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Pulsed Electric Fields (PEF) as hot air drying pre-treatment: effect on quality and functional properties of saffron (*Crocus sativus* L.)

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Abstract

This study aimed to investigate the use of pulsed electric fields (PEF) (2 kV cm^{-1} ; 1.5 KJ kg^{-1}) as pre-treatment to the hot air drying process for the improvement of quality and functional properties of saffron. Analysis of flavouring, aroma and colouring strength ($E1\%_{\lambda_{\text{max}}}$), crocins pattern (UHPLC-MS/MS), total polyphenol content (TPC) and antioxidant activity (AOA) were performed after processing and during aging at room temperature. Microstructure of fresh and processed stigmas was also assessed.

PEF decreased ($\approx -41\%$) the flavour, aroma and colouring strength of stigmas and negatively influenced the safranal production during drying; nevertheless, stigmas pre-treated by PEF (PD) after 3 months of aging showed quality properties similar to those of not pre-treated sample (D). Analysis of crocins by UHPLC-MS/MS highlighted a negative effect of PEF on trans-cis crocins ratio and showed poor correlation with colour strength. PEF did not influence the TPC of fresh stigmas but after drying and aging PD showed the highest value among the samples. PEF negatively influenced (-24%) the AOA of fresh stigma while favored its increase during drying. Loss of AOA during aging (up to 86%) was processing independent. Microstructure analysis showed that PEF treatment strongly impaired the stigmas' cells, thus enhancing the extractability of intracellular compounds.

Keywords: saffron, Pulsed Electric Field, quality, crocin isomers, bioactive compounds, microstructure.

1. Introduction

Crocus sativus L. is a perennial bulbous plant belonging to the Iridaceae family with flowers presenting three stigmas, which since ancient times have been widely used as herbal medicine and spice with colouring and flavouring properties (Singh et al., 2010; Del Campo et al., 2009).

Saffron, known as “red gold”, is one of the most expensive spice in the world. It is commonly cultivated in many different countries such as Greece, France, Spain, Italy, Afghanistan, Azerbaijan, Egypt, China, Mexico, Morocco, Turkey (Mansouri et al., 2012) and above all in Iran, which ranks first for cultivated area and production with more than 80% of the total world production (Rameshrad et al., 2018). In the food sector, it is mainly used for its colouring power, bitter taste and unique aroma, attributed primarily to crocins (group of water-soluble carotenoids, originated from the carotenoid zeaxanthin by enzymatic cleavage), picrocrocin (glycoside terpenoid) and safranal (terpenic aldehyde), respectively.

The final content of the aforementioned secondary metabolites is strictly dependent on post-harvesting activities, drying process and storage conditions (Del Campo et al., 2010). Drying, in particular, is an important step in saffron production for both stability and quality purposes since it decreases the moisture content and reduces the water activity to a very low level, induces physical changes, and promotes chemical and enzymatic reactions that lead to the production of the key compounds (i.e. safranal from picrocrocin and crocetin esters) that overall are responsible for the conversion of *Crocus sativus* L. stigmas to the “saffron” spice (Carmona 2006 and 2007).

Different drying methods such as drying at room temperature (Demiray and Tulek, 2012), toasting (Alonso et al., 1998), infrared drying, vacuum drying, microwave drying, convective

drying, and freeze drying (Celma et al., 2008; Maghsoodi et al., 2012), along with the effects of process conditions (e.g. temperature) on saffron secondary metabolites have been variously investigated (Rocchi et al., 2018; Gregory et al., 2005; Del Campo et al., 2010; Maghsoodi et al., 2012; Acar et al., 2015; Carmona et al., 2005). In particular, drying at high temperature (90-110°C) has been showed to increase to a larger extent the saffron coloring strength compared to treatments carried out at lower (70 °C) temperatures. As reported by Carmona et al. (2005), in saffron cells a high amount of secondary metabolites (~20%), is located in chromoplasts so the high temperature employed during dehydration could facilitate crocin liberation, while allowing the most effective breakage to be carried out with the compound with highest glycosylation and degree of symmetry, *trans*-crocin 4.

