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Pulsed electric field combined with preheating to preserve mildly extracted pea protein

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ABSTRACT

Innovative extraction methods involving dry fractionation and aqueous phase separation have been recently developed to obtain plant-based protein ingredients with high texturing functionality. Such a process was applied to yellow peas, and the microbial quality of the extract was assessed. *Pseudomonas trivalis* and *Erwinia gerundensis* were identified as critical microbial contaminants due to their high growth capacities (at 10 °C). As a preservation strategy, pulsed electric field (PEF) treatment (24 kV/cm, 50 Hz, 126 μs) was employed, combined with different product inlet temperatures (25–45 °C). Ohmic heating (ΔT = 20 °C) was measured. At 25–30 °C, the microbial reduction reached 3 logs, attributed solely to electrical effects. At 40 °C, the reduction exceeded 4 logs, where thermal effects dominated. Further increase to 45 °C resulted in thermal modification of pea proteins, potentially compromising its gelling capacity.

Additionally, the growth of PEF-treated contaminants at 5 and 10 °C were monitored, revealing rapid cell adaptation and proliferation. Finally, the extracts native microbiota showed an extension of the lag phase (4 days at 5 °C) after PEF treatment, accompanied by a pH stability of 10 days. The findings indicate improved microbial stability of the pea extract following PEF treatment.

1. Introduction

The shift from an animal-based to a plant-based diet is recognized as a key strategy for creating a more sustainable and healthy food system (Agyemang et al., 2022; Smetana et al., 2023). The development of plant-based meat substitutes, designed to mimic the taste, texture, and ease of preparation of meat, aims to facilitate this transition (Michel et al., 2021). The desired texture in these substitutes is typically achieved through extrusion, a thermomechanical process involving denaturation and intermolecular binding of biopolymers (proteins and potentially polysaccharides) at high temperatures combined with high shear stress. Van der Sman & Van der Goot (2023) and Guan et al. (2024) described the formation of fibrous structures through a multi-layer structuring theory, highlighting the critical role of the raw materials' viscoelastic properties in controlling gelation during extrusion.

Pea is a promising source for meat substitutes because of its low allergenicity, complete amino acid profile and the presence of proteins (predominantly globulins (~80%) and albumins (~20%)) that exhibit strong gelling capacities (Lu et al., 2020). Additionally, substituting beef with pea protein has been shown to impact both human health positively and reduce carbon footprint (Saget et al., 2021). Typically, pea derivates as food ingredients come as powdered pea protein isolates (PPIs) with a protein content of 75–90% (Schutyser et al., 2015). PPIs are produced via wet extraction methods involving alkaline solubilization, isoelectric point precipitation, and mechanical separation followed by spray drying. While this process yields relatively pure protein ingredients, it comes at the cost of diminished protein nativity and high water, energy, and chemical consumption (Lie-Piang et al., 2021; Reinkensmeier et al., 2015; Schutyser & van der Goot, 2011). Additionally, this process removes pea albumins, which are water-soluble at the isoelectric point of pea globulins, and which contribute to firmer and more elastic gels than pea globulins (Kornet et al., 2021a; Möller et al., 2022). The extraordinary texturizing effect of albumins was justified with its high content of sulfur-containing amino acids (Higgins et al., 1986), participating in disulfide binding and protein network formation at extrusion

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Abbreviations

DSC	Differential scanning calorimetry
MMRS	Modular microfluidic reactor system
PEF	Pulsed electric fields
PPC	Pea protein concentrate
PPE	Pea protein extract
PPI	Pea protein isolate

cooking.

Novel mild processing techniques, such as dry fractionation, are emerging as sustainable alternatives that prioritize protein functionality over purity (Kornet et al., 2021b; Möller et al., 2022; Pelgrom et al., 2015). Dry fractionation involves air classification, a physical separation of protein from starch, based on size and density, resulting in a pea protein concentrate (PPC) (Schutyser et al., 2015). Typically, PPC contains ~50% of proteins (Reinkensmeier et al., 2015; Rodriguez & Beyrer, 2023). An additional step of aqueous phase separation and subsequent centrifugation can increase this protein content to ~60%, yielding pea protein extract (PPE) that contains both albumins and globulins (Lie-Piang et al., 2021; Möller et al., 2022; Pelgrom et al., 2015; Rodriguez & Beyrer, 2023). The higher functionality of these extracts compensates for their lower protein content in comparison to PPI. Indeed, Kornet et al. (2021b) showed that a lower degree of fractionation leads to an increased gelling capacity of the ingredient. Moreover, Rodriguez and Beyrer (2023) observed that partially substituting PPI with PPE enhanced the elasticity of the resulting gel. They also demonstrated that PPE could be directly used in the production of meat substitutes without the need for additional drying or concentration.

For storage and transportation purposes, ensuring PPE's microbiological stability is crucial. Pulsed electric fields (PEF) technology emerges as a sustainable alternative to thermal preservation. Despite showing higher estimated cost (0.037 EUR/L versus 0.015 EUR/L), PEF offers a non-denaturing, and low-energy preservation method suitable for heat-sensitive liquids at industrial scale (Jadhav et al., 2021; Sampedro et al., 2014; Toepfl et al., 2006). By subjecting the liquid to electric pulses of high voltage and short duration, the inactivation of microbial vegetative cells is possible at temperatures below 60 °C. The microbial inactivation is explained primarily by cell membrane permeabilization and can be facilitated by the additional thermal effect when combined with mild preheating (30–50 °C) (Alirezalu et al., 2020; Sharma et al., 2014; Yan et al., 2021; Zheng et al., 2024). The effectiveness of PEF depends strongly on factors such as electric field strength E_{el}, treatment time t_t, substrate inlet temperature T_{in}, pH, conductivity, composition, and microbial cell characteristics (Raso et al., 2016). PEF can be successfully used for the microbial reduction of low viscosity and low electrical conductivity food liquids like fruit juice and milk (Cavalcanti et al., 2023; Nowosad et al., 2021; Schottroff et al., 2019; Sharma et al., 2014; Timmermans et al., 2022; Šalaševičius et al., 2021). Recent studies have also explored the application of PEF on plant protein ingredients such as microalgae (De Gol et al., 2024), soy (Alzahrani et al., 2022), and oat milk (Thamsuaidee et al., 2024), as well as oat-based beverages enriched with pea protein (Horlacher et al., 2024). The authors highlighted challenges related to the protective effect of proteins and the high conductivity of the substrate (Schottroff et al., 2019). Conversely, PEF treatment not only achieves microbial inactivation but also has the potential to enhance protein solubility, thereby improving gelling characteristics (Malik et al., 2024; Taha et al., 2022). This dual benefit makes PEF an appealing option for preserving plant-based ingredients intended for extruded meat substitutes.

