



Towards waste meat biorefinery: Extraction of proteins from waste chicken meat with non-thermal pulsed electric fields and mechanical pressing

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ABSTRACT

Meat waste has significant economic and environmental costs in the food supply chain. New approaches for meat waste conversion to products are needed. In this work, we developed a pulsed electric field-based process for functional chemicals extraction from waste chicken breast muscle. We show that a two-step protocol, which consists from high voltage, short pulses followed by low voltage long pulses, with the total invested energy of $38.4 \pm 1.2 \text{ J g}^{-1}$, of initial waste meat, enables extraction of $12 \pm 2\%$ of the initial waste chicken biomass to the liquid fraction. The protein content of the extract fraction was $78 \pm 8 \text{ mg mL}^{-1}$. *In silico* analysis also suggested that the extracted proteins could have antioxidant properties. This was corroborated experimentally with DPPH and ABTS assays. Process parameters analysis with Taguchi methodology showed that long pulse duration played the most important role in liquid extraction from the waste chicken breast muscle, followed by short pulses duration, number of long and short pulses. Our study suggests that pulsed electric fields combined with mechanical pressing can be used for extraction of functional molecules from the waste meat biomass using non-thermal, chemical-free process. Such a strategy could provide an additional income and thus, stimulate farmers and meat processors to reduce waste and waste-related environmental pollution.

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1. Introduction

Population growth leads to the increasing demand for food production. However, approximately 1.3 billion tonnes of food, worth \$780 billion although produced, never reaches the consumer because it gets lost or wasted. Although less meat (20% of production) is wasted in comparison with cereals (30% of production) and root crops (40–50% of production), meat waste has the highest negative environmental impacts estimated by greenhouse emissions (Costello et al., 2016; Tonini et al., 2018).

Although meat processing facilities target to decrease the amount of waste, residuals from meat and meat processing water, are still major environmental challenges (Woon and Othman, 2011). As landfill dumping of large quantiles of meat can lead to surface water contamination, odors and increased greenhouse emission, technologies such as anaerobic digestion, acid hydrolysis, and direct incineration were proposed for waste meat treatment on

site and simultaneously generate energy (Franke-Whittle and Insam, 2013). The shift from solid waste disposal to its use as feedstock for energy production provides an opportunity for the industry to reduce its operating costs (Bujak, 2015), as has been seen in bagasse combustion in sugar and ethanol refineries (Wang et al., 2013). Additional methods have been proposed to recover energy from the waste meat including direct combustion, pyrolysis or gasification in fluidized or fixed bed reactors (Bujak, 2015), pelletizing by immersion frying (Hamawand et al., 2017), and anaerobic digestion to biogas (Ware and Power, 2016) and fat extraction for biodiesel production (Banković-Ilić et al., 2014). However, as has been observed in biofuels systems based on lignocellulose (Arora et al., 2018) and algae (Chew et al., 2017), energy production alone is not always sufficient for the economic viability of the processes; therefore, there is a need to diversify the product range in the processing facilities.

A co-production of energy with other products from the same feedstock is coined biorefinery (Cherubini, 2010). Although in the past decade most of the work has been focused on technologies for vegetation based biorefineries, recent studies suggested the use of the biorefinery concepts for waste meat processing (Okoro et al.,

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2017; Shahzad et al., 2017). However, a major challenge of bio-refineries is to develop a portfolio of energy efficient technologies for products separation from biomass (Mussatto, 2016). One of such technologies is high voltage, non-thermal pulsed electric fields (PEFs) (A Golberg et al., 2016a).

When high voltage PEF is applied on a biological cell, it can lead to the increase of membrane permeability to usually non-permeable molecules, a phenomenon known as electroporation (A Golberg et al., 2016a). Currently, electroporation describes the formation of aqueous pores in the lipid bilayer that enable molecular transport (Kotnik et al., 2012; Spugnini et al., 2007; Weaver and Chizmadzhev, 1996). Thermodynamic considerations led to a current electroporation model, which describes the formation of aqueous pores as started by penetration of water molecules into the lipid bilayer of the membrane. Such a penetration leads to the reorientation of the adjacent lipids with their polar headgroups towards the polar water molecules (Weaver and Chizmadzhev, 1996). Unstable pores with nanosecond lifetimes can form even in the absence of an external electric field, but the presence of an external field induces an additional voltage across the lipid bilayer, reducing the energy required for penetration of water into the bilayer (Weaver and Chizmadzhev, 1996). This activation energy decrease increases both the probability of pore formation and the pores' average lifetime, resulting in a larger number of pores formed in the bilayer per unit of area and per unit of time, with the pores more stable than in the absence of the electric field.

Electroporation-based technologies are used in multiple medical, food and biotechnology applications (A Golberg et al., 2016a; Kotnik et al., 2015; Yarmush et al., 2014). Although the impacts of PEF on the mass transport have been investigated for biomedical application, such as electrochemotherapy and gene electro transfer (Golberg and Rubinsky, 2013; Granot and Rubinsky, 2008), and in the food industry for multiple plant tissues (Knorr, 2018; Puértolas et al., 2012; Vorobiev and Lebovka, 2011), and agricultural plant waste (Poojary et al., 2017), and meat treatment (Alahakoon et al., 2017; Arroyo et al., 2015; Bhat et al., 2018; Cummins and Lyng, 2016; Topfl, 2006).

Previous published works, recently reviewed in (Arroyo and Lyng, 2016), showed the impact of PEF on meat structure and properties and on the enhancing of mass transport to accelerate drying, accelerate the uptake of molecules substances in marinating/curing processes, enhance the water-binding characteristics through the diffusion of water binding molecule (Arroyo and Lyng, 2016). However, the reports on applications of PEF on animal waste tissues, although proposed by some authors as future application of PEF, are scarce (Gudmundsson and Hafsteinsson, 2001; Rocha et al., 2018). In addition, to be best of our knowledge there are no works that describe the use of PEF to extract proteins from waste meat.

The goal of this work is to use PEF as a new strategy to extract functional proteins from waste meat, to identify the PEF-extractable proteins and identify their functional properties. The chicken breast was chosen as it is a common output of meat processing facilities, which is wasted daily in the production lines because of various reasons, including wrong packaging and contamination concerns. In Table 1 we summarized previous works on processing chicken breast meat. In this work, we show that it is possible to generate an additional to energy products stream during waste meat processing by separation of water-soluble proteins with PEF combined with mechanical pressing. To characterize the extract, we have identified the extracted proteins fraction using liquid-chromatography coupled with mass spectrometry (LC/MS/MS) annotating the peptide sequences using available databases. To further characterize the extracted proteins, we created a ranked, according to the MaxQuant quantification in the extract (Cox and Mann, 2008; Schwahnüsser et al., 2011), list of PEF extracted proteins genes and used *GORilla* to identify enriched Gene Ontology (GO) terms, which consist of three hierarchically structured vocabularies (ontologies) that describe gene products in terms of their associated biological processes, cellular components and molecular functions (Eden et al., 2009, 2007). Finally, we show experimentally that PEF extract has antioxidant properties. Our results provide new information that suggests that PEF can be used for waste meat biorefineries to provide additional, protein-rich, functional products. Such an approach is expected to increase the motivation of farmers and meat processors for waste recycling.