However, regardless to the drying methodology applied, the current internationally recognised ISO rules (ISO 3632-2:2010 and ISO 3632-1:2011) deal with compositional and quality standards that allow the saffron classification and its grading in three quality categories (category I: high quality; category II: medium quality, category III: low quality) based on the final moisture content (at or below 12 %, computed as % on wet weight), and presence and concentration of saffron crocins and picrocrocin according to simple spectrophotometer evaluations.

Pulsed electric field (PEF) is a novel technology that is increasingly applied in the food sector for non-thermal processing of foodstuff. Although its main application concerns food safety (Toepfl et al., 2014), it is becoming largely used by food industries to assist and improve the efficiency of conventional processes (freezing, drying, freeze-drying and osmotic dehydration through the acceleration of mass transports), and to increase yield and selectivity of extraction of compounds with high nutritional value upon extraction processes (Raso et al., 2016). The

application of electrical pulses to plant tissues can lead in fact to electrical breakdown and structural damage of cell membranes, which increases their permeability (Tylewicz et al., 2017). In particular, for electric field intensities in the range of 0.1–1 kV/cm the result is a reversible electroporation, in the range 0.5–3 kV/cm there is an irreversible electroporation of plant and animal tissue, and between 15 and 40 kV/cm or higher, there is an irreversible permeabilization of microbial cells (Jaeger et al., 2012).

The effectiveness of PEF to increase the extraction yield has been widely proved in previous studies conducted on plant materials such as red beet (Fitzmaurice et al., 2004; Nowacka et al., 2019), sugar beet (Jemai and Vorobiev, 2003), chicory (Loginova et al., 2010), apple (Jemai and Vorobiev, 2002), paprika (Ade-Omowaye et al., 2001), red cabbage (Gachovska et al., 2010), carrot (El-Belghiti and Vorobiev, 2005) and tea leaves (Zderik et al., 2013).

With the same objective, Pourzaki et al. (2013) carried out PEF treatments on saffron, but these authors applied the treatment directly on the dried stigma and evaluated only the extraction yield of the three key secondary metabolites, i.e. crocin, picrocrocin and safranal.

To the best of our knowledge, no information is available in literature on the effect of the PEF treatment on the chemical and physical properties of the fresh stigmas, as well as on its effects on the content and extractability of the main secondary metabolites and antioxidants after drying and aging.

Thus, the aim of this study was to contribute to fill in this knowledge gap and to investigate the potential use of pulsed electric fields as pre-treatment to conventional hot air drying for the improvement of saffron quality and the enhancement of its colouring, flavouring and functional properties. Chemical and functional analysis were carried out on aqueous extracts of the stigmas before and after drying, and up to 10 months of storage at room temperature.

Crocin isomers have been shown to be reliable markers of saffron quality, process and traceability (Rocchi et al., 2018) therefore their evaluation in the saffron samples differently processed and stored has been carried out to assess the impact of PEF on saffron quality. Moreover, microstructural analysis of fresh and dried stigma was also performed.

2. Materials and Methods

2.1 Materials

Fresh saffron stigmas farmed in Lombardy (Italy) and picked up in November 2018 were provided by a local producer (Zafferanami Srl, Varedo, Italy).

All chemicals and standards were purchased from Sigma-Aldrich (Steinheim, DE).

2.2 Experimental plan and samples preparation

All samples were prepared from a single batch of saffron stigmas and produced within 8 hours from the stigmas harvest. Upon arrival, fresh stigmas (F) were immediately aliquoted and either subjected to drying (D), or to Pulsed Electric Field treatment followed by drying (PD). In order to distinguish the effect of PEF from the one determined by the drying treatment, an aliquot of fresh stigmas was subjected only to the PEF treatment (P). Since PEF involves the immersion of the sample in water for the correct conduction of electricity, a sample represented by saffron stigmas dipped in water (DIP) was also prepared. PD, D, P and DIP, sample were obtained by combining the stigmas resulting from 5 replicated treatments.