This study aims to 1) identify relevant spoilage microorganisms able to grow in PPE under refrigerated conditions, 2) optimize PEF-induced inactivation of identified bacterial species at different product inlet temperatures, and 3) define a PEF treatment window for microbial inactivation and shelf-life extension of PPE.

2. Material and methods

2.1. Pea protein extract (PPE)

Air classified PPC was obtained from yellow pea (*Pisum sativum)* (GMSA, Switzerland). PPE was produced from PPC by aqueous extraction. PPC was dispersed in demineralized water (15 g PPC/100 g) and stirred at 200 rpm for 4 h at room temperature. This process avoided the use of solvents to maintain the natural pH of the PPE, thereby preserving protein functionalities. Moisture content was determined using a rapid moisture analyzer (HC103 Mettler-Toledo, Switzerland) at the conditions of 120 °C, initial mass of 2 g, and stop criteria of 1 mg/50 s. The pH and conductivity were measured using a pH/conductometer 914 (Metrohm, Switzerland). The extraction was done at lab scale in triplicate. The composition analyses and the microorganism selection (sections 2.1.1, 2.1.2, 2.2 and 2.3) were done on multiple PPEs. The preservation treatments (sections 2.5 to 2.6) were applied in biological triplicate on the same PPE, produced at pilot scale.

2.1.1. Sugar analysis by HPLC

Carrez I and Carrez II reagents were prepared from potassium hexacyanoferrate (II) (Sigma-Aldrich GmbH, Germany) and zinc sulphate (Sigma-Aldrich GmbH, Germany), respectively, and 200 μL of each reagent was added to the PPE (1 mL). After vortexing and incubation (10 min), the mixture was centrifuged (2000×*g*, room temperature, 5 min) using a microcentrifuge (Roth AG, Switzerland). The resulting supernatant was subjected to different purification steps: dilution with demineralized water (1:1), sonication for 10 min, centrifugation (7500×*g*, 20 °C, 8 min) and filtration through a 0.45 μm filter (Macherey-Nagel, Germany). Standards glucose, fructose and saccharose (Sigma-Aldrich GmbH, Germany) were dissolved in ultrapure water to prepare the stock solutions (10 g/L). The chromatographic analysis was conducted using an HPLC system 1220 Infinity LC system (Agilent Technologies, USA) equipped with the Aminex HPX-87H column (300 mm × 7.8 mm) from Bio-Rad Laboratories (USA) in conjunction with a Bio-Rad pre-column. Sulfuric acid (Sigma-Aldrich GmbH, Germany) was used as the eluent solution (5 mmol/L) in isocratic mode and with a 0.6 mL/min flow rate. The sample injection volume was 5 μL and the column was maintained at 35 °C. Detection was accomplished using a Refractive Index Detector (RID) at a wavelength of 210 nm. The peak areas were measured at 7.4, 8.8 and 9.6 min for saccharose, glucose and fructose, respectively. Four measurements were performed on multiple PPEs.

2.1.2. Protein content

The crude nitrogen content of the PPE was determined using the Kjeldahl method. The protein content was calculated using a conversion factor of 5.5, which is appropriate for pea proteins (Schlangen et al., 2022). The measurements were done in duplicate.

2.2. Microorganism isolation and identification

Fresh PPE was stored at 10 °C in a constant climate chamber (ICH260 Memmert, Germany), and was microbiologically analyzed at days 0, 3 and 6. For that, 1 mL of sample was decimally diluted using maximum recovery diluent (MRD, 1.0 g enzymatic digest of casein and 8.5 g sodium chloride in 1 L water) (Biolife, Italy), and different dilutions were plated on plate counting agar (PCA) (Biokar Diagnostics, France). The plates were incubated at 10 °C for up to 9 days. The dilution showing an appropriate number of colonies was considered, and every single colony on the plate was isolated and purified for identification by three subsequent streaking on PCA plates and incubation (IPP 110^{PLUS} Memmert, Germany) at 30 °C for 24 h. The plates containing pure colonies were sealed to avoid cross-contamination and sent externally for

identification (Mabritec SA, Switzerland) using MALDI-TOF MS technology. The spectra were analyzed using the SARAMIS SuperSpectra (SSp) tool and matched with the putative assigned protein masses for the identification database (PAPMID). Finally, the isolated species were grown in tryptic-casein soy broth (TSB) (Biokar Diagnostics, France) at 30 °C, 150 rpm for 24 h, mixed with glycerol at a ratio 1:1 and stored at −80 °C for further use in challenge testing. The procedure was done on three different PPEs.

2.3. Microorganism selection

Based on the identification results (section 2.2.), the three dominating species (*Erwinia gerundensis*, *Pseudomonas trivalis* and *Enterobacter amnigena*) were pre-selected and used for further growth tests. The isolated species were freshly inoculated on PCA plates. Single colonies were picked, transferred and grown in TSB at 30 °C, 150 rpm for 24 h, and the cell concentration was estimated at 10^9 colony forming units (CFU)/mL by microscopy (Olympus XC10, Carl Zeiss, Germany) using a Neubauer chamber 0.02 mm depth and 0.0025 mm^2 (Assistant, Germany). The inocula were diluted into autoclaved (120 °C, 15 min) PPE and incubated at 10 °C, 150 rpm for 6 days in a shaking incubator (Excella E24 New Brunswick Scientific, USA). The microbial growth was monitored by sampling the PPE on days 0, 3 and 6 by preparing decimal dilution series and plating on PCA, that were incubated at 30 °C for 24 h. Two microorganisms were selected for challenge tests based on their capacity to adapt and grow in refrigerated conditions.