2. Materials and methods

2.1. Meat biomass

The chicken breast meat was purchased in a local supermarket. The meat was kept at 4 °C for 3 weeks to simulate spoiled meat, not useful for cooking (Chouliara et al., 2007). Three weeks were chosen based on the previous study which showed the shelf-life of 5–6 days for an untreated fresh chicken meat kept at 4 °C; this shelf-life was increased to 20–25 days by various packages (Chouliara et al., 2007). After 3 weeks at 4 °C, all the samples were frozen and were thawed at least 2 h before the experiments.

2.2. Pulsed electric field setup for liquid-solid separation of waste meat

The schematic design of the process for liquid from the solid separation of the chicken biomass is shown in Fig. 1a. Digital image of the system is shown in Fig. 1b. The electroporation system consisted of a pulse generator (BTX 830, Harvard Apparatus, MA),

Table 1
Products and processes for chicken muscle processing.

Source	Product	Method of extraction	References
Boneless chicken leg meat	Angiotensin-converting enzyme (ACE)-inhibitory peptides	Blending and hydrolysis with Pepsin	(Terashima et al., 2010)
Spent hen meat hydrolysate	Antihypertensive and Oxidation inhibitory peptides	Blending followed by extraction with alkali/acid and hydrolysis with pepsin/pancreatin	(Udenigwe et al., 2017)
Spent hen meat hydrolysate	Anti-inflammatory peptides	Homogenisation followed by enzymatic hydrolysis	(Yu et al., 2018)
Chicken meat residue	Gelatin (yield of 16.03%)	Acid-alkali process	(Babji et al., 2012)
Chicken breast meat	Angiotensin-converting enzyme (ACE)-inhibitory peptides	Boiling followed by hydrolysis with <i>Aspergillus</i> proteases	(Saiga et al., 2003)
Chicken breast and leg meat	Carnosine (Antioxidant properties)	Homogenisation, Acid hydrolysis, and extraction ultrafiltration	(Maikhunthod and Intarapichet, 2005)
Extract of chicken essence	Antioxidant properties (prevented oxidation of linoleic acid)	Acid/alkali hydrolysis	(Wu et al., 2005)

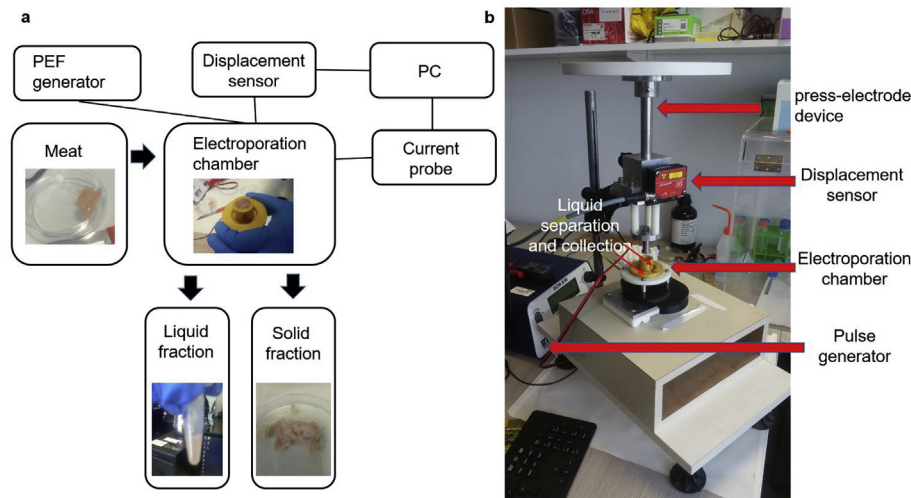


Fig. 1. Pulsed electric field-based process for waste meat biomass fractionation. **a.** Schematic diagram of the process. **b.** Digital image of the custom-made gravitation driven press electrode device.

which delivers square pulses, and a custom-made electroporation cell (Fig. S1) combined with gravitation-press electrode device (1604 g). Currents were measured in vivo using a PicoScope 4224 Oscilloscope with a Pico Current Clamp (60A AC/DC) and analyzed with Pico Scope 6 software (Pico technologies Inc., UK).

The electroporation cell is designed to hold the biomass in the interelectrode space during the application of the pulsed electric field. It also allowed separating the meat biomass into liquid and solid simultaneously with the application of electric fields and mechanical pressure. The electroporation cell consists of a plastic body (Teflon) of a cylindrical shape and the positive electrode, located at the lower part of the cylinder. In the lateral part of the electroporation cell, there are narrow slit-like openings for the outlet of the liquid fraction during the electroporation of the biomass. The extracted liquid is collected and discharged through a groove at the base of the cell (Fig. 1b).

The gravitational press-electrode device is shown in Fig. 1b. This device is needed for the active separation of the waste biomass into liquid and solid fractions. With the help of the free sliding top electrode, an inter-electrode pressure is created, the value of which is established prior to electroporation and is constant during its conduct. At the upper end of the rod is fixed the load-receiving platform and at its lower end a movable negative electrode, which can freely move inside the electroporation cell. A load weighing up to 10 kg can be placed on the load-receiving platform to create the necessary inter-electrode pressure on the biomass. A displacement sensor (optoNCDT, Micro-Epsilon, NC), is installed on the sliding rod to monitor volume change of the biomass during electroporation (Fig. 1b).

2.3. Pulsed electric fields coupled with the mechanical press for liquid-solid separation of the waste chicken biomass

Five gram of chicken breast muscle was cut, weighed and loaded

in the electroporation cell (2.5 cm diameter). The cell was closed with the sliding top electrode of the custom-made pulse generator. The distance between the two electrodes was measured continuously with the displacement sensor. The voltage was applied to the chamber using the custom-made pulse generator. The currents were measured continuously. Immediately after the PEF treatment, the biomass was weighted again. Here we used the PEF protocol which consisted of a combination of high-voltage, short pulses, followed by low-voltage long pulses (Andre et al., 2008; Yao et al., 2017). We used a three-step protocol for extraction as shown in Table 2. The extracted liquid yield (LY) was calculated as appears in Eq. (1).

$$\%LY = \left(\frac{M_{PEF} - M_0}{M_0} \right) \times 100 \quad (1)$$

where M_{PEF} is the weight of the sample (g) after the PEF treatment and M_0 (g) is the initial biomass weight.