All the samples were analysed just after their preparation (t_0); D and PD samples were also analysed after 3 (t_3) and 10 months (t_{10}) of storage at dark and room temperature, i.e. conventional conditions used to promote the aging process and the development of the typical quality properties of saffron. Flow chart of samples preparation is reported in Figure 1.

2.3 Pulsed Electric Field (PEF) treatment

PEF treatment was carried out on an aliquot of saffron stigmas (0.2 g) in a cubic cell (5×5×5 cm), with two stainless steel electrodes (contact surface: 5×5 cm) placed above and on the bottom of cell. For the correct conduction of electricity, the cell was filled with tap water with an electrical conductivity equal to 463 $\mu\text{S}/\text{cm}$, value determined by EC-Meter basic 30+ conductivity meter (Crison Instruments, s.a., Barcelona, Spain). The cell was filled with 0.2 g of the sample and 30 g of water (product/water ratio of 1:150) and then placed inside an insulated plexiglass chamber with a safety switch. The electric current was supplied in a quantity of 23 A by an alternating electric pulse generator (Power supply S-P7500 60A 8kV, Alintel srl, Bologna, Italy); the control and measurement of the pulses was guaranteed by a software on a PC connected to the instrument. The instrument has been set to 50 rectangular shaped pulses with a length of 20 μm , for a total treatment time of 1 millisecond. The intensity of the electric field was set at 2 kV/cm^2 at a temperature of 25 °C and the energy input on the product was of 1.5 KJ kg^{-1} . The PEF parameters has been optimized in preliminary trials, taking as reference data reported in the literature (Pourzaki et al. 2012) and the limitations of the settings of the available equipment.

After the treatment, the samples were removed from the treatment chamber and quickly, but gently dapped with paper to eliminate the excess of water.

2.4 Dipping

An aliquot (0.6 g) of fresh saffron was dipped in distilled water (product/water ratio of 1:150) within the PEF chamber for 5 min, time that corresponds to the total immersion time of stigmas in water for the fulfilment of the PEF treatment.

After the treatment, the samples were removed from the treatment chamber and gently dapped with paper to eliminate the excess water.

2.5 Hot Air Drying

Aliquots (6.5 g) of fresh and PEF pre-treated stigmas were placed in aluminium pans and dried at 45 °C for 120 min (air velocity of 2 m/s and air renewal fee of 50%) by using an air dryer (POL-EKO-APRATURA SP.J., PL). These drying conditions were preliminarily assessed to achieve a moisture content of samples below the 12 % on wet basis, according to the ISO 3632-1 protocol (ISO 3632-1:2011).

2.6 Quality evaluation

Saffron samples were analysed according to the ISO 3632-2:2010 protocol with some modifications as reported by Paredi, Raccani, and Mozzarelli (2014). According to Rocchi et al. (2019) this procedure allows the classification of saffron in three “ISO-like” quality categories referred to spectrophotometric parameters. Briefly, an aliquot of each samples (10 mg) was dispersed in 2 mL distilled water and sonicated in ultrasound bath (Labsonic LBS1, Falc, Italy) for 15 min at $T=25$ °C. Then, the extracts were centrifuged at 10000 rpm for 10 min and analysed by using a spectrophotometer (Perkin-Elmer Lambda 25 UV/Visible, US). The absorbance of the extract, diluted at 1% w/w in water, was evaluated in a 1 cm pathway quartz cell at three fixed wavelengths corresponding to the λ_{max} of the three secondary metabolites of saffron (picrocrocin: $\lambda_{max} = 257$ nm; safranal: $\lambda_{max} = 330$ nm; and crocins: $\lambda_{max} = 440$ nm).

