Korean J. Chem. Eng., 36(2), 272-280 (2019)
DOI: 10.1007/s11814-018-0191-9

pISSN: 0256-1115
eISSN: 1975-7220

The potential use of pulsed electric field to assist in polygodial extraction from Horopito (*Pseudowintera colorata*) leaves

Joanna Nadia, Marliya Ismail, Kaveh Shahbaz, and Mohammed Farid†

Department of Chemical and Materials Engineering, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

(*Received 30 July 2018 • accepted 14 November 2018*)

Abstract−Horopito (*Pseudowintera colorata*) contains polygodial as an active compound that has many health beneficial properties. The potential of applying a continuous pulsed electric field (PEF) as a pretreatment step prior to solvent extraction of polygodial from Horopito leaves was studied. Horopito leaves suspended in water were subjected to PEF at electric field intensity ranging from 5 to 25 kV/cm and pulse frequencies from 200 to 800 Hz. The interaction between electric field intensity and pulse frequency was found to have a significant role in extraction. Both electro-permeabilization and temperature increase from treatment caused some polygodial leaching from the leaves prior to solvent extraction. The study revealed that PEF at low electric field intensity and high frequency is the most effective way to achieve higher solvent extraction yield while minimizing the effect of leaching. The maximum improvement was obtained when PEF at 5 kV/cm and 800 Hz for 348 μs were applied, giving a polygodial extraction yield of about 16.6% higher than that of non-PEF treated leaves.

Keywords: Extraction, Horopito (*Pseudowintera colorata*), Polygodial, Pulsed Electric Field

INTRODUCTION

Horopito (*Pseudowintera colorata*), a native plant of New Zealand, is traditionally used by the indigenous inhabitants of New Zealand, the Māori, and European settlers for a range of medicinal purposes, such as analgesic, antiseptic, digestive tonic, and treatment for skin infections [1]. The plant belongs to Winteraceae family and is recognized as a New Zealand pepper tree due to its aromatic and pungent taste [2]. Sscientific studies on Horopito revealed that the healing property of the plant was due to the presence of polygodial, which is mainly located in the essential oil of the plant leaves. Polygodial is also present in certain other plant species, such as *Polygonum hydropiper* or *Persicaria hydropiper*, *Drimys winteri*, *Tasmania lanceolata* and T. *stipitata*, *Warbugia stuhimannii* and W. *ugandensis*, and *Spilanthes acmella* [3-6]. Polygodial plays important role in antifungal, antibacterial, antifeedant, insecticidal, and anthelmintic activities [5,7-12]. Among these reported characteristics, polygodial is best known as a potent antifungal agent, which have made it a valuable bioactive compound globally. With the prevalence of Horopito in New Zealand, several local companies have developed products containing polygodial extracted from this plant matter. As the application areas of polygodial are widening and thereby its demand, it becomes crucial to investigate and develop an efficient process to extract polygodial from Horopito.

Essential oil containing polygodial can be conventionally extracted from the plant materials by maceration or stirring in organic solvents [8,13-16], infusion using olive oil, Soxhlet extraction using organic solvent [5,17], hydrodistillation [18], and steam distillation [19]. However, the heat sensitive nature of polygodial makes conventional extraction processes that apply heat, such as in steam distillation, unfavorable. On the other hand, the use of organic solvents at lower temperature also has many drawbacks, including long processing time, low extraction yields and selectivity, and the requirement of a large amount of high purity solvent [20,21]. These limitations led to the investigation of new techniques to improve the extraction process of this low-polarity and heat-sensitive compound.

In this sense, authors have previously shown that newly synthesized deep eutectic solvents (DESs) are selective to extracting polygodial from Horopito. Although the yield from DES is comparable to ethanol, it was revealed that the extracted polygodial using DES displayed superior stability and the solvents were reusable for a larger number of cycles compared to ethanol [22]. Elsewhere, supercritical carbon dioxide and pressurized hot water extraction methods have been introduced to improve the extraction yield of polygodial from Horopito and Tasmanian native pepper (*Tasmannia lanceolata*), respectively [23-25]. Both technologies were found to be successful in improving the extraction yield of polygodial from biomass, which opens a window of opportunity to explore the potential of other emerging technologies in this field, such as pulsed electric field (PEF) technology.

PEF technology has emerged as one of the alternative methods to potentially improve conventional extraction of bioactives and natural colorants, nutraceuticals and cosmeceuticals products [26]. During PEF treatment, electric pulses with electric field intensity (E) between 10 to 80 kV/cm are applied to the material to create pores on the treated cells, which is known as electroporation. Thereby, this technology has been found to increase mass transfer rate during

†To whom correspondence should be addressed.
E-mail: m.farid@auckland.ac.nz

extraction by disturbing the membrane structure of the treated cell wall [27]. While the technology is mostly used to enhance the extraction of water-soluble compounds, such as polyphenols and plant pigments [28-30], it can also be applied in non-water soluble compound extraction.