2.4. Cultivation and viable cell count after PEF and thermal treatments

The selected species were freshly inoculated on PCA plates. Single colonies were grown in TSB at 30 °C, 150 rpm for 24 h, and inoculated in previously diluted PPE (dry matter content (dm) of 3 g/100 g sample) to reach an initial microbial load of approximately 10^6 CFU/mL, noted as N_0. The samples were used to determine the PEF and thermal resistance of the microorganisms (sections 2.5 and 2.6). The treated samples were stored on ice and plated within 2 h following the treatment. The decimal dilutions were prepared with MRD, and at least three dilutions (50 µL) were plated. The colonies were grown on PCA plates at 30 °C for 24 h. Enumeration of colonies allowed us to determine the inactivation level for each treatment, calculated as $\log_{10}(N/N_0)$, where N and N_0 are the microbial concentrations (CFU/mL) in the treated and untreated samples, respectively. OriginLab® 2022b (OriginLab Corporation, USA) generated log-reduction graphs. Cultivations, sample preparation, inactivation treatments, and microbiological analysis were done in triplicate.

2.5. PEF treatments

During PEF treatment, the power required to apply a specific treatment is highly dependent on the conductivity of the substrate (Raso et al., 2022). Increased conductivity results in higher current in the PEF chamber, which can disrupt the generation of square pulses at high voltage and consequently decrease the intensity of the electric field strength. In this study, the conductivity of the PPE was reduced from 6 to 2 mS/cm by dilution with demineralized water to reach an $E_{el} > 20$ kV/cm. The PEF system was described by De Gol et al. (2024). Briefly, the setup included a pilot-scale PEF machine (HVP-5, ELEA, Germany) with a co-linear flow cell with one high-voltage electrode in the middle and two ground electrodes (spacers of 1 cm), and a 1.57 mL treatment chamber. A screw pump (Mono pump 3NU10, Socsil-Inter SA, Switzerland) regulated the flow rate (F) of the diluted PPE (dm of 3 g/100 g sample, 2 mS/cm) at 45 L/h, corresponding to a residence time in the PEF cell $t_r = V_{chamber}/F$ of 0.126 s. A customized tubular heat exchanger, with temperature control via a thermoregulator (HB120, Huber Kältemaschinenbau AG, Germany), was used for heating (T_{in} = 25, 30, 35, 40, 45 °C) and cooling the PPE. The time for the PEF-treated liquid to reach the cooling zone was 27 s at 45 L/h. Temperatures were monitored and recorded with PT100 TMR31 temperature sensors (Endress + Hauser Group Services AG, Switzerland) and LabView 2015 software. Square pulses of width τ 20 µs and pulse repetition rate f 50 Hz were generated, with an output voltage set at 90%, resulting in an electric field strength E_{el} of 24 kV/cm and a treatment time $t_t = t_r \bullet f \bullet \tau$ of 126 µs. An oscilloscope (TDS 2024C Tektronix, USA) was connected to register the pulse shape. The pulse amplitude (voltage U (V) and current I (A)), the electric field strength E_{el} (kV/cm), the specific energy input W_s (kJ/kg) and the outlet temperature T_{out} (°C) were monitored after 1 min treatment to ensure process stability. Cultivations, sample preparation, PEF treatments and microbiological analysis were done in biological triplicate using the same PPE. The detection limit was 20 cells/mL sample.

2.6. Thermal treatments

As described by De Gol et al. (2024), in addition to the PEF resistance, the thermal resistance of microorganisms was studied to distinguish between electrical and thermal inactivation effects. The temperature profile of both processes is similar. A modular micro reaction system (MMRS) (Ehrfeld Mikrotechnik GmbH, Germany) with a total treatment volume of 2.5 mL was used. The liquid was pumped (Nikkiso pump, LEWA GmbH, Switzerland) at a flow rate of 5.5 mL/min (mini-Cori-Flow M14-ABD-22-0-S, Bronkhorst, Switzerland), leading to a total retention time of 27 s, corresponding to the holding time in the PEF system from the PEF cell to the cooling zone (section 2.5). The temperatures and flow rate values were monitored using the software LabView. Cultivations, sample preparation, thermal treatments and microbiological analysis were performed in biological triplicates. The detection limit was 1 cell/mL sample. The critical temperature T_{crit} was defined as the minimal temperature required to observe inactivation. It was calculated using equation (1) on the straight part of the inactivation curve. The extracted T_{crit} mean value was used to determine the confidence intervals (95% confidence level) using the Student's t-distribution.

$$\log_{10} N / N_0 = a \bullet (T - T_{crit}) \quad (1)$$

2.7. Thermal degradation of PPE – Differential scanning calorimetry (DSC)

Heat-induced denaturation was determined by DSC using a µDSC7 evo (Setaram Instrumentation, France), as explained by Rodriguez and Beyrer (2023). Briefly, the samples were scanned from 20 °C to 100 °C at a heating rate of 0.5 °C/min, held at 100 °C for 10 min, and subsequently cooled to 20 °C at the same rate. The Thermal Analysis® Software package V. 1.46 (Setaram Instrumentation) was used to extract the reaction enthalpy ΔH and peak maximum temperature T_{peak} values. The measurements were done in triplicate on the reference and diluted PPE (dm of 3 g/100 g sample) and the PEF-treated PPE (T_{in} = 30, 40, 45 °C).

2.8. Storage control

P. trivalis and *E. gerundensis* were inoculated in diluted and pasteurized (85 °C, 5 min) PPE. Their microbial growth following PEF treatment (24 kV/cm, 50 Hz, 126 µs and 40 °C) was studied during cold storage (5 and 10 °C, 14 days). Additionally, the growth of the native microbiota of PPE (no pasteurization) after PEF treatment was also explored. Monitoring of the pH was done using a pH-meter 914 (Metrohm, Switzerland). Cultivations, sample preparation, PEF treatments and storage control were done in biological triplicates. The detection limit was 20 cells/mL sample.

Similarly to De Gol et al. (2024), the growth curves were modelled using the primary reparametrized Gompertz model (equation (2)) (Garre et al., 2023; Zwietering et al., 1990):

Table 1
Microbial and pH evolution during incubation of PPE (dm of 10 g/100 g sample) at 10 °C for 6 days. Species (sp) identification was achieved through MALDI-TOF MS. Data are averages of biological triplicates (n = 3) ± SD [95 % confidence intervals]. % indicates the proportion in % of total detected species.