The moisture and volatile content was evaluated on saffron stigmas after grinding with pestle and mortar. An aliquot (15 mg) of each sample was weighted and put in an oven at 103°C for

16 h and moisture and volatile content (W_{MV}) were calculated according to the following equation [1]:

$$W_{MV} = \frac{(m_0 - m_1)}{m_0} * 100 \quad [1]$$

Where:

m_0 = initial weight of the sample;

m_1 = final weight of the sample.

The ISO-like quality indexes were obtained using the equation [2] (ISO 3632-2:2010):

$$E_{\lambda_{max}}^{1\%} = \frac{D-10000}{m*(100-w_{MV})} \quad [2]$$

Where: D is the specific absorbance at 253, 350 and 440 nm; m is the mass of the saffron sample (g); w_{MV} is the moisture and volatile content of the sample, expressed as a mass fraction. For each sample, analyses were carried out in duplicate.

2.7 Extraction and analysis of crocins by UHPLC-MS/MS

Crocins extraction and analysis were carried out according to Rocchi et al. (2018). Before analysis, P, DIP and fresh stigmas (F) were freeze-dried for water removal by using a ScanVac CoolSafe 95–15 Pro freeze dryer (Lyngø, Denmark). In particular, the samples were frozen at -40 °C and freeze-dried for 24 h at a pressure of ≈ 0.5 hPa and by setting the shelves temperature at 0 °C.

Extraction was carried out on 5 mg of dried saffron powder dispersed in 1.25 mL of MeOH/H₂O (50:50, v/v) in a 1.5 mL Eppendorf by sonication in ultrasound bath (Labsonic LBS1, Falc, Italy) for 15 min at T=25 °C. Thereafter, the extract was centrifuged and filtered

through a 0.22 μm PTFE (polytetrafluoroethylene) syringe filter with a diameter of 4 mm and put in an autosampler vial for UHPLC-MS/MS analysis. An UHPLC equipment composed by a Nexera LC20AD XR apparatus, with autosampler, vacuum degasser and column oven (Shimadzu, Tokyo, Japan) coupled with a 4500 Qtrap mass spectrometer (Sciex, Toronto, Canada) equipped with a Turbo V ESI source, was used for UHPLC-MS/MS analysis.

The target analytes were separated using a reverse-phase Kinetex C18 column from Phenomenex (Torrance, CA, USA) packed with Core Shell particles (1.7 μm , 2.1 \times 100 mm). The mobile phases were 5 mM formic acid in water (phase A) and 5 mM formic acid in acetonitrile (phase B); flow rate was 0.3 mL min^{-1} entirely transferred into the ion source. The analyte separation was performed using a gradient elution: for 0.1 min, phase B was maintained at 5%, then, increased to 99% in 13 min and kept at 99 % for 4 min; the system is brought back to the initial conditions in 3.30 min. The analyte separation occurs in 10 min. All the substances were detected in negative ionization with a capillary voltage of -4500 V, nebulizer gas (air) at 50 psi, turbo gas (nitrogen) at 40 psi, and 450 $^{\circ}\text{C}$.

Source and instrumental parameters for the analytes of interest were optimized by using crocin standard (Sigma Aldrich, St. Louis, USA) dissolved in methanol/water (50:50, v/v) at a concentration of 10 ng mL^{-1} at a flow of 10 $\mu\text{L min}^{-1}$ by a syringe pump. Peak areas for selected ions were determined using MultiQuant Software 3.0 (Sciex, Foster City, CA).

2.8 Determination of total phenolic content (TPC) and antioxidant activity (AOA)

Total polyphenols content (TPC) and antioxidant activity (AOA) were evaluated on aqueous extracts obtained as indicated in section 2.6.