PEF at low electric field intensity (E<10 kV/cm) is commonly used as a pre-treatment step to extraction [31]. Low electric field intensities cause only minor temperature increases, and hence no detrimental effect on bioactives in the treated materials [20,31]. The findings are attributed to the ability of PEF to damage cell membrane of plant tissue at minimum increase in temperature [20]. For this reason, PEF treatment is a promising alternative for the extraction of thermolabile compounds, specifically plant bioactives, which usually degrade during solvent extraction at high temperature.

Numerous studies have been published on the application of PEF for pre-treatment of plant materials, indicating that this emerging technology has been well known for its efficacy in enhancing extraction process. Most of these PEF treatments were conducted in a batch mode and targeted the extraction of water-soluble bioactive compounds [28-30,32-35]. The application of PEF pre-treatment for enhancing the extraction of water-insoluble products, such as lipid of microalgae, olive oil, and patchouli oil, were also reported in the literature [36-39]. While most of the existing PEF-assisted extraction methods have focused on batch mode, a continuous process is more favorable to treat product in large quantities. To our knowledge, continuous PEF treatment to enhance the extraction of non-polar bioactive compounds contained in the essential oil of plant leaves has not been investigated before. Hence, the aim of this work was to investigate the potential of PEF pre-treatment to cause electroporation in Horopito leaves suspended in water, using a continuous PEF unit. In this study, the effects of process-related parameters (electric field intensity and number of pulses) were investigated to enhance the extraction yield of polygodial.

MATERIALS AND METHODS

1. Materials

Dried and milled horopito leaves were provided by Forest Herbs Research Ltd., New Zealand. Particles with size ranges of d ≤0.2 mm (referred to as powder) and 0.2 mm<d≤0.85 mm (referred to as coarse sample) were separated for the experiments using RETSCH sieve shaker and test sieves. The samples were stored under refrigerated conditions until use.

Chemicals used in this research were ethanol (>99.4%, ECP LabChem, Auckland, New Zealand), chloroform (>98%, Avantor, Center Valley, PA, USA), and polygodial standard for HPLC analysis (≥98%, Sigma-Aldrich, St. Louis, MO, USA).

2. The PEF Unit

The PEF unit at the Department of Chemical and Materials Engineering, University of Auckland consists of a high-voltage pulse generator, treatment chamber, data acquisition system, fluid handling and cooling system, and a multimeter, as described in detail by Alkhafaji and Farid [40]. The configuration of the PEF system is shown in Fig. 1.

The PEF treatment chamber has a treatment zone having 12.8 mm in diameter that includes a pair of mesh electrodes made of food grade stainless steel (Fig. 2) [41]. This chamber allows the generation of bipolar square waves for product feed of low electrical conductivity in the treatment zone where the electric field is concentrated. Due to the low electrical conductivity of the slurry used in this work, the treatment zone diameter was increased to 12.8 mm from the original design of 5.0 mm. This innovative cell design kept the high electric field to be far from the electrodes to avoid their corrosion. Additionally, the electrodes aid product mixing inside the chamber [40].

3. Selection of Sample Particle Size

In this work, it was necessary to select the most suitable parti-

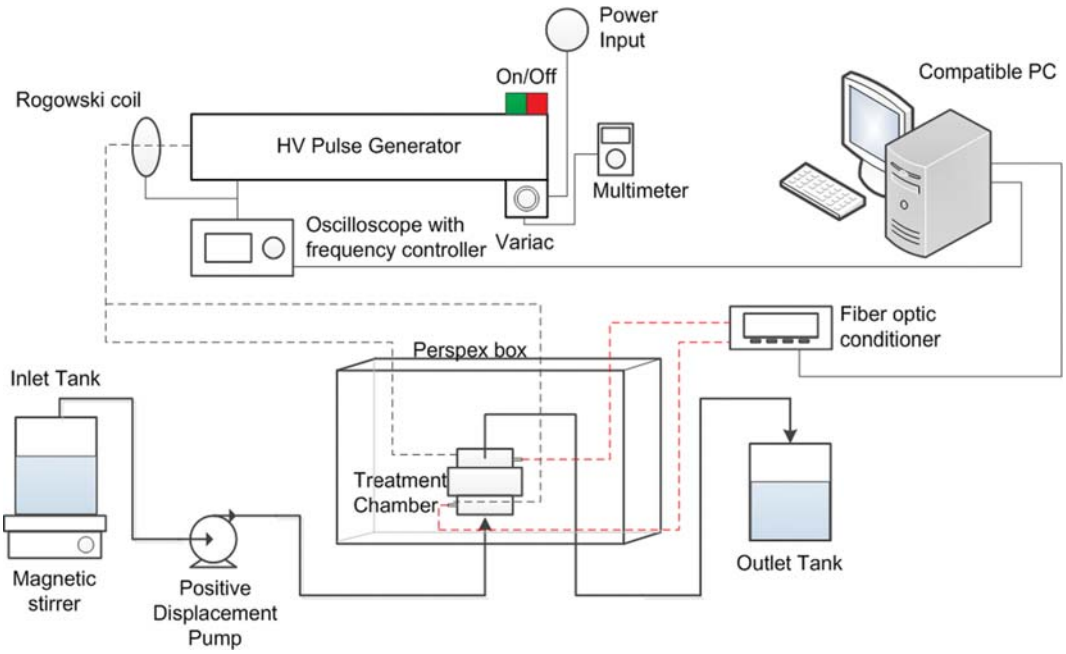

Fig. 1. Configuration of the PEF unit for suspension handling.