2.4. Protein quantification in the crude PEF extract

Protein content was determined by a modified version of Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as a protein standard, as recommended by a previous study that evaluated the different extraction and quantification methods of protein for marine macro- and microalgae (Barbarino and Lourenço, 2005). Briefly, using a 96-well plate, 100 μ L of either diluted samples or diluted standard solutions was added, followed by 200 μ L of biuret reagent (a mixture of 0.5 ml of 1% cupric sulfate with 0.5 mL of 2% sodium potassium tartrate and 50 mL of 2% sodium carbonate in 0.1N NaOH). After extensive mixing by pipetting, the plate was incubated at room temperature for 10–15 min. Next, 20 μ L of Folin & Ciocalteu's reagent (Sigma-Aldrich, Israel) diluted twice (final concentration of 1N) was added and mixed by pipetting. Finally, the

Table 2
Three step protocol used for liquid extraction from waste chicken meat.

Extraction step	Applied Voltage	Pulse duration	Pulses number	Additional Load
1	1000 V	50 μ s	100	–
2	500 V	1 ms	50	2 kg
3	200 V	5 ms	50	2 kg

plate was incubated at room temperature for 30 min before reading the absorbance at 750 nm using a Tecan spectrophotometer (Infinite 200 Pro, Tecan, Switzerland).

2.5. Taguchi orthogonal array to determine the impact of pulse duration and number on the extracted liquid yield

The goal in these series of experiments was to determine the effects of pulsed electric field parameters such as duration of the short pulse (t_s), duration of the long pulse (t_l), number of short pulses (N_s) and number of long pulses (N_l) on the liquid from solid separation yield in the chicken breast muscle. The range of parameters and their combinations is large; therefore, to decrease the number of experiments but still be able to evaluate the impact of each parameter independently, we applied the Taguchi robust design method for the experimental design (Kacker et al., 1991; Taguchi, 1988). The key feature of the Taguchi method is the design of the experiment where process factors are tested with orthogonal arrays (Jiang et al., 2016).

We tested the impact of the following range PEF settings using L9 Taguchi matrix: t_s of 25, 50, 75 μ s, t_p of 2.5 ms, 5 ms, 7.5 ms, N_s of 25, 50, 75 and N_l of 75, 150, 300. The voltage in all experiments was 1000 V for short pulses and 50 V for long pulses. The interval between pulses series was the 30s, delivered with a frequency of 3 Hz. Table S1 summarizes the experiments conducted for the L9 orthogonal Taguchi array which is needed to determine the individual effects of each of the tested parameters on the extracted juice yield. At least 3 repeats were performed for each experimental condition. Analysis for maximizing the best target function, following (A. Golberg et al., 2016), was done using Minitab 18 (Minitab Inc, USA).

In Taguchi design of experiment algorithm, the best parameter setting is determined using signal-to-noise ratio (SN). In our experiments, we used the algorithm of “the larger the better” type. The ratio SN is determined independently for each of the process outcomes (OUT_max) to be optimized (Jiang et al., 2016). In this study, the single process outcome is the mass of PEF liquid extract. Maximizing SN corresponds to obtaining the maximum mass of PEF extract. The ratio SN was calculated by:

$$SN^{OUT_max}(j) = -10 \cdot \log \left[\frac{1}{\#R} \sum_{R=1}^{\#R} \frac{1}{(m_{Rep})^2} \right] \quad 1 \leq j \leq K \quad (2)$$

where K is the number of experiments (in our case $K = 9$; $\#R$ is the number of experiment repetitions (in our case $\#R = 3$) and m_{Rep} is the measurement of the process outcome (OUT) in the specific repetition R of experiment j .

Considering a process parameter P (t_s , t_p , N_s , N_l) and assuming that P has a value of V in $n(P, V)$ experiments. Let $J(P, V)$ be the set of experiments in which process parameter P was applied at level V . Let:

$$SN^{OUT}(P, V) = \frac{1}{n(P, V)} \sum_{j \in J(P, V)} SN^{OUT}(j) \quad (3)$$

be the average ratio SN for concrete level V of parameter P . The sensitivity (Δ) of each outcome (OUT) with respect to the change in a parameter P is calculated as:

$$\Delta^{OUT}(P) = \text{Max}\{SN^{OUT}(P, V)\} - \text{Min}\{SN^{OUT}(P, V)\} \quad (4)$$

Ranking (on the scale of 1–4, where 1 is the highest) was assigned to the process parameters according to the ranges obtained.

2.6. Extracted proteins identification quantification with LC-MS/MS

2.6.1. Proteolysis

The samples were added to 8M urea, 400 mM ammonium bicarbonate, 10 mM DTT, vortexed, sonicated for 5 min at 90% with 10–10 cycles, and centrifuged, based on the protocols in previous reports (Bourdetsky et al., 2014; Levitan et al., 2015; Michael et al., 2017). Protein amount was estimated using Bradford readings. 20 μ g protein from each sample was reduced at 60 °C for 30 min, modified with 37.5 mM iodoacetamide in 400 mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digested in 2 M Urea, 100 mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio, overnight at 37 °C. An additional second digestion with trypsin was done for 4 h at 37 °C.

2.6.2. Mass spectrometry analysis

The tryptic peptides were desalted using C18 tips (Harvard), dried and re-suspended in 0.1% Formic acid. The peptides were resolved by reverse-phase chromatography on 0.075 \times 180-mm fused silica capillaries (J&W) packed with Repronil reversed phase material (Dr. Maisch GmbH, Germany). The peptides were eluted with linear 180 min gradient of 5–28%, 15 min gradient of 28–95% and 25 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 μ L min⁻¹. Mass spectrometry was performed by Q-Exactive plus mass spectrometer (Thermo) in a positive mode using repetitively full MS scan followed by collision-induced dissociation (HCD) of the 10 most dominant ions selected from the first MS scan.

The mass spectrometry data from all the biological repeats were analyzed using the MaxQuant software 1.5.2.8 (Mathias Mann's group) vs. the *Gallus gallus* proteome from the UniProt database with 1% FDR. The data were quantified by label-free analysis using the same software, based on extracted ion currents (XICs) of peptides enabling quantitation from each LC/MS run for each peptide identified in any of the experiments. We also identified the functional groups of the extracted proteins using Gene Ontology (GO) analysis with GOrilla (Eden et al., 2009), annotating the ranked gene list to the mouse genome.

2.7. Antioxidant assays

2.7.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH assay was first reported by Blois (1958) and measures the decoloration of the DPPH radical in the presence of antioxidant radical scavengers. The DPPH radical is considered a stable free radical due to the delocalization of the unpaired electron (Molyneux, 2004). This radical chromogen absorbs strongly within the visible spectrum between 515 and 528 nm. However, when a compound with the potential to transfer an electron, such as an antioxidant, reacts with the DPPH radical it becomes reduced to diphenyl picrylhydrazine (pale yellow) and the absorption of DPPH between 515 and 528 nm is reduced (Brand-Williams et al., 1995) due to the loss of its electron paramagnetic resonance (EPR) free radical signal (Blois, 1958; Papariello and Janish, 1966). This led us to choose the wavelength of 515 nm for our studies on DPPH assay.