TPC was estimated using the Folin Ciocalteu's reagent according to Singleton and Rossi, (1965). Gallic acid (GA) was used as reference standard (0.03 – 0.19 mg/mL) and the results are expressed as mg GA Equivalents/g dry weight matrix.

AOA was measured according to the method described by K. et al. (1999) with some modifications. ABTS^{•+} (2,2'-azino-bis- (3-ethylbenzothiazolin-6-sulfonic) (Fluka, Buchs, Switzerland) was dissolved in water to a 7 mM concentration; the ABTS radical was formed by reacting ABTS stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS radical solution was diluted with deionised water to reach an absorbance of 0.70 ± 0.02 at 734 nm. The absorbance at 734 nm was evaluated by a Perkin Elmer (Boston, MA, USA) Lambda 25 UV/VIS spectrophotometer. The reaction was started by the addition of 10 μ L of extract to 990 μ L of ABTS^{•+} solution. The time of analysis was of 7 min. This time of analysis was chosen after preliminary tests and represented the time necessary to reach at least 80% of the overall inhibition of the ABTS radical at a given concentration. For each sample, the percentage of inhibition (I%) was plotted as a function of concentration and the TEAC (Trolox Equivalent Antioxidant Capacity) calculated as the ratio of the linear regression coefficient of the sample to that of the Trolox standard (Fluka). Results are expressed as micromoles of Trolox equivalents per gram of dry matter. The coefficient of variation of the method was below 5%.

2.9 Laser Scanning Confocal microscopy

Saffron stigmas were halved, placed on microscope slide and covered with cover slips prior to analysis. Microstructure images of the stigmas were collected by using a Nikon A1r laser confocal scanning microscope, equipped with a Plan Apo λ 60X Oil objective (numerical aperture: 1.4; Refractive Index: 1.515), detector Galvano with a pinhole size of 39.6 μ m. For Z Stack scanning the specific function Nikon A1 Piezo Z Drive at 0.15 μ m of Z-step was used, in channel series, as follows:

Channel 1: DAPI: $\lambda_{exc} = 404$ nm; $\lambda_{em} = 450/50$ nm at 7.6% of the maximum laser power

Channel 2: FITC: $\lambda_{exc} = 488$ nm; $\lambda_{em} = 525/50$ nm, at 3.1% of the maximum laser power

Channel 3: TRITC: $\lambda_{exc} = 561.5$ nm; $\lambda_{em} = 595/50$ nm at 3.1% of the maximum laser power

2.10 Statistical Analysis

Data are expressed as mean and standard deviation and additionally analysed by one-way ANOVA analysis. Significant differences between means were computed by Least Significant Difference (LSD) test at a significance level of 0.05. Data were processed using STATISTICA for Windows (StatSoftTM, Tulsa OK, USA) software.

3. Results and discussion

3.1 Quality evaluation of saffron by conventional indices

Colouring, aroma and flavour strength of saffron samples were determined by evaluating the $E^{1\%}$ of aqueous extracts at 440, 330, and 257 nm. These indices contribute to define the saffron quality, with the flavour strength ($E^{1\%}_{257}$) relating to picrocrocins, aroma strength ($E^{1\%}_{330}$) to safranal and colour strength ($E^{1\%}_{440}$) to crocins. In order to evaluate the effect of PEF and to distinguish it from that of drying, beside conventional dried saffron (D) and PEF

pre-treated dried saffron (PD), quality indices were evaluated also on fresh (F), dipped (DIP) and PEF (P) stigmas and all the results are reported in Table 1.

PEF pre-treatment negatively affected the quality of fresh stigmas by inducing a significant loss ($\approx -41\%$) of the flavour, aroma and colouring strength. Since F and DIP samples showed overall similar $E^{1\%}$ values, this decrease can be ascribed to the leaching of picrocrocin, safranal and crocins in the treatment medium (water) as an effect of electroporation phenomena induced by PEF on the cell membranes. This effect was also evidenced by Pourzaki et al. (2013) who found an increase of picrocrocin, safranal and crocins in the treatment medium (water) after PEF treatment of saffron samples (dried *Crocus sativus* stigmas).