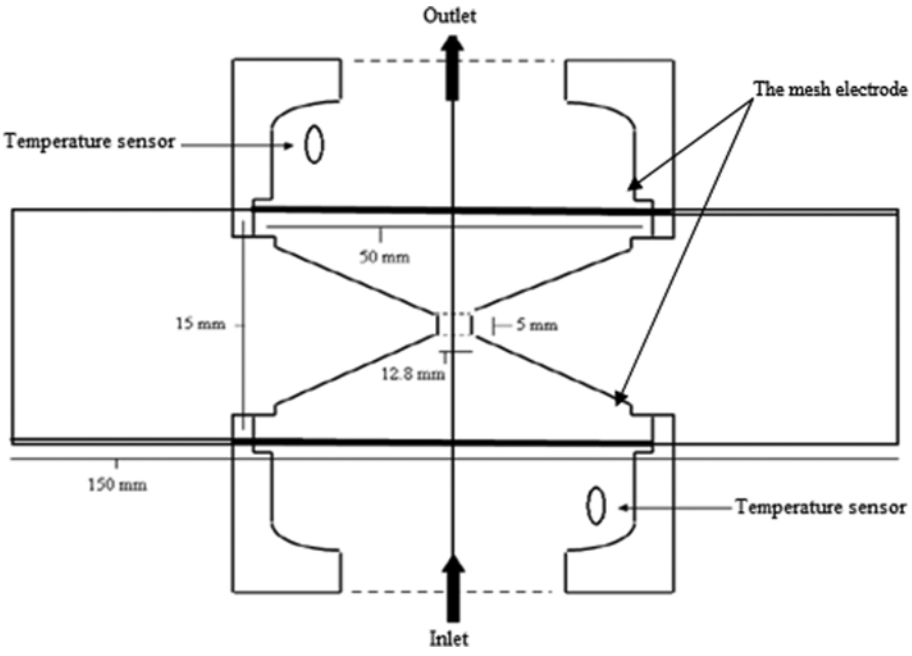

Fig. 2. Schematic diagram of the PEF treatment chamber [41].

cle size range for PEF pre-treatment that enables the particles to be suspended in water, giving a nearly consistent and a continuous flow. The experiments were carried out with stirring at 250 rpm and without stirring (maceration) in solvent. 50 mL of solvent (ethanol) was added to 5 g of Horopito fine ($d \leq 0.2$ mm) and coarse ($0.2 \text{ mm} < d \leq 0.85$ mm) samples separately in a glass beaker. Samples of 0.5 mL solid/liquid mixture were withdrawn after 0.5, 10, 20, 30, 45, 60, 90, 120, 150, and 180 min of contact time, then filtered through 0.45 μm microfilter for subsequent HPLC analysis. For maceration condition, the mixture was shaken gently prior to sampling, to ensure homogeneous polygodial distribution in the liquid phase.

4. PEF-assisted Polygodial Extraction

4-1. PEF Pre-treatment of Horopito Leaves

15 g of coarse sample suspended in 450 mL milli-Q^{TM} water (solid : liquid ratio=1 : 30 (w/v)) was introduced to the PEF unit in each experiment at a flow rate of 5 mL/s. The suspension was agitated constantly while in the feed tank during operation of the PEF unit to ensure proper solid distribution in water. It had a typical electric conductivity of 0.86 mS/cm.

PEF treatment time was calculated using the following equation:

$$T_t = N_p \times N_c \times \tau \quad (1)$$

where N_p, N_c, and τ are the number of pulses, number of circulations, and the pulse width (which is 1.7 μs for the available PEF unit).

The residence time (R_t) and number of pulses (N_p), both in seconds, were calculated based on the following equations:

$$N_p = R_t \times f \quad (2)$$

$$R_t = v/Q \quad (3)$$

where v, f and Q are the volume of the treatment chamber (mL), the frequency of the pulse (Hz), and the flow rate of the product (mL/s), respectively.

The volume of treatment chamber (v, mL) was calculated using Eq. (4) by ignoring the inlet and outlet regions of low electric field:

$$v = \frac{\pi d_t^2 h_t}{4} \quad (4)$$

where d_t and h_t are the diameter (cm) and the depth of the treatment zone (cm), respectively.