Description	Storage time (days)		
	0	3	6
N° colonies identified (–)	24	15	30
Total count (log CFU/mL)	3.4 ± 0.1	7.2 ± 0.1	9.1 ± 0.1
pH (–)	6.7 ± 0.0	6.7 ± 0.0	5.8 ± 0.5
Erwinia sp. (%)	64 ± 13 [47–82]	39 ± 8 [28–50]	n.d.
Pantoae sp. (%)	23 ± 14 [4–43]	14 ± 4 [8–19]	n.d.
Enterobacter sp. (%)	n.d.	n.d.	65 ± 14 [46–84]
Pseudomonas sp. (%)	n.d.	25 ± 19 [9–41]	25 ± 12 [9–41]
Other sp. (%)	12 ± 7 [2–22]	22 ± 8 [11–33]	10 ± 6 [2–18]

n.d.: not detected.

$$\log_{10} N = \log_{10} N_0 + A \bullet \left(\exp\left(-\exp\left(\frac{e \bullet \mu}{A}(\lambda - t) + 1\right)\right)\right) \quad (2)$$

With the microbial concentration N (CFU/mL), the difference between $\log_{10} N_{max}$ and $\log_{10} N_0$ A, the storage time t (d), the maximum growth rate μ ($\log_{10}$ d^{-1}) and the lag phase λ (d).

2.9. Statistical analysis

All the analytical measurements were done in duplicate (n = 2) or triplicate (n = 3). Results were expressed as individual data points for duplicates, while triplicates were expressed as average ± standard deviation (SD). Regarding statistical analysis, first, normality distribution (Shapiro-Wilk test) and equal variance (Chi-square test for variance) were checked (p = 0.05). Statistical significance was estimated by one-way ANOVA and Tukey's post hoc test. Dunn's test and Welch's ANOVA were used when normality and equal variance were rejected, respectively. P-values < 0.05 were considered statistically significant. All statistical tests were performed using OriginLab® 2022b (OriginLab Corporation, USA).

3. Results and discussion

3.1. Chemical properties of the substrate PPE

The dm content and pH of PPE were 10.6 ± 0.1 g/100 g and 6.7 ± 0.0, respectively. The protein content was 6.1 g/100 g PPE on a wet basis, corresponding to an average value of 57.7 g/100 g PPE on a dry basis. Free sugar analysis revealed saccharose, glucose and fructose concentrations at 3.1 ± 0.0, 0.5 ± 0.0, and 3.8 ± 0.3 g/L PPE, respectively. The substrate contains all the necessary nutrients (water, carbon and nitrogen sources, minerals and vitamins) for microbial growth, supported by its neutral pH (Campos-Vega et al., 2010; Millar et al., 2019; Vidal-Valverde et al., 2003). The dilution of the extract with demineralized water aiming to reduce the conductivity from 6 to 2 mS/cm and reach $E_{el} > 20$ kV/cm during PEF treatment led to a decrease of the dm to 3 g/100 g sample.

3.2. Microbial contaminants of PPE

3.2.1. Identification of marker microorganisms

The identification of bacterial species in PPE after 3 and 6 days was conducted to select the main microbial contaminants during cold storage (10 °C). A total of 69 species were purified and identified, leading to the detection of 20 different species, including *Erwinia* (*E. persicina, E. gerundensis, E. aphidicola*), *Pseudomonas* (*P. poae, P. trivalis, P. sivasensis, P. canadensis., P. reactans, P. synxantha, P. fluorescens*), *Pantoea* (*P. anthopila, P. agglomerans, P. pleuroti*) and *Enterobacter* (*E. cowanii, E. cloacae complex, E. amnigena*) species. *Myroides odoratimimus, Lysinibacillus capsici, Providencia stuartii* and *Bacillus cereus* were also detected. *E. persicina, E. gerundensis, P. agglomerans, P. fluorescens, E. amnigena, Lysinibacillus* and *B. cereus* were previously identified in pea (Chartrel et al., 2021; Godebo et al., 2020; Kyrylenko et al., 2023). During cold storage, the microbial load increased significantly from 3.4 to 9.1 logs CFU/mL, accompanied by a decrease in pH from 6.7 to 5.8, conceivable due to the production of organic acids (Table 1). Initially, *Erwinia* species dominated, comprising 64% of the microbial population on day 0 and 39% on day 3. However, by day 6, *Enterobacter* species became dominant, accounting for 65% of the population. *Pseudomonas* species were detected after 3 days of storage, constituting 25% of the microbial community at days 3 and 6.

3.2.2. Growth capacity of marker microorganisms

To eliminate competing effects between species, the three dominating species, i.e., *E. gerundensis, E. amnigena* and *P. trivalis,* were individually inoculated into autoclaved PPE and incubated at 10 °C for 6 days. The log increase after 3- and 6-days storage is presented in Fig. 1. *P. trivalis* (2.5 and 2.9 log increase after 3 and 6 days, respectively) showed the highest growth capacity and was selected for the challenge tests. A second bacterial species (*E. gerundensis*) was also included in the study. Similarly, Chartrel et al. (2021) explored the microbial community composition of *Pisum sativum* after 5 days of soaking at 4 °C, identifying the *Proteobacteria* class (including *Erwinia, Pantoea, Pseudomonas and Sphingomonas*) as the most abundant, comprising 57% of the total community.

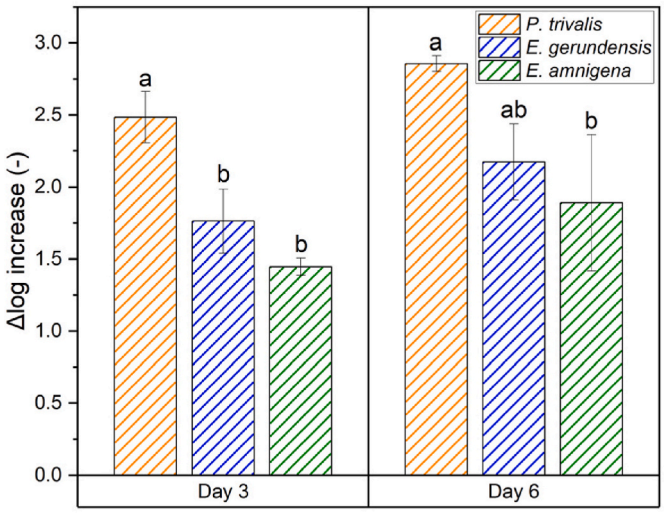

Fig. 1. Growth kinetics of *P. trivalis, E. gerundensis* and *E. amnigena* shown as Δlog increase after 3- and 6-days storage at 10 °C. Different letters indicate significant differences (p < 0.05).