DPPH radical scavenging activity of PEF extracted chicken meat liquid was determined according to the method reported by (Goupy et al., 1999) with slight modification for use in a microtiter approach. First, the neat absorbance of the PEF liquid extract (100 μ L) dilutions was recorded at 515 nm using a UV-VIS microplate reader (Tecan, Switzerland).

The dilutions were mixed with 100 μ L of a methanolic solution of DPPH (0.18 mM). The test solution was then thoroughly mixed and allowed to stand in the dark for 30 min. The absorbance of the test solution was then recorded at 515 nm. Methanol was used as

the control instead of the hydrolysate and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard. The DPPH radical scavenging activity (%) of the test sample was calculated by using the following equation (control was devoid of any chicken juice):

$$\%I = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad (5)$$

where A_0 = absorbance of DPPH control reaction minus the absorbance of the methanol blank after 30 min. A_1 = absorbance of the sample reaction minus the neat sample absorbance after 30 min.

2.7.2. ABTS (2,2'-and-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay

ABTS radical scavenging assay was performed according to the method suggested by Owen Kenny and Brunton, 2015 and was slightly modified to for use in a microtiter approach (Kenny and Brunton, 2015). The ABTS^{•+} radical was generated by a solution of ABTS (7 mM) with a solution of potassium persulfate (2.45 mM). The mixture was incubated in the dark for 12–16 h in order to generate ABTS^{•+} radicals. The ABTS^{•+} solution was further diluted to an absorbance of 0.7 ± 0.02 AU, measured using a UV–VIS spectrophotometer (Infinite 200 Pro, Tecan, Switzerland) at 734 nm. The PEF liquid extract ((100 μ L) dilutions were mixed with 100 μ L ABTS^{•+} solution in a microtiter plate and the absorbance was recorded at 734 nm after 6 min of incubation at 30 °C. Water was used as the control instead of the juice and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard. The ABTS radical scavenging activity (%) of the test sample was calculated by using the following equation (control was devoid of any chicken juice):

$$\%I = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad (6)$$

where A_0 = absorbance of ABTS control reaction minus the absorbance of the water blank after 6 min. A_1 = absorbance of the sample reaction minus the absorbance of the water blank after 6 min.

2.8. Statistical analysis

Statistical analysis was performed using R-studio, fitdistrplus, ggplot2 and dplyr packages (RStudio: Integrated development environment for R (Version 1.1.383) [Windows]. Boston, MA).

3. Results and discussion

3.1. Extraction and identification of proteins from the waste chicken muscle biomass

The results from a combined high/low voltage PEF extraction protocol appear in Table 3. Using the described protocol 12 + 2% of the initial waste chicken biomass was extracted to the liquid fraction (0.62 ± 0.11 g of liquids from 5.14 ± 0.16 g of the initial meat biomass). The invested energy was 37.6 ± 4.69 J g^{-1} . The protein

concentration in the crude extract was 78 ± 8 mg mL^{-1} . These results are consistent with previous work which showed that meat waste high content of proteins (Paritosh et al., 2017). However, it is important to mention that multiple inferences to the Lowry quantification could take place in the crude PEF extract. In addition, it is important to mention that using initial material which has a different from 3 weeks at 4 °C storage history could lead to different protein extraction yield and different functional properties of the recovered proteins. The resistivity of the samples reduced from 5.29 ± 0.14 Ohm·cm during the first step of the treatment to 3.71 ± 0.12 Ohm·cm at the end of the process, indicating that the biomass was electroporated (Golberg et al., 2011, 2010; Polikovskiy et al., 2016). The electric field strength at the first step was 60.4 ± 2.5 V mm^{-1} and the invested energy was 28.00 ± 0.50 J. The electric field strength at the second step was 30.9 ± 1.6 V mm^{-1} and the invested energy was 81.67 ± 11.55 J. The electric field strength at the third step was 15.7 ± 0.8 V mm^{-1} and the invested energy was 83.33 ± 5.77 J. The total invested energy in the process was 38.4 ± 1.2 J g^{-1} of initial waste meat and 3.95 ± 0.12 J mg protein⁻¹.

Most of the previous studies used a combination of thermal (boiling), chemical (low or high pH) and enzymatic methods for processing of waste chicken breast (Table 1); therefore, direct comparison of energy consumption of the PEF process to other studies is difficult. Furthermore, a recent literature review indicated that are very limited studies in literature focused on the energy consumption for meat cooking (Pathare and Roskilly, 2016). One of the reported methods for protein extraction used 3.5 h boiling to extract proteins from muscle (Saiga et al., 2003). Previous studies showed energy consumption of 211 kJ–3.6 MJ kg^{-1} for meat boiling, while our process used 38.4 ± 1.2 kJ kg^{-1} .

One of the advantages of the developed technology is its potential scalability. Over 40 years of research results in recent years for the development of industrial PEF systems, it is possible to apply up to 80 kW power per module (A Golberg et al., 2016a), with up to 240 kW systems reported (Sarathy et al., 2016; Toepfl, 2011). As electroporation is a threshold phenomenon, the goal of scaling up design is to keep the same parameters of electric field strength, a number of pulses and pulse duration as optimized at laboratory level devices (Kempkes, 2016). In industrial continuous systems, the power required is predetermined by the conductivity of the fluid, from which currents and energy can be calculated for a given field strength and product flow rate (Kempkes, 2016). The power required increases linearly with flow rate and conductivity (Kempkes, 2016). As power increases by the square of the electric field strength, optimizing the minimum electric field strength will lead to minimum consumed power in the process (Kempkes, 2016). Current industrial systems apply 50–200 kJ kg^{-1} for permeabilization of plant tissue (Toepfl, 2011), which is higher than 38.4 ± 1.2 kJ kg^{-1} we found are needed for protein extraction from the chicken breast, suggesting that the existing pulsed power generators can be used for this application after a chamber for continuous treatment of meat is developed. Currently, commercial scale systems with the capacity to treat up to 10 t h^{-1} of sugar beets have been constructed (A Golberg et al., 2016b), suggesting a potential adaptation of this technology to waste meat treatment.

Table 3
Multistep PEF protocol for liquid extraction from waste chicken meat biomass (5.14 ± 0.16 g).