Drying treatment increased the flavouring, aroma and colouring strength of fresh stigmas respectively of 60, 145 and 59 %. When the heat treatment was carried out on PEF pre-treated stigmas (sample PD) different effects were observed and, in particular, the increase of the saffron flavouring and colouring strength respectively of 177 and 40%, and no significant variation of the aroma strength. Compared to the F sample, the PD showed higher flavouring strength (58 %), but lower aroma and colouring strength (-39% and -15%, respectively).

Overall, these results indicate a negative effect of PEF pre-treatment on the chemical pathways that lead to the formation of safranal during drying. During this process, in fact, the β -glucosidase hydrolyses picrocrocin to give the aglycone, 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC), which, in turn, is transformed by dehydration to safranal (Lozano et al., 2000).

The different increase of the flavouring and colouring power observed in D and PD samples can be dependent both on the different extractability of picrocrocin and crocins, as an effect

of the different extent of the structural damages impaired on the stigmas' cells by both the PEF and drying treatment as well as to the different concentration of these compounds, and/or of their precursors, in the corresponding not dried samples (fresh and PEF pre-treated stigmas).

It is important to highlight that according to the ISO-like classification, sample D could be classified as saffron of category I (i.e. best quality), whilst due to an aroma strength lower than 20 units (minimum value) PD samples resulted not classifiable.

Aging at room temperature positively affected D and PD saffron samples by increasing both the flavouring and aroma strength (Table 1). The increase of the aroma strength can be attributed to the production of safranal as an effect of the increase of safranal's precursors during drying according to a mechanism still not clear (Alonso et al., 1996). Colouring power increased after three months of aging due to increase of crocins; however, higher aging times led to the decrease of these compounds. This effect, observed also by Raina et al. (1996), is due to autoxidation of crocins. However, these results confirm that drying and aging contribute largely to the final concentration of picrocrocin, crocin and safranal in saffron and thus, to its quality (Rocchi 2019; Carmona, et al., 2005).

Moreover, by comparing PD and D sample it is possible to note as the former, despite its significant lower initial (t_0) flavouring, aroma and colouring strength, after 3 months of aging showed quality properties similar ($p>0.05$) to the D sample. This result can be related to the highest increase of picrocrocin, crocin and safranal content in the PD sample as an effect of the damages and decompartmentation induced by PEF on the cell structures (Tylewicz et al., 2017); these structural modifications, in turn, could have favoured the release of carotenoids from chromoplasts and their interaction with other substrates and enzyme involved in the biogenesis of these colouring and flavouring bioactive compounds (Mannozi et al., 2018). It is

known, in fact, that the bio-oxidative cleavage of zeaxanthin by zeaxanthin cleavage dioxygenase lead to the production of crocetin dialdehyde and picrocrocin and to the glucosylation of the generated zeaxanthin cleavage products by glucosyltransferase enzyme (Namin et al., 2009; Frusciante et al., 2014).

Basing on the kinetics of $E^{1\%}_{330}$ variation during aging (data not shown) and according to the ISO-like classification, PD and D samples resulted classified as saffron of I class up to about 8 months while higher storage times led to a no classification of both the samples due to the overcoming of the ISO fixed $E^{1\%}_{330}$ maximum limit of 50 unit.

3.2 Crocins pattern determined by UHPLC-MS/MS

Crocins are crocetin esters with different glycosyl moieties, such as glucose, gentiobiose, or triglucose and they can exist in cis- and trans- isomeric forms. Briefly, crocin-4 is esterified in both terminal carboxylic groups with gentiobiose. Crocin-3 exhibits mixed glucosyl–gentiobiosyl structure. Crocin-2 and crocin-2II have identical molecular mass, but different structure. Crocin-2 is the gentiobiosyl derivative of crocetin, and crocin-2II contains two glucosyl moieties, while crocin-1 is esterified with only one glucose molecule (Rocchi et al., 2018).