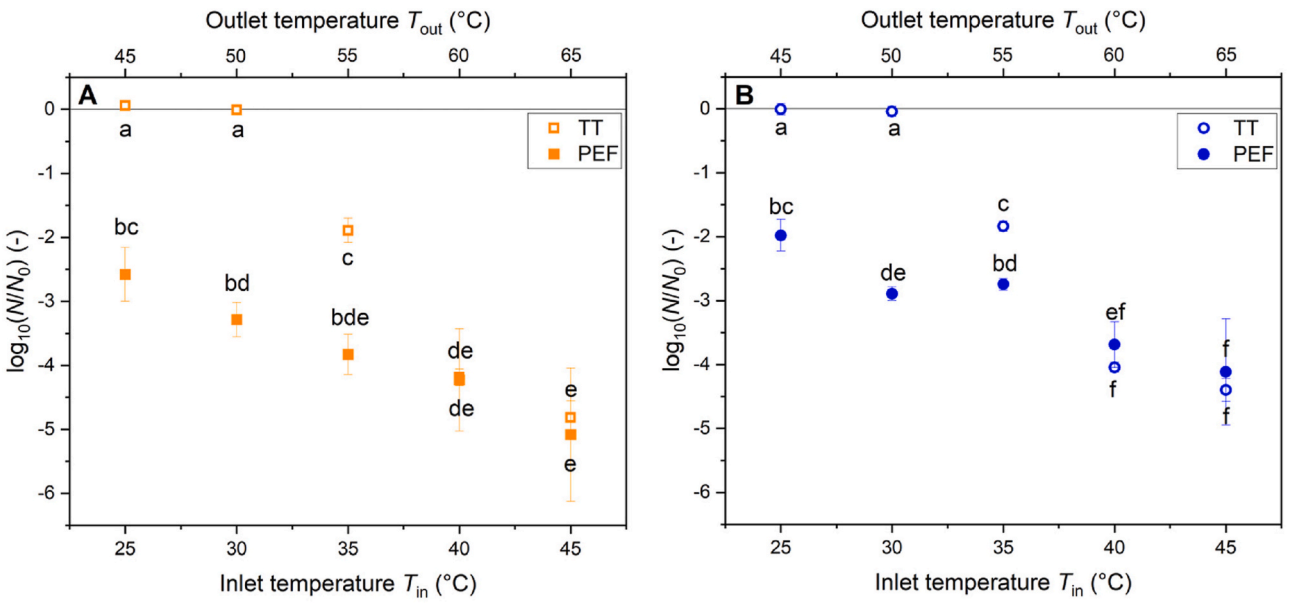

Fig. 2. Influence of inlet temperature T_{in} on inactivation effects of *P. trivalis* (■, A) and *E. gerundensis* (●, B) for a PEF treatment of 24 kV/cm, 50 Hz, 126 µs in a PPE substrate of 2 mS/cm and pH 6.7. The initial count N_0 was ~10^6 CFU/mL. Data points are averages of three individual measurements (n = 3) ± SD. Filled and open symbols represent PEF (section 2.5) and purely thermal (section 2.6) inactivation effects, respectively. Different letters indicate significant differences ($p < 0.05$).

Table 2
Thermal properties of the substrate PPE at a dm of 10 g/100 g sample, after dilution to a dm of 3 g/100 g sample and after PEF treatments (T_{in} = 35, 40 or 45 °C). Data are averages of triplicates (n = 3) ± SD. Different letters indicate significant differences ($p < 0.05$).

Ingredients	ΔH_1 (J/g dm)	$T_{peak,1}$ (°C)	ΔH_2 (J/g dm)	$T_{peak,2}$ (°C)
PPE (dm of 10 g/100 g sample)	0.8 ± 0.2^{a}	53.8 ± 1.3^{a}	4.2 ± 0.7^{a}	73.9 ± 1.3^{a}
Diluted PPE (dm of 3 g/100 g sample)	0.6 ± 0.2^{ab}	54.4 ± 1.3^{a}	4.9 ± 1.1^{a}	77.3 ± 0.3^{a}
PEF-treated (T_{in} = 35 °C)	0.2 ± 0.1^{b}	55.3 ± 1.3^{a}	3.3 ± 0.3^{ab}	73.7 ± 0.5^{a}
PEF-treated (T_{in} = 40 °C)	n.d.	n.d.	3.0 ± 0.4^{ab}	72.9 ± 1.0^{a}
PEF-treated (T_{in} = 45 °C)	n.d.	n.d.	2.0 ± 0.7^{b}	72.6 ± 4.0^{a}

n.d. not detected.
ΔH_1 and ΔH_2 refer to the thermal events at 50–61 °C and 61–82 °C, respectively.

3.3. PEF and thermal sensitivity of marker microorganisms

P. trivalis and *E. gerundensis* were inoculated into diluted (dm of 3 g/100 g sample, 2 mS/cm, pH 6.7) and pasteurized (85 °C, 5 min) PPE, and then subjected to PEF and thermal treatments. After optimization (data not shown), the selected PEF parameters were E_{el} = 24 kV/cm, f = 50 Hz, τ = 20 µs, t_t = 126 µs, corresponding to a measured specific input energy of W_s = 144 kJ/kg and reliable square-shape pulses (Figure A.1). Various substrate inlet temperatures (T_{in} = 25, 30, 35, 40, and 45 °C) were tested. An increase in temperature of 20 °C due to ohmic heating was measured under these conditions. Therefore, thermal treatments at 45, 50, 55, 60, and 65 °C were conducted to study the thermal contribution during PEF treatments starting at inlet temperatures of 25, 30, 35, 40, and 45 °C, respectively.