Extraction step	Applied Voltage (V)	Pulse duration	Pulses number	Additional Weight (kg)	Current (A)	Resistivity (Ohm·cm)	Invested Energy (J)
1	1000	50 μ s	100	–	5.6 ± 0.1	5.29 ± 0.14	28.00 ± 0.50
2	500	1 ms	50	2 kg	3.27 ± 0.46	4.73 ± 0.42	81.67 ± 11.55
3	200	5 ms	50	2 kg	1.67 ± 0.12	3.71 ± 0.12	83.33 ± 5.77

3.2. Identification of pulsed electric field extracted proteins

Although previous works reported on the protein extraction from meat (Hrynets et al., 2010; Omana et al., 2010; Tahergorabi et al., 2012; Zhao et al., 2017) to increase the value, the proteins were extracted as bulk. These works identified some useful properties of the extracted proteins for the food and material industry such as gel formation, emulsion formation, foam formation, texture and thermal denaturation (Hrynets et al., 2010; Omana et al., 2010; Tahergorabi et al., 2012; Zhao et al., 2017); these works related to proteins as bulk. However, the identification of the extracted proteins could allow better prediction of their function and also allow for the planning of the downstream processes for protein bulk separation into different functional groups. The list of proteins identified in the PEF extract liquid appears in Table S2.

Using semi-quantitative proteomic data, we calculated the molecular weight (MW, Fig. S2a), the normalized intensity for each sample (LFQ, Fig. S2b), and intensity (Fig. S2c) and normalized within sample intensity (iBAQ, Fig. 2a). Using these quantitative data, we selected the list of most abundant proteins with $iBAQ > 10^8$ for further analysis. The histogram (Fig. 2b), box-plot and density function (Figs. S2d and e) suggested the PEF extracted proteins have a not-normal and skewed to the right distribution function. The

skewness and kurtosis plots of log MW (Fig. S2f) suggested that logMW has lognormal, gamma or Weibull distributions. Histogram of the fitted densities, Q-Q plot, CDF and P-P plots of these three distributions appear in Fig. 2c, d, e, f respectively. The goodness of fit analysis (Table 4) suggests that log of MW of the most abundant proteins extracted by PEF is closer to lognormal distribution (smallest statistics for all checked criteria).

The parameters and the uncertainty in the parameters (confidence interval) for the lognormal distribution function were determined using bootstrapping (Efron and Tibshirani, 1993) and appear in Table 5.

Table 4

The goodness of fit analysis of highly abundant PEF extracted proteins ($iBAQ > 10^8$).

	Weibull	lognormal	gamma
Goodness-of-fit statistics			
Kolmogorov-Smirnov statistic	0.1007094	0.04542676	0.05319504
Cramer-von Mises statistic	0.5333880	0.07737214	0.08566754
Anderson-Darling statistic	3.4982083	0.46848494	0.58610532
Goodness-of-fit criteria			
Akaike's Information Criterion	161.4041	130.1844	130.7228
Bayesian Information Criterion	168.0107	130.1844	137.3294

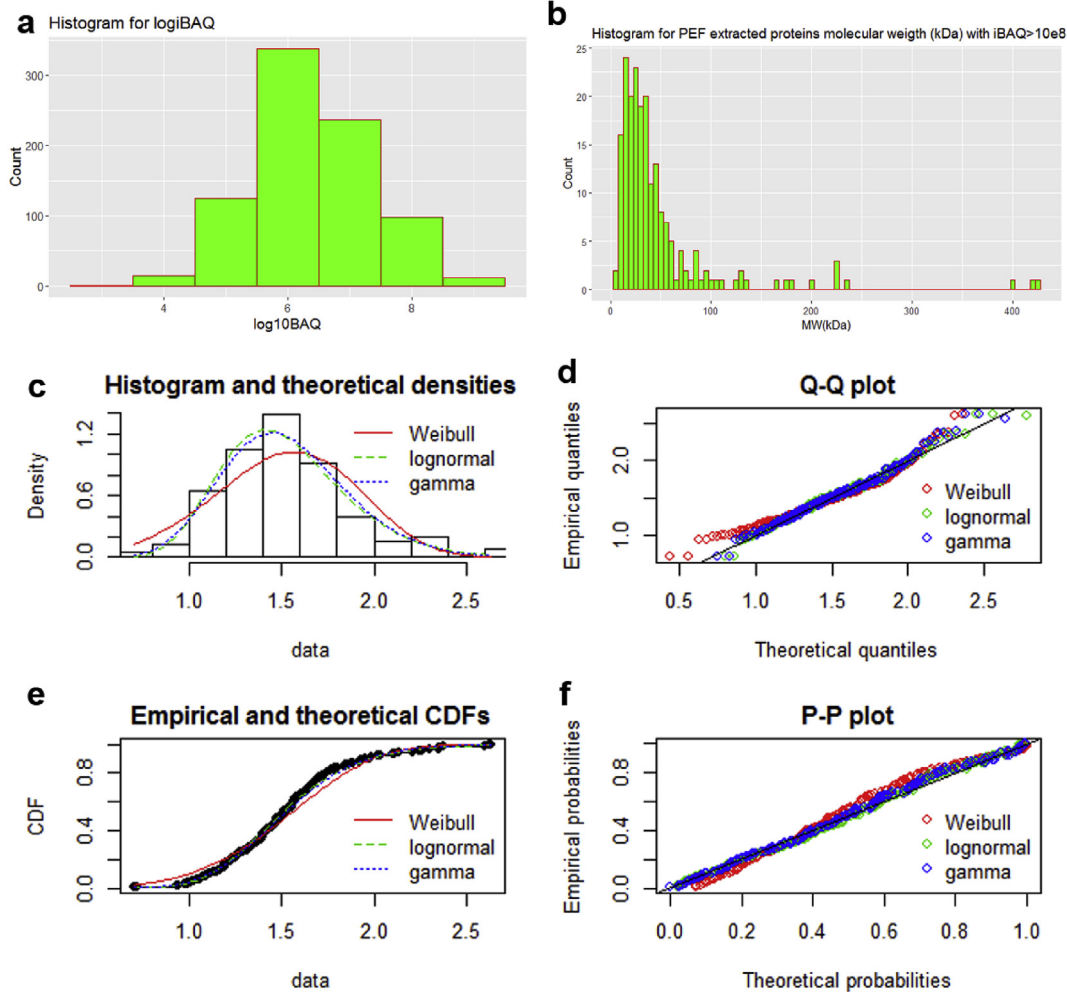


Fig. 2. Molecular weight analysis of the PEF extracted proteins. **a.** Histogram of $\log_{10} iBAQ$ of the extracted proteins as quantified by MaxQuant. **b.** Histogram of extracted proteins molecular weight after cutoff $iBAQ$ of 10^8 . **c.** Histogram of fitted densities. **d.** Q-Q plot of the fitted molecular weight distributions. **e.** CFD of the fitted molecular weight distributions. **f.** Q-Q plot of the fitted molecular weight distributions.

Table 5
Parametric bootstrap medians and 95% percentile CI for lognormal distribution of the log of MW of PEF extracted proteins.

