incubation.
Ethanol extraction of free lipophilic compounds.
PEF treatment for cell electroporation.
cell wall
cytoplasmic membrane
endogenous enzyme
high molecular weight compound
hydrolysed compound
bound compound
ethanol