The levels of PEF inactivation increased with higher inlet temperatures for both *P. trivalis* (from 2.6 ± 0.4 to 5.1 ± 1.0 log reduction) and *E. gerundensis* (from 2.0 ± 0.3 to 4.1 ± 0.8 log reduction), as displayed in Fig. 2. No thermal inactivation was detected at temperatures ≤ 50 °C for either species. However, PEF treatments at inlet temperatures T_{in} of 25 and 30 °C (resulting in an outlet temperature T_{out} of 45 and 50 °C, respectively) caused significant inactivation (≥ 2 logs), attributed solely to electrical effects. Both species exhibited thermal inactivation at 55 °C (1.9 ± 0.2 and 1.8 ± 0.1 log reduction for *P. trivalis* and *E. gerundensis*, respectively). They demonstrated similar thermal sensitivity, with critical inactivation temperatures T_{crit} calculated at 50.9 ± 0.4 °C [95% confidence interval: 49.7–52.0] for *P. trivalis* and 50.8 ± 0.2 °C [95% confidence interval: 50.3–51.4] for *E. gerundensis*. Therefore, the inactivation observed at T_{in} = 35 °C can be attributed to both thermal and electrical effects. Previous studies also noted combined effects in the temperature range of 50–55 °C (Alirezalu et al., 2020; Schottroff & Jaeger, 2020; Sharma et al., 2014). At higher inlet temperatures (40 and 45 °C), the observed inactivation levels are ≥ 4 logs and thermal effects dominate electrical effects.

Horlacher et al. (2024) recently investigated a similar system with 5.1% plant-based protein content (oat and pea), a conductivity of 2.3 mS/cm and pH 7.0. They applied moderate field strengths (9–10 kV/cm, 50 Hz, 20 µs) combined with mild preheating (35, 40 and 45 °C) to a cocktail of surrogate bacteria (*E. coli* and *Listeria* sp). For a W_s of 96–102 kJ/L and an increase in temperature of ~15 °C, inactivation levels of 0.9, 1.6, and 4.8 logs were achieved for T_{in} of 35, 40 and 45 °C, respectively. At 35 and 40 °C, the reduction was likely attributed solely to electrical effects, with reduction levels (0.9 and 1.6 logs) slightly lower than those observed in the current study. These differences can be explained by the lower PEF intensity applied. At 45 °C and an increase of ~15 °C, the substantial jump to a similar reduction level (4.8 logs) as obtained in our study is most probably attributed to added thermal effects. Thamsuaidee et al. (2024) applied PEF treatment (24 kV/cm, 100 kJ/L, 30 °C) on an oat-based beverage (10 g/100 g, 2 mS/cm, pH 6.3) inoculated with *Lactiplantibacillus plantarum* and achieved higher reduction level (5.6 logs) compared to this study. Finally, Sharma et al. (2014) studied the effect of T_{in} (4, 30, 40, 45, 50 and 55 °C) on PEF-induced inactivation of *Pseudomonas aeruginosa* in whole milk (3.9 mS/cm). Using treatment parameters of 23 kV/cm, 60 Hz, 20 µs and 101 µs, they achieved lower reduction levels (0, 1.2, 2.3 and 3.0 log at 4, 30,

40 and 45 °C, respectively). The most probable explanation is the lower E_{el} and t_t values compared to this study.

3.4. Thermal properties of the substrate PPE before and after PEF

The DSC thermogram of the reference PPE reveals three distinct endothermic peaks (Figure A.2): a minor peak ΔH_1 (0.8 ± 0.2 J/g dm) detected at temperatures between 50 and 61 °C, a major peak ΔH_2 (4.2 ± 0.7 J/g dm) between 61 and 82 °C, and a second minor peak ΔH_3 (0.2 ± 0.1 J/g dm) between 82 and 88 °C (Table 2). Ma et al. (2017) analyzed DSC characteristics of yellow pea flour and coarse fibers, reporting a major peak (0.68 ± 0.05 J/g) at 81.9 ± 0.4 °C and a minor peak (0.02 ± 0.00 J/g) at 86.2 ± 0.2 °C for the flour. Specifically for the coarse fibers, next to a peak at 86 °C, an additional peak at 50–60 °C was detected. Therefore, in Figure A.2, the first peak (ΔH_1) corresponds to fibers or pea starch gelatinization, whereas the second peak (ΔH_2) is associated with protein denaturation (Zink et al., 2024). Moreover, Kuang et al. (2023) performed DSC analysis on PPI and attributed two characteristics denaturation endothermic peaks at 71 and 84 °C for 7S and 11S globulins, respectively. Similarly, O'Kane et al. (2004) reported denaturation temperatures of 69.9–71.8 °C and 87 °C for 7S and 11S globulins, respectively, and Pelgrom et al. (2015) found 87.7 ± 0.8 °C for PPI (NUTRALYS® F85G). Variations in temperature values may arise from different pea varieties or heating rates used during analyses (Li & Ganjyal, 2017; Mession et al., 2013).

Table 2 compares the thermal properties of PPE before and after dilution and PEF treatments. During PEF treatment, a temperature increase $\Delta T = 20$ °C was measured due to ohmic heating. Consequently, PEF treatments at $T_{in} = 35$, 40 or 45 °C were selected for DSC analysis under the assumption that PEF at $T_{in} = 25$ and 30 °C would not significantly impact the thermal properties of PPE ($T_{out} \leq 50$ °C), PEF at $T_{in} = 35$ °C ($T_{out} = 55$ °C) would partially affect the first peak, PEF at $T_{in} = 40$ °C ($T_{out} = 60$ °C) would substantially affect the first peak, and PEF at $T_{in} = 45$ °C ($T_{out} = 65$ °C) would further impact the second peak. As expected, dilution had no significant impact on the thermal properties of PPE. Similarly, PEF treatment at $T_{in} = 35$ °C showed no significant influence. However, PEF treatment at $T_{in} = 40$ °C and 45 °C caused significant decreases in the reaction enthalpy values ΔH_1. Additionally, PEF treatment at $T_{in} = 45$ °C resulted in a significant reduction in ΔH_2 (–39%), likely due to a $T_{out} = 65$ °C exceeding 61 °C, coupled with an observed decrease in gelling capacity during rheology tests (data not shown). Based on these findings, PEF treatment starting at a T_{in} of 40 °C was selected for further tests, showing high microbial inactivation (*P. trivialis*: 4.3 ± 0.8 log reduction and *E. gerundensis*: 3.7 ± 0.4 log reduction), while preserving the thermal properties of the PPE protein fraction (ΔH_2).

Table 3
Growth rate μ and lag phase duration λ after PEF treatment $T_{in} = 40$ °C, extracted from the biogrowth simulation as shown in Figs. 3 and 4. Values are presented as averages of triplicates (n = 3) ± SD. Different letters indicate significant differences ($p < 0.05$).