	Median	2.5%	97.5%
meanlog	0.3971035	0.3682178	0.4274519
sdlog	0.2226903	0.2007680	0.2448714

3.3. Gene ontology analysis of the extracted proteins

As 844 proteins were identified using unlabeled proteomics, we performed gene ontology analysis for the associated genes using GOrilla (Eden et al., 2009), annotating the ranked gene list to the mouse genome. Analysis of the component resulted in five significant cell components (Fig. 3a) including contracting fibers, a troponin complex, proteasome core complex, myelin sheath and phosphopyruvate hydratase complex (Table 6). All the detected components are strongly associated with a muscle tissue, our initial biomass. Process analysis of gene ontology (Fig. S3 and Table S3) suggested the extracted protein have a strong association with energy processes in the tissue (P-value up to $1.54E-14$).

Next, we analyzed the gene ontology by function. Six significant functions were identified (Fig. 3b): threonine-type endopeptidase, threonine-type peptidase, intramolecular transferase, fatty acid binding, calcium-dependent protein binding, phosphopyruvate hydratase activity (Table 7). Previous studies suggested that peptidases and proteases possess antioxidant properties, as they inactivate reactive oxygen species, scavenge free radicals, reduce hydroperoxides and chelate prooxidative transition metals (Fang et al., 2002; Garner et al., 1998; Guiotto et al., 2005; Seth and Mahoney, 2001; Trujillo et al., 2016). Therefore, we analyzed the crude PEF extracts for antioxidant properties biochemically.

3.4. Anti-oxidant properties of the PEF extracted liquid phase

Different concentrations of the PEF extract ($1-6 \text{ mg protein mL}^{-1}$) were tested for the antioxidant capacity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-and-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assays. The results

are shown in Fig. 4. The PEF extract showed varied antioxidant activity as compared to the standard Trolox. Both the assays showed a dose-dependent activity where the higher concentration of the extract showed a higher antioxidant activity. The highest antioxidant activity (%) using DPPH was $25.13 \pm 1.13\%$ while the highest radical scavenging capacity of the PEF extract using $\text{ABTS}^{\bullet+}$ radical was $26.93 \pm 0.56\%$. The scavenging ability of standard Trolox was higher than that of extract samples. Similar studies focused on chicken meat hydrolysates have found their antioxidant capacities in a similar range with our studies (Crush, 1970; Jang et al., 2008; Maikhunthod and Intarapichet, 2005; Wu et al., 2005). The majority of the studies have suggested that the antioxidant capacity of chicken meat is due to the presence of a dipeptide carnosine (Crush, 1970; Kopeć et al., 2013). Carnosine is present in the skeletal connective tissues of animals along with another dipeptide called anserine (Wu and Shiau, 2002). The chicken extract used in our studies was the raw extract after PEF treatment of the meat. The antioxidant capacity could be increased by purification of the dipeptide from the raw extract. These dipeptides, in turn, could be used as nutritional supplements and as endogenous antioxidants in meat essences. Our results show that PEF can extract liquids with functional properties from the waste meat. Nevertheless, additional studies are needed to show if the PEF extracts with antioxidant properties are valuable for applications in agriculture.

3.5. Impact of pulse duration and a number of liquid extractions from the chicken muscle biomass in a combined high-voltage, short pulses, and low-voltage long pulses protocol

Finally, we determined the impact of various PEF parameters on the liquid extraction yield. The studied PEF experimental protocol parameters were: 1) electric field strength ($E, \text{V mm}^{-1}$), 2) pulse duration ($t_p, \mu\text{s}$), and 3) number of pulses (N). The optimum combination of factors depends on the starting biomass as well as the required product. The traditional method of “one-factor-at-a-time” (OFAT) optimization requires time and resources. Moreover, since these parameters need to be applied simultaneously during the PEF treatment, the individual role of each of the parameters in the

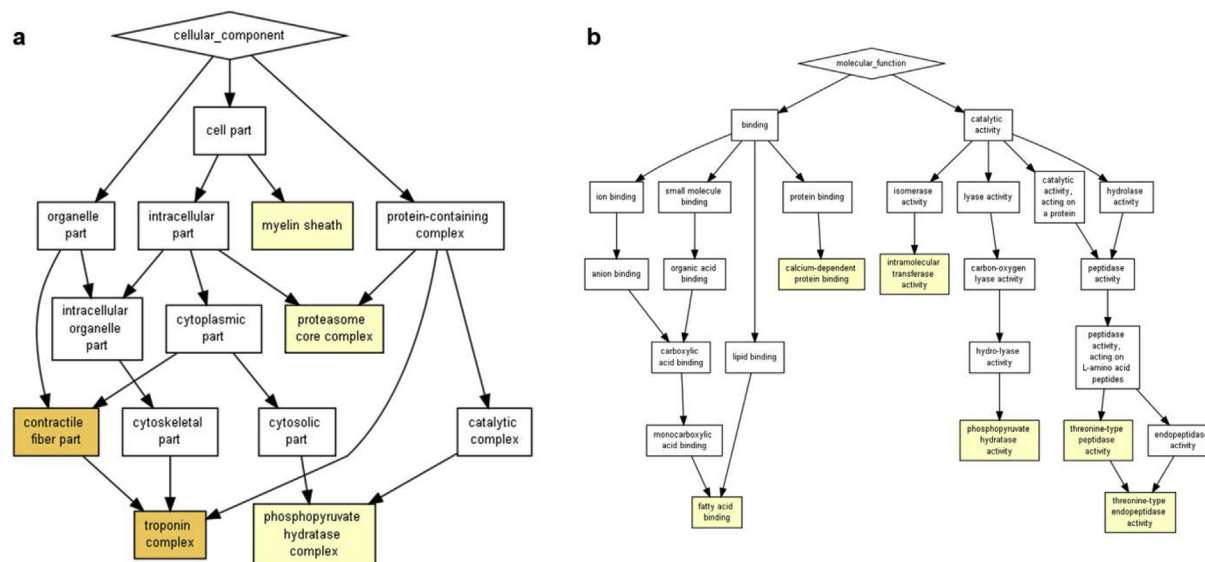


Fig. 3. Annotation of the identified proteins to cellular component **a.** and function **b.** The annotation was done on all identified proteins (Table S2) by GOrilla (Eden et al., 2009) using a ranked of the iBAQ list.

Table 6

Gene ontology by a component of the PEF waste meat extract mapped with GOrilla (Eden et al., 2009).