Preliminary experiments were carried out at different treatment times and showed no significant effect on extraction for treatment times beyond 348 μs. Therefore, 348 μs was taken as the treatment time for all experiments. Within this treatment time, sample suspension was subjected to different PEF parameters (f=200 and 800 Hz, E=5, 15, and 25 kV/cm). At a specific frequency, the treatment time was maintained at 348 μs by applying more than one recirculation of the sample. After the PEF pre-treatment, the solids (more than 95%) were collected at the outlet port. The remaining solids from the system were flushed with water.

4-2. Extraction of Polygodial from PEF-pretreated and Control Sample

Solid particles after PEF treatment were separated from the water by means of vacuum filtration, and then were mixed with 150 mL ethanol (solid/liquid ratio=1/10 (w/v)) under a stirring at 250 rpm. Ethanol was selected as the extraction solvent in this work to enable immediate HPLC analysis after sampling. 1 mL of the mixture was removed at different extraction times to quantify the amount of polygodial extracted.

The amount of polygodial that would have become diffused in the water (polygodial is sparingly soluble in water) during PEF treatment was also quantified by collecting the first 450 mL of the filtered water obtained during the collection of solid particles. Polygodial in the water was recovered by liquid-liquid extraction using

chloroform, which was proven to have good ability to extract polygodial based on our preliminary experiments, followed by subsequent solvent removal under vacuum condition and dilution of the crude extract using ethanol. The amount of polygodial extracted from solvent extraction and polygodial recovered from the water collected after PEF treatment were quantified separately using HPLC.

For non PEF-treated (control) sample, the same amount of horopito coarse particles/water was prepared and agitated at 250 rpm similar to what was done during PEF treatment; hence polygodial in the solid and liquid parts was extracted according to the same procedure explained.

4-3. Determination of Kinetic Parameters of Polygodial Extraction

To study the kinetics of polygodial extraction, the experimental data of polygodial obtained during post-PEF solvent extraction were fitted to Eq. (5) [42]. It is an exponential model for solid-liquid extraction of different intracellular compound that relates solute diffusion with solvent contact time t (min):

$$Y=Y_{max}(1-e^{-kt}) \quad (5)$$

where Y is polygodial extracted to the solution at time t (mg/g dried leaves); Y_{max} is the equilibrium polygodial concentration, at t=∞ (mg/g dried leaves); and k is the extraction parameters-dependent constant (min^{-1}).

5. Polygodial Quantification

The concentration of polygodial in the extracts was measured using a Shimadzu LC-20AT HPLC unit equipped with LabSolutions software for the data acquisition and analysis. A ZORBAX Eclipse XDB C-18 column 4.6×150 mm, 5 μm column, guarded with a C-18 guard column was used for the compound separation at a controlled temperature of 25 °C. Milli-Q™ water and HPLC-grade acetonitrile (50 : 50, v/v), set at a flow rate of 1 mL/min, were used as the mobile phase. Peak area of polygodial in the samples (measured in UV absorbance units, AU) was quantified at 230 nm with an injection volume of 20 μL. A set of standard solutions of five different polygodial concentrations in ethanol, 0.01, 0.1, 0.2, 0.4, and 0.8 mg/mL, was prepared to establish the calibration curve with an R^2=0.9999.

6. Statistical Analysis

The experiments were repeated at least twice and each sample was analyzed with HPLC in duplicates. Mean values were compared at confidence level of 95% (p≤0.05). Minitab®17 Software was used to perform the statistical analysis, as well as to build the fitted curves for the extraction kinetics study. The variability of the data was expressed as standard deviation, which was presented as error bar on results reported in figures.

RESULTS AND DISCUSSION

1. Particle Size Selection for PEF Pre-treatment

Prior to studying PEF extraction of polygodial, selection of the particle size range of Horopito leaves for PEF treatment was imperative. Particle size of solids plays a considerable effect on the kinetics of extraction. In this sense, it was important to understand polygodial extraction kinetics with different particle sizes. The extraction kinetics of fine (d≤0.2 mm) and coarse (0.2 mm< d≤0.85 mm) dried Horopito particles with stirring at 250 rpm and without stirring (maceration) in ethanol are shown in Fig. 3. There was significant difference in the amount of polygodial extracted in the four conditions.

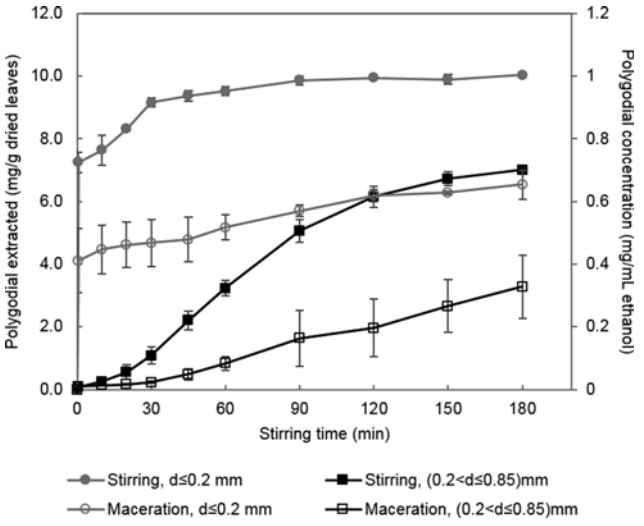

Fig. 3. Polygodial extraction kinetics curves of fine and coarse horopito particles in ethanol.