Isolates	*5 °C storage*		*10 °C storage*	
	μ ($\log_{10}d^{-1}$)	λ (d)	μ ($\log_{10}d^{-1}$)	λ (d)
P. trivialis	1.5 ± 0.0^{a}	1.6 ± 0.2^{a}	2.5 ± 0.1^{a}	0.7 ± 0.0^{a}
E. gerundensis	1.2 ± 0.1^{b}	1.8 ± 0.2^{a}	2.6 ± 0.0^{a}	1.0 ± 0.1^{b}
Native microbiota	1.1 ± 0.1^{b}	4.0 ± 0.1^{b}	2.0 ± 0.0^{b}	1.3 ± 0.0^{c}

3.5. Storage control and extract stability

The microbial growth of *P. trivialis* and *E. gerundensis* following PEF treatment (T_{in} = 40 °C) and subsequent storage under refrigerated conditions (5 and 10 °C for 14 days) is depicted in Fig. 3. Growth parameters extracted from the model (equation (2)) are summarized in Table 3.

As expected, the growth rate of both bacterial species increased at 10 °C in comparison to 5 °C, and a prolonged lag phase at lower temperatures was observed (Table 3). At 10 °C, both species exhibited similar growth rates and reached the spoilage level (i.e. 7 log/mL) within 3 days (Fig. 3). In contrast, at 5 °C, *P. trivialis* grew faster and reached the spoilage level one day earlier than *E. gerundensis*. Previous studies have demonstrated the efficacy of PEF in extending the shelf-life of food liquids. For instance, Timmermans et al. (2016) applied PEF (24 kV/cm, 100 Hz, 55 kJ/kg, 40 °C, ΔT = 18 °C) to yeast in fruit juice (3 mS/cm, pH 3.6) and observed an extension of the lag phase by 8.4 and 16 days at 7 and 4 °C, respectively. Additionally, De Gol et al. (2024) applied PEF (28 kV/cm, 120 Hz, 226 µs, 115–120 kJ/kg, 30 °C) to bacteria inoculated into *Chlorella vulgaris* suspensions (2 g/100 g, 0.7

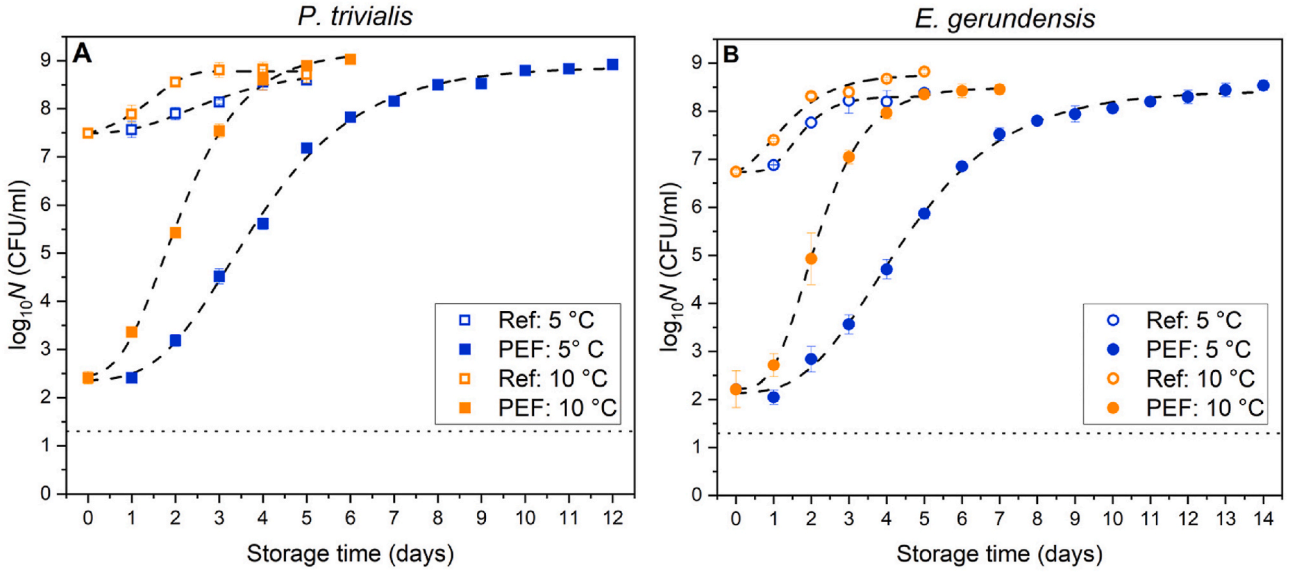

Fig. 3. Microbial growth of untreated versus PEF-treated PPE inoculated with *P. trivialis* (A) and *E. gerundensis* (B) at T_{in} = 40 °C. Incubation was done at both 5 and 10 °C for 14 days. PEF treatments conditions were 24 kV/cm, 50 Hz, 126 µs in a PPE substrate of 2 mS/cm and pH 6.7. The initial count N_0 was ~10^6 CFU/mL, and the detection limit was 1.3 log (dot lines). Data points are averages of three individual measurements (n = 3) ± SD. Open and Filled symbols represent references and PEF-treated samples, respectively. For visualization purposes, growth fitting was done on the average data points with biogrowth (dashed lines, section 2.8).

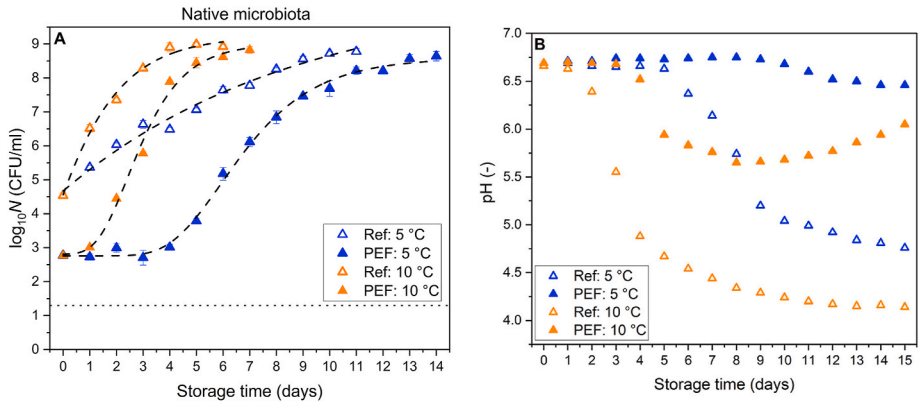

Fig. 4. A: Microbial growth of untreated versus PEF-treated (T_{in} = 40 °C) PPE native microbiota. Incubation was done at both 5 (▲) and 10 °C (▲) for 14 days. PEF treatments conditions were 24 kV/cm, 50 Hz, 126 μs in a PPE substrate of 2 mS/cm and pH 6.7. The initial count N_0 was ~10^6 CFU/mL, and the detection limit was 1.3 log (dot lines). Data points are averages of three individual measurements (n = 3) ± SD. Open and filled symbols represent references and PEF-treated samples, respectively. For visualization purposes, growth fitting was done on the average data points with biogrowth (dashed lines, section 2.8). B: pH evolution during storage at 5 and 10 °C for 15 days.