GO term	Description	P-value*	FDR q-value**	Enrichment (N, B, n,b)***
GO:0044449	contractile fiber part	5.8E-6	4.24E-3	3.16 (625,52,76,20)
GO:0005861	troponin complex	7.66E-6	2.8E-3	20.83 (625,4,30,4)
GO:0005839	proteasome core complex	1.37E-5	3.34E-3	2.93 (625,12,213,12)
GO:0043209	myelin sheath	7.18E-5	1.31E-2	3.72 (625,56,42,14)
GO:0000015	phosphopyruvate hydratase complex	9.74E-4	1.42E-1	31.25 (625,2,20,2)

P-value** is the enrichment p-value computed according to the mHG or HG model. This p-value is not corrected for multiple testing of 731 GO terms.*FDR q-value** is the correction of the above p-value for multiple testing using the Benjamini and Hochberg (1995) method.Namely, for the *i*th term (ranked according to p-value) the FDR q-value is (p-value * a number of GO terms)/*i*.*****Enrichment (N, B, n, b)** is defined as follows:

N - is the total number of genes.

B - is the total number of genes associated with a specific GO term.

n - is the number of genes in the top of the user's input list or in the target set when appropriate.

b - is the number of genes in the intersection.

Enrichment = (b/n)/(B/N).

Table 7

Gene ontology by the function of the PEF waste meat extract mapped with GOrilla (Eden et al., 2009).

GO term	Description	P-value*	FDR q-value**	Enrichment (N, B, n,b)***
GO:0004298	threonine-type endopeptidase activity	3.85E-05	4.61E-02	2.93 (625,11,213,11)
GO:0070003	threonine-type peptidase activity	3.85E-05	2.31E-02	2.93 (625,11,213,11)
GO:0016866	intramolecular transferase activity	1.02E-04	4.08E-02	14.29 (625,5,35,4)
GO:0005504	fatty acid binding	6.07E-04	1.82E-01	4.05 (625,8,135,7)
GO:0048306	calcium-dependent protein binding	7.59E-04	1.82E-01	2.68 (625,13,197,11)
GO:0004634	phosphopyruvate hydratase activity	9.74E-04	1.94E-01	31.25 (625,2,20,2)

P-value** is the enrichment p-value computed according to the mHG or HG model. This p-value is not corrected for multiple testing of 731 GO terms.*FDR q-value** is the correction of the above p-value for multiple testing using the Benjamini and Hochberg (1995) method.Namely, for the *i*th term (ranked according to p-value) the FDR q-value is (p-value * a number of GO terms)/*i*.*****Enrichment (N, B, n, b)** is defined as follows:

N - is the total number of genes.

B - is the total number of genes associated with a specific GO term.

n - is the number of genes in the top of the user's input list or in the target set when appropriate.

b - is the number of genes in the intersection.

Enrichment = (b/n)/(B/N).

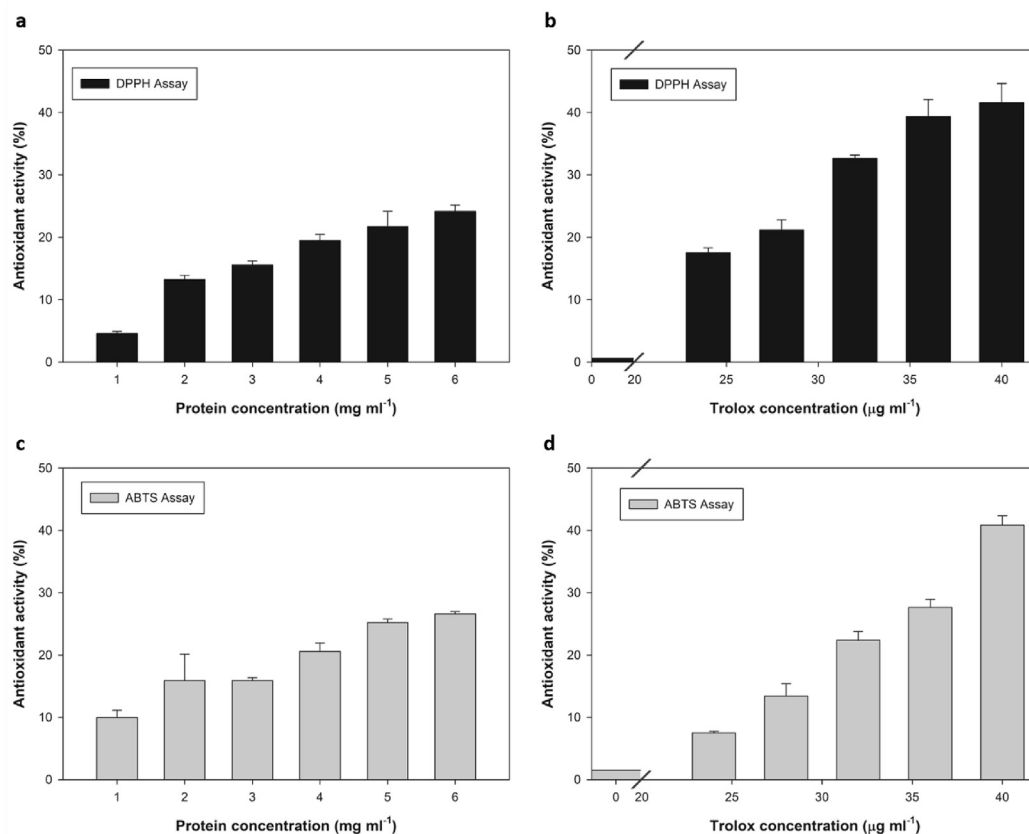
**Fig. 4.** Antioxidant properties of the PEF extract in comparison to standard Trolox. **a.** PEF extract DPPH assay. **b.** Trolox DPPH assay. **c.** PEF extract ABTS assay. **d.** Trolox ABTS assay.

Table 8
Response Table for Signal to Noise Ratios and ranking of PEF parameters using the Taguchi approach.

Level	t_l (ms)	t_s (μ s)	N_l	N_s
1	33.80	30.33	26.39	22.32
2	19.46	20.02	22.14	27.73
3	23.41	26.31	28.13	26.62
Δ	14.34	10.31	6.00	5.41
Rank	1	2	3	4

process is unclear. Therefore, the goal of the present study was to the combined effect of pulse duration and pulse number for

extraction of liquid from the chicken muscle biomass.

Initial studies suggested a combination of high-voltage, short pulses, and low-voltage, long pulses could extract liquid from chicken muscle biomass. The impact of pulse duration and number was yet to be studied. Therefore, to decrease the number of experiments but still delineate the impact of each parameter independently, we applied the Taguchi Orthogonal Array to the experimental design (Golberg et al., 2015).