It is obvious from Fig. 3 that a large amount of polygodial was released in a short time (0.5 min) as soon as the solvent was in contact with the fine particles, whereas the concentration of polygodial extracted from coarse particle increased gradually over time in the solvent. As expected, equilibrium concentrations were reached in shorter time with stirring, and the final concentration achieved with fine particles was higher than that of coarse particles. It has been proven in many studies on the extraction of essential oils from plant materials that particle size controls the diffusion of solute to solvent [20,42-45]. Smaller particle size increases the mass transfer surface area, decrease diffusion path, and also allows oil to be released easily from the broken cells [43,45,46]. Particularly, for this research, the fine particle size (d≤200 μm) is within the oil cell size of Horopito leaves, which is approximately 50 μm [47]. Hence, it was indeed possible that some of the oil cells in the sample were already broken during the grinding process. The stirring in this experiment also added extra driving force for releasing the polygodial-containing essential oil, as the extraction was controlled by diffusion process. It also allowed the solute to be more homogeneously distributed in the solvent, which is indicated by the smaller error bars compared to those of maceration.

In PEF-assisted extraction, neglecting the effect of particle size, could lead to a false conclusion about the benefit of PEF pre-treatment. While it is true that PEF has been proven to enhance the extraction of solutes from plant materials by selectively damaging biological tissue resulting in cell disintegration [48], mechanical grinding at an increased intensity can also produce high cell disintegration index. This can be seen in the polygodial extraction yield achieved by fine particles (around 10 mg/g dried leaves) that is higher than the value achieved by coarse particles (around 7 mg/g dried leaves). Additionally, it was reported that the effect of PEF was negligible on the cell disintegration index of apple and carrot mash when the size of particle was less than or the same as the tar-

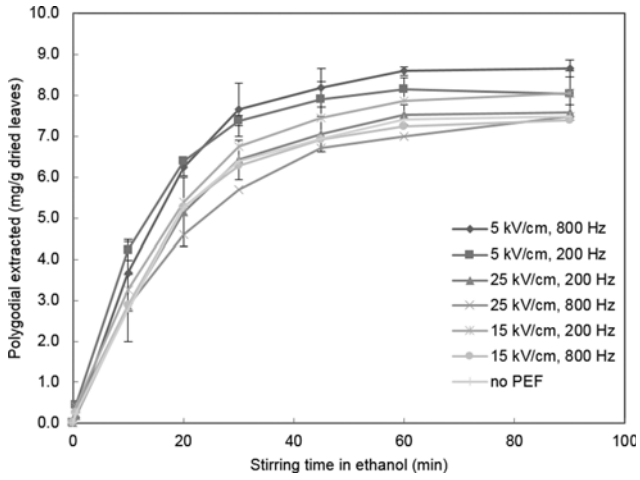

Fig. 4. Extraction kinetics curves of polygodial in ethanol (solid/liquid ratio=1 : 10 (w/v)), subsequent to PEF pre-treatment time for 348 μs.

geted cell size [49]. Accordingly, coarser leaf particles (0.2 mm<d≤ 0.85 mm) at 250 rpm stirring condition were selected to study the effect of PEF on extraction kinetics.

2. PEF-assisted Extraction of Polygodial

2-1. Effect of PEF Pre-treatment on Extraction Kinetics

In Fig. 4, polygodial extraction kinetics curves of non PEF-treated and PEF treated Horopito samples under different conditions (E= 5, 15, and 25 kV/cm and f=200 and 800 Hz) are presented. As mentioned in section 2.4.1, the PEF treatment time for this experiment was set at 348 μs. This was based on the result of a preliminary experiment that showed longer treatment time (1,044 μs) at PEF conditions E=5 kV/cm and f=800 Hz did not increase polygodial content any further. El-Beghiti, et al. [50] and Brunton and Luengo [51] also revealed that the response of PEF-treated cells depends strongly on electric field strength and rather weakly on treatment time. Consequently, the investigations were carried out only to explore the effect of electric field intensity and the number of pulses subjected per second (which is expressed by the frequency) on polygodial extraction while keeping treatment time constant. However, the actual contact time (or residence time) for PEF experiments of lower frequency was longer than high frequency PEF, due to more recirculation applied to reach the targeted treatment time.