mS/cm, pH 6.5). They noted an increased lag phase for *Pseudomonas guariconensis* and *Lactococcus lactis* during storage at 10 °C. In the current study, the PEF treatment on *P. trivalis* and *E. gerundensis* appears to be ineffective for extending the lag phase and reducing the growth rate. Tests on reference samples with an initial cell concentration of 2–3 logs are recommended for improved comparability.

In addition to controlled challenge tests on *P. trivalis* and *E. gerundensis*, PEF treatment and storage control were also performed on the native microbiota of the PPE (Fig. 4), with growth parameters shown in Table 3. The lag phase duration was significantly longer compared to the isolates. During storage at 5 °C, no growth was observed for the 4 first days, and the pH was stable for 10 days (Fig. 4). Additionally, the growth rate during storage at 10 °C was significantly lower than the data obtained for the isolates. The positive impact of PEF treatments on preservation was therefore more pronounced for the native microorganisms, making the psychotrophic, substrate-isolated *P. trivalis* and *E. gerundensis* species relevant fast-growing candidates for challenge tests.

4. Conclusion and perspective

Mildly extracted and native pea proteins have significant potential for use in plant-based meat substitutes. However, they exhibit rapid spoilage even under refrigerated conditions. In this study, pilot-scale PEF treatments were applied to inactivate psychotrophic spoilage microorganisms isolated from pea protein extract. The two studied species exhibited similar resistance at purely thermal or electrical treatments. PEF treatment effectively reduced microbial counts by approximately 3 logs, at processing peak temperatures below 50 °C, through purely electrical effects and without thermally modifying the pea proteins. However, to achieve inactivation levels higher than 4 logs, a processing peak temperature of 60 °C was necessary, where the inactivation was mainly attributed to thermal effects. Thus, the advantage of PEF over mild thermal treatment (at 60 °C) for higher inactivation levels remains to be demonstrated. While PEF may preserve or even improve protein functionality, further investigations are needed to confirm this potential benefit.

Additionally, pea protein extracts with the native microbiota were treated with PEF to validate the system in practical application. The lag phase of the native microbiota and general substrate stability were more extended compared to substrates inoculated with the isolated species, suggesting their suitability for challenge tests. Mild extraction, followed by temporary preservation of protein extracts, indicated energy savings and retention of protein functionality compared to traditional intense extraction and spray drying methods. Future research should focus on optimizing PEF treatments for higher concentration and higher electrical conductivity substrates, which is technically still challenging and essential for industrial scale implementation.

CRediT authorship contribution statement

Cora De Gol: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Nicolas Etschmann:** Validation, Investigation, Formal analysis. **Marcel H. Zwietering:** Supervision. **Heidy M.W. den Besten:** Writing – review & editing, Supervision. **Michael Beyrer:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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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Appendices

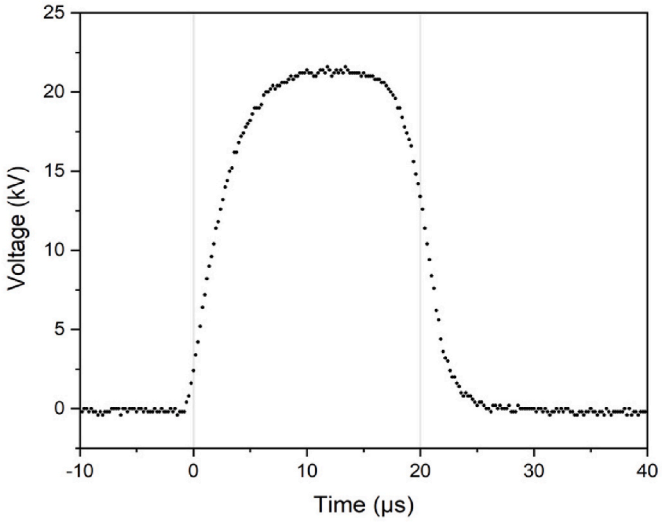

Fig. A.1. Monitoring of the pulse shape during PEF treatment at $E_{el} = 24$ kV/cm, $f = 50$ Hz, $\tau = 20$ μs, $t_t = 126$ μs on the PPE substrate (dm of 3 g/100 g sample, 2 mS/cm, pH 6.7).

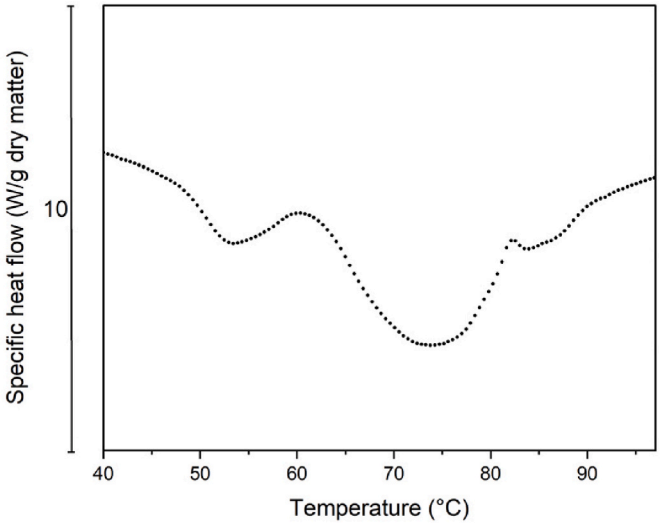

Fig. A.2. DSC thermogram (scanning from 20 °C to 100 °C at a heating rate of 0.5 °C/min) of the reference PPE (n = 3).

Data availability

Data will be made available on request.

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