According to Taguchi ranking, the parameter of long pulse duration (t_l) has the most prominent impact on liquid extraction (Taguchi Rank 1, Table 8). Increasing the pulse duration might increase the liquid extraction from chicken muscle biomass due to the creation of more pores from which the liquid may come out

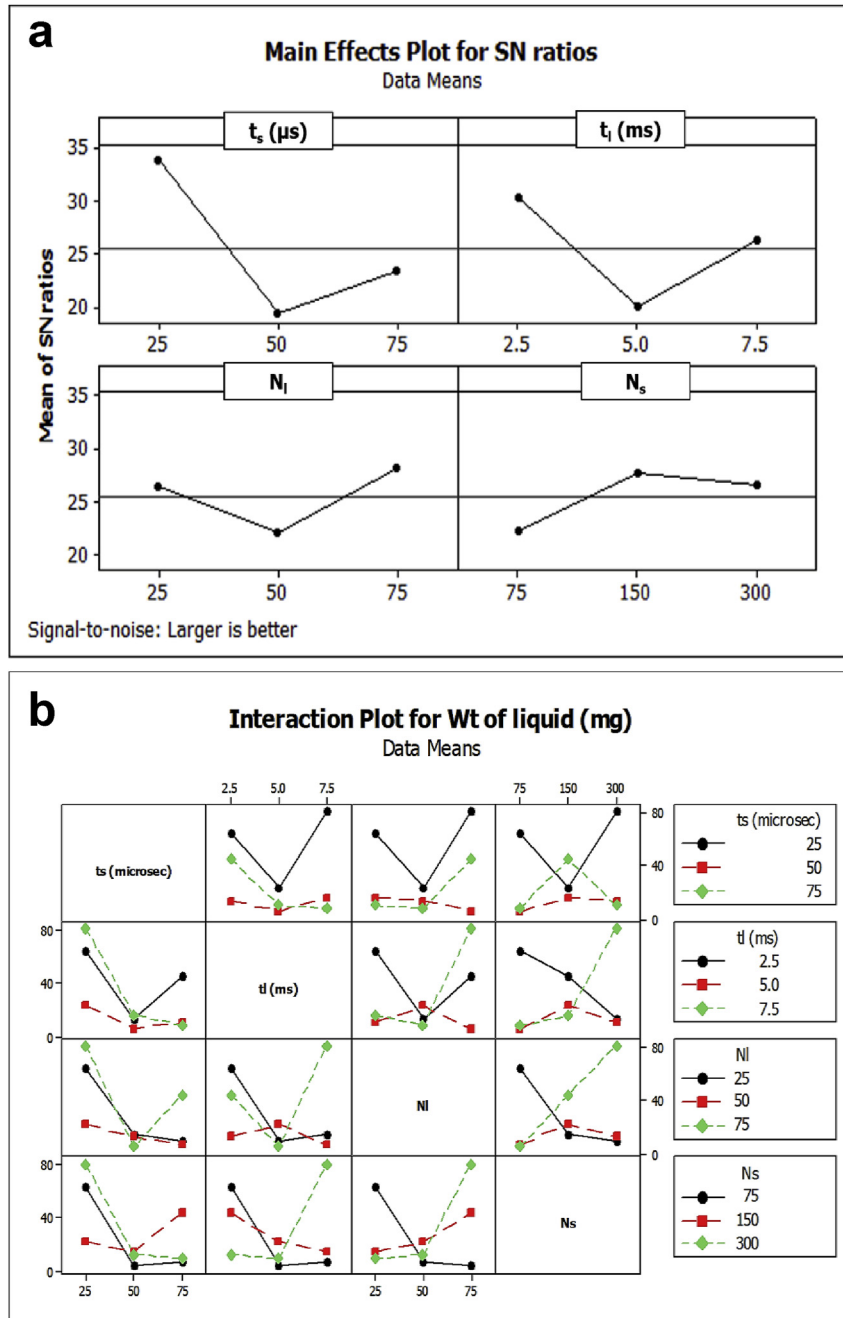


Fig. 5. a. Taguchi analysis of the impact of 4 PEF parameters on the liquid phase extraction from the waste chicken breast biomass. The effects of pulsed electric field parameters such as duration of the short pulse (t_s), duration of the long pulse (t_l), number of short pulses (N_s) and number of long pulses (N_l) were tested. **b.** Interaction plots for effect of different factors on the liquid extraction from waste chicken meat using Pulsed Electric fields.

Table 9

Optimum protocol for liquid extraction from waste chicken meat calculated based on Taguchi method.

Extraction step	Applied Voltage	Pulse duration	Pulses number
1	1000 V	25 μ s	50
2	50 V	2.5 ms	300

easily from the biomass. Alternatively, longer pulses could generate chemical species by electrolysis, which assist in tissue lysis. The quantification of electrolytic events and their impact on the extraction still requires further analysis.

Short pulse duration (t_s) is ranked second (Taguchi Rank 2) in significance on increase in juice content followed by the number of long pulses (N_l , Taguchi Rank 3). The number of short pulses (N_s) has the smallest effect on increasing collagen level elevation (Taguchi Rank 4).

We also analyzed the sensitivity of the total liquid release during PEF to the tested process parameters that can be controlled (Fig. 5). The time of pulsed significantly influences the process (Fig. 5). The variation in signal to noise ratio (S/N) shows the influence of duration for short and long pulses on the process. Therefore, controlling the duration of pulses (both long and short) may lead to an increase in the yield of liquid from the chicken muscle biomass. The number of pulses did not show a significant effect on the process which was also observed in the ranking table for Taguchi design (Fig. 5a). The interaction plots that show the impact of various tested parameters on the extracted juice yield appears in Fig. 5b.

The optimum parameters for the maximum liquid extraction (the maximum SN ratio) were determined as 1000 V, 25 μ s, 50 pulses followed by 50 V, 2.5 ms, 300 pulses. (Tables 8 and 9).

4. Conclusions

Meat waste has the highest economic and environmental costs in the food production. In this work, we show that electroporation, based on the pulsed electric field coupled with mechanical pressing provides a technology platform for functional chemicals extraction for additional to energy generation uses. We show that a two-step protocol, which consists from high voltage, short pulses followed by low-voltage long pulses, with the total invested energy of $38.4 \pm 1.2 \text{ J g}^{-1}$, of initial waste meat, enables extraction of the liquid fraction which is $12 + 2\%$ of the initial weight. The protein content of the extract fraction was $78 \pm 8 \text{ mg mL}^{-1}$. The logarithm of the molecular weights of the extracted proteins was found to have a lognormal distribution. Gene ontology analysis of identified PEF extracted proteins suggested that extracts include proteins belong to contracting fibers, troponin complex, proteasome core complex, myelin sheath, phosphopyruvate hydratase complex. This analysis also suggested that the extracted proteins participate in threonine-type endopeptidase, threonine-type peptidase, intramolecular transferase, fatty acid binding, calcium-dependent protein binding, and phosphopyruvate hydratase activities. Antioxidant properties were corroborated with DPPH and ABTS assays. Process parameters analysis showed that long pulse duration played the most important role in liquid extraction from the waste chicken breast muscle, followed by short pulses duration, number of long and short pulses. Our study suggests that pulsed electric fields can be used for extraction of functional molecules from the waste meat biomass using non-thermal, chemical-free process. Such a strategy could provide an additional income and thus, stimulate farmers and meat processors to reduce waste and waste-related environmental pollution.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jclepro.2018.10.037>.

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