The trends of the extraction kinetic curves are the same for all conditions in general, as shown in Fig. 4. It agrees with the previous investigation on batch PEF pre-treatment of borage leaves, which was followed by aqueous extraction of polyphenols [28]. The investigators reached a conclusion that PEF treatments did not affect the shape of polyphenols extraction curves; all of them fitted an exponential model asymptotically rising with time to a maximum value for all treatment conditions, similar to the non PEF-treated sample. Nevertheless, the application of a PEF pre-treatment to the borage leaves increased the polyphenols yield and reduced the time required to reach a given yield [28]. A similar trend was observed in this experiment, where E=5 kV/cm, f=800 Hz conditions of PEF made a reduction in the time needed to achieve a specific concentration as well as the final yield when equilibrium was attained.

Further, all of the data points from the extraction kinetics study up to 90 min were fitted to Eq. (5), to analyze the data in more detail. Accordingly, this equation enabled the evaluation of the extraction rate constant (k) and the maximum concentration of polygodial reached at equilibrium (Y_{max}), considering the boundary conditions of t=0 to t and Y=0 to Y=Y_{max}. The goodness-of-fit of the data is expressed as the standard error of regression (S), with a value that should be ≤2.5 to produce sufficiently narrow 95% prediction interval. Despite the extraction curves fitting well to the equation, k in this research is a not reaction-related constant; it is a mass transfer coefficient directly proportional to the diffusivity that is affected by the molecular shape and size, and the properties of the solvent [42,52].

Results presented in Table 1 show the mathematical model that was adjusted to Eq. (5) has a good fit to the experimental data (0.22<S<0.43). Games-Howell's pairwise comparison on the *k*-values obtained indicated no statistically significant difference between the extraction rate constants. This observed trend was in agreement with El-Beghiti et al. [50] and Segovia et al. [28], who also reported that there was no significant effect of the electric field intensity on rate constants of sugar extraction from sugar beets or polyphenols from borage leaves, probably because the cell membranes treated with PEF were already electro-permeabilized at lower

Table 1. Extraction kinetic parameters of non PEF-treated and PEF-treated Horopito leaves

E (kV/cm)	f (Hz)	$Y_{max,\ average}$ (mg/g dried leaves)	Y_{max} range*	$k_{average}$ (min^{-1})	k range*	SSE	MSE	S
5	200	8.13	7.74-8.53	0.076[a]	0.067-0.084	0.77	0.06	0.24
	800	8.84	8.71-8.96	0.061[a]	0.055-0.067	1.73	0.13	0.36
15	200	8.16	8.11-8.21	0.054[a]	0.050-0.058	0.66	0.05	0.23
	800	7.55	7.34-7.77	0.057[a]	0.050-0.065	1.00	0.08	0.28
25	200	7.87	7.79-7.94	0.064[a]	0.053-0.075	2.01	0.16	0.39
	800	7.59	7.19-7.98	0.048[a]	0.032-0.061	2.26	0.17	0.42
Control		7.65	7.60-7.70	0.054[a]	0.052-0.057	0.80	0.06	0.25

E: electric field strength, f: frequency, SSE: sum of squared errors of prediction, MSE: mean squared error, S: standard error of the regression

*The confidence level of the range reported was 95% (95%CL)

Values with the same superscript letters are not significantly different (p≤0.05)

experimented electric field intensities.

On the other hand, our results contradict the results reported by Puértolas et al. [30] on the extraction of anthocyanins and total phenols in crushed Cabernet Sauvignon and Merlot grape varieties. The authors reported a significant increase of the *k*-values at electric field intensities higher than 2 kV/cm, which may be due to the particle size of the PEF-treated product. In their study, crushed grapes without controlled particle size were used. While in fact, particle size plays an important role in the effective diffusion of the solute to solvent during extraction as shown in section 3.1. According to Fick's Law, diffusivity is inversely proportional to the square of the particle size [53]. Therefore, the value of *k* will increase significantly as particle size is reduced. Compared to their work, the size range of leaf particles that was used in this study was above the size of the oil cell of horopito, which can be a possible reason for *k*-values that were almost independent of either electric field intensity or frequency in this case.

2-2. Effect of PEF Variables on the Extraction Yield of Polygodial

To study the effect of PEF pre-treatment on the final extraction yield, the amount of polygodial extracted after one hour of stirring PEF-treated horopito leaves in ethanol was compared, as presented in Fig. 5. One hour was selected for the comparison because the maximum yield for different pre-treatments was already reached in this duration for most of the studied conditions. The mean values from duplicate experiments were then compared with Games-Howell's pairwise comparison test at a 95% confidence interval ($p \leq 0.05$). When the sample was subjected to electric field of low intensity (5 kV/cm), PEF created a significant increase in polygodial extraction yield from 7.360±0.075 (non PEF-treated sample) to 8.134±0.284 and 8.585±0.100 mg/g dried leaves at pulse frequencies of 200 Hz and 800 Hz, respectively. However, at f=800 Hz, the results became significantly lower when the electric field intensity was elevated to 15 and 25 kV/cm. On the other hand, at f=200 Hz, the extraction yields between 5, 15 and 25 kV/cm were not significantly different. This observed trend indicates that it was neither electric field intensity nor frequency as a single factor that affected the result, but more likely the interaction of the two factors. As predicted, the result of ANOVA presented in Table 2 shows that it was the interaction between electric field intensity and frequency that was significant to the extraction yield ($p \leq 0.05$). It therefore explains why the increase in frequency at 15 and 25 kV/cm created significant difference in yield, whereas for 5 kV/cm the effect was not significant.