In Table 2, the content of crocin isomers expressed as relative % on the total crocin area (TCA), TCA and trans/cis isomers ratio (T/C) in fresh and differently processed stigmas at time 0 and after 3 and 10 months of aging, are reported. In general, in all samples the trans crocin 4 (trans-4) was the most abundant isomer (ca. 86 %, on average) followed by the cis crocin 4 (cis-4), trans crocin 2 (trans-2) and trans crocin 3 (trans-3). Rocchi et al. (2018) found a similar pattern as for dried stigmas, while Carmona et al. (2005) observed different isomers

percentages with trans-4>trans-3>trans-2>cis-4, but a similar T/C ratio. The higher content of trans- than cis-isomers found on saffron samples is to ascribe to the lower energy of the formers and thus, to their higher stability (Zechmeister, 1944; Rocchi et al., 2018).

Drying did not affect ($p>0.05$) the total content of crocins as indicated by the TCA values and this same result was obtained also by Raina et al. (1996) on stigmas dried under different methods and thermal conditions. Similarly, PEF pre-treatment did not affect the total content of crocins while it caused a significant ($p<0.05$) change of the crocin profile, by decreasing the T/C ratio as an effect of the increase of cis-isomers. This result could be dependent on local heating which can be induced by PEF, depending on processing conditions (electric field intensity, total specific energy, frequencies and pulse width) due to Joule effect (Raso et al., 2016).

Aging increased to a similar extent ($p<0.05$) the TCA of D and PD samples, showing the highest values in the 3 months-aged saffron samples. As regards the T/C ratio, after 3 months of aging both the D and PD samples showed a drastic decrease, however, after 10 months, compared to the corresponding non-aged sample, sample D showed no difference and PD even higher value.

Correlation analysis between TCA and $E_{440}^{1\%}$ led to a poor significance ($R^2 = 0.51$) and no correlation was found also by taking into account the sum of trans crocins. As explained by Del Campo et al. (2010), statistical differences in colouring strength against no differences in total crocetin or trans crocetin esters is due to the different contribution of trans and cis crocetin esters to the saffron colouring strength: cis esters, in fact, have a lower extinction coefficient, ϵ , at 440 nm ($63\,350\text{ mol}^{-1}\text{ dm}^3\text{ cm}^{-1}$) in respect to that of the trans esters ($89\,000\text{ mol}^{-1}\text{ dm}^3\text{ cm}^{-1}$) and, thus, a lower colouring strength. Moreover, as reported by Rocchi et al. (2018), the presence in saffron of other interfering substances with absorbance at 440 nm can

be responsible for this finding. In this regard, it can be assumed that Maillard's reaction products contribute to the colouring strength of saffron as shown with respect to the aroma (Cadwallader, 2002).

3.3 Total phenolic content

The total polyphenols content (TPC) of fresh and differently processed stigmas was determined both after processing (Figure 2a) and upon aging (Figure 2b) on aqueous extracts by using the Folin Ciocalteu assay. PEF treatment did not influence the TPC of fresh stigmas while drying led, in general, to the increase of this parameter with PD and D samples showing similar values. The data reported are also in accordance with those shown by Baba et al. (2015) and Karimi et al. (2010). After 3 months of aging no effects ($p>0.05$) was observed on TPC of saffron samples, while after 16 months a drastic increase was observed with PD sample being the most ($p<0.05$) affected (+116%).

The increase of TPC during aging was observed also by Moratella-Lopez et al. (2016) on dehydrated floral bio-residues of *Crocus sativus* with RH% <23%. Storage at non-freezing temperatures has been shown to induce a TPC increase also in other plant matrices (Spanos and Wrolstad, 1