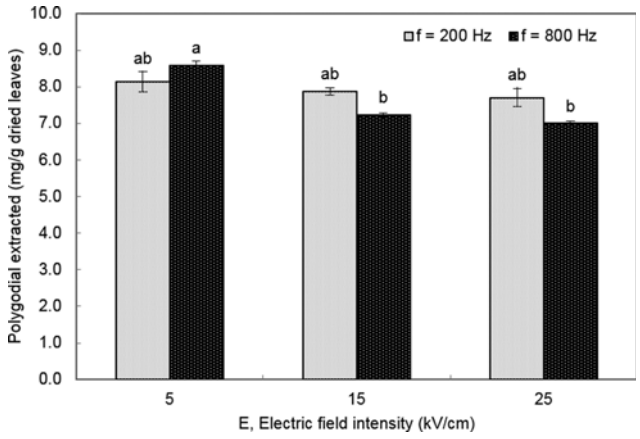

Fig. 5. Effect of electric field intensity and frequency of PEF treatment (treatment time=348 μs) to the extraction yield of polygodial from horopito leaves reached after 1 hour of extraction in ethanol. Different letters above the bars indicate significant differences between the mean values ($p \leq 0.05$).

Many studies on the application of PEF as pre-treatment prior to solvent extraction reported that higher electric field strength resulted in higher cell disintegration index or higher extraction yield [28-30,54,55]. However, it does not necessarily mean that higher electric field intensity always contributes positively in PEF-assisted extraction. Note that the former studies were conducted in batch mode at electric fields of no more than 5 kV/cm. PEF at low intensity (0.1-5 kV/cm) was known to be sufficient to create pores on plant cells, resulting in non-thermic extraction of bioactives from solid food. This was attributed to the larger size (≈100 μm) of plant cell compared to microbial cell (≈1-10 μm) [56]. Particularly for Horopito leaves, the oil cells are ellipsoidal with volume ranging from approximately 100,000 to 150,000 μm^3 [47], which corresponds to cell size of less than 100 μm, but still much bigger than microbial cell. In this study, it is possible that PEF at 5 kV/cm was sufficient to make a significant improvement compared to the non PEF-treated sample because the critical transmembrane potential was attained at that electric field strength. Thus, it is not necessary to treat with higher electric field strength, as other additional effects, such as electrical breakdown or degradation of the target compound might occur.

Table 2. Analysis of variance of the first-order model for the extraction of polygodial from PEF pre-treated Horopito leaves in ethanol (factor(s) with $p \leq 0.05$ are significant)

Source		DF	Adj SS	Adj MS	F-value	p-Value
Regression		3	3.0035	1.0115	14.21	0.001
	E (kV/cm)	1	0.0023	0.0023	0.03	0.863
	f (Hz)	1	0.2241	0.2241	3.15	0.114
	E (kV/cm)*f (Hz)	1	0.6659	0.6659	9.35	0.016
Error		8	0.5695	0.0712		
	Lack-of-fit	2	0.4093	0.2046	7.67	0.022
	Pure error	6	0.1602	0.0267		
Total		11	3.6040			

Table 3. Temperature data during PEF pre-treatment (total treatment time=348 μs)

f (Hz)	E (kV/cm)	T_{inlet} (°C)	T_{outlet} (°C)	ΔT (°C)
200	5	23.0±0.5	23.5±0.5	0.5^{e}
	15	20.6±0.8	25.0±0.9	4.4^{d}
	25	23.0±0.6	41.1±0.2	18.1^{b}
800	5	23.1±1.2	23.9±0.9	0.8^{e}
	15	22.2±1.5	30.4±2.5	8.2^{c}
	25	21.6±1.9	49.3±4.0	27.7^{a}

Values with the same superscript letters are not significantly different ($p \leq 0.05$)

2-3. Effect of Temperature During PEF on Extraction Yield

As shown in Fig. 5, the yield of polygodial got interestingly reduced when both frequency and electric field were increased. Therefore, it was worthwhile to investigate the PEF pre-treatment step and the solvent extraction step further. In this study, water acted as the suspending medium of the particles in the continuous PEF system while ethanol was used as the extraction solvent. It was hypothesized that temperature that was generated due to ohmic heating during PEF pre-treatment could have an effect on extraction yield. Table 3 shows the temperature increment during the different PEF conditions. Temperature clearly increases considerably when both electric field and frequency increase. This implies that water could have dissolved polygodial partially, and any significant temperature during PEF treatment would enhance the release of a small part of polygodial to water. In addition, increase in temperature during PEF treatment can also cause degradation of heat sensitive compounds like polygodial.

The amount of polygodial that got leached out to water and extracted to ethanol is presented in separate columns in Table 4. At 5 kV/cm, the temperature increment of treatment done at 200 Hz and 800 Hz was not significantly different, and the amount of polygodial that leached to the water was also not too different than the non-PEF-treated sample. As a result, there was no release of polygodial, which has limited solubility and stability in water as the suspending medium. The results also explain why the amount of polygodial that was recovered by ethanol at E=25 kV/cm, f=800 Hz was significantly lower than that of PEF treatment at milder conditions. Thus, when higher frequency and electric field intensity were applied, the electroporation was followed by the dissolution and degradation of polygodial to the water due to temperature rise, and consequently less polygodial was available in the PEF-treated leaves when extraction with ethanol was done. These observations are also supported by Goettel et al. [36] who reported that PEF treatment led to the spontaneous release of soluble components when they applied PEF at 23-43 kV/cm and 1.0 to 5.5 Hz in continuous mode to disrupt the cells of fresh water microalgae suspended in water. This also agrees with a study on the ion leakage from onion tissue as a result of electroporation, where the authors concluded that PEF pre-treatment with high electric field intensity applied with lower number of pulses was more efficient to increase the rate of plant tissue permeabilization in extraction processes [57].

Table 4. Amount of polygodial recovered from horopito leaves after 1 hour of extraction in ethanol and fraction that leached to water during suspension in water

E (kV/cm)	f (Hz)	Polygodial recovered (mg/g dried leaves)	
		Extraction in ethanol	Leaching to water
5	200	8.134±0.284^{ab}	0.0103±0.0048^{cd}
	800	8.585±0.1^{a}	0.0078±0.003^{cd}
15	200	7.859±0.097^{ab}	0.0123±0.0038^{cd}
	800	7.231±0.053^{b}	0.0093±0.003^{cd}
25	200	7.684±0.23^{ab}	0.0324±0.0015^{c}
	800	6.981±0.072^{b}	0.0098±0.0039^{cd}
Control		7.36±0.075^{b}	0.0039±0.0006^{d}

Values with the same superscript letters are not significantly different ($p \leq 0.05$)

On the other hand, direct, continuous PEF extraction at high electric field intensity has been reported to improve the extraction yield of bioactive compounds, as long as the suspending medium is the solvent itself. The first high intensity PEF extraction to extract non-polar compound contained in white rot fungus (*Inonotus obliquus*), with electric field intensity from 10 to 80 kV/cm and pulse frequency of 600 Hz, showed that the extraction yield kept increasing as the electric field intensity was increased up to 60 kV/cm at treatment time of 4 μs. Therefore, there is a high possibility that, if the PEF treatment was integrated with the extraction step by replacing water with solvent in which polygodial has high solubility, higher extraction yield will be observed at high electric field intensities. This hypothesis is supported by ANOVA done on the leaching effect of PEF (data not shown), which shows that electric field intensity becomes a significant variable to the leaching effect, along with the interaction between electric field intensity and frequency. However, the heat-sensitive nature of polygodial has to be taken into account when horopito leaves are to be treated with PEF at both high electric field intensities and frequencies.

CONCLUSIONS

The results of the investigation showed that continuous PEF is a potential technique to pre-treat coarse horopito leaves prior to solvent extraction to enhance the extraction yield. Continuous PEF pre-treatment at low electric field intensity (5 kV/cm) and high frequency (800 Hz) was found to be the best combination to improve the extraction yield by 16.6%. The interaction between the electric field intensity and the pulse frequency was the significant factor that affected the extraction yield in ethanol. The extraction yield was negatively affected at both high electric field intensity and pulse frequency, possibly due to the concurrent increase of temperature that caused diffusion of polygodial into the water prior to solvent extraction. Moreover, electric field intensity also contributed significantly to leaching of polygodial to the suspending medium during continuous PEF treatment. It is therefore suggested to operate the PEF unit at low electric field intensity with either high or low frequency, although low frequency is preferred in many

cases to minimize heating.

In addition, it is worthwhile to explore PEF application at high electric field intensity and frequency, considering the temperature increase up to a certain extent as a positive attribute. In this case, it is also imperative to consider an appropriate solvent in the PEF treatment that is conducive for polygodial extraction than suspending in water.

ACKNOWLEDGEMENTS

The authors would like to thank Forest Herbs Research Ltd. for providing the leaf samples and Indonesia Endowment Fund for Education (LPDP RI) for the financial support given for the first author during her postgraduate study. Further, this research was carried out as part of the Food Industry Enabling Technologies (FIET) program funded by the New Zealand Ministry of Business, Innovation and Employment (contract MAUX1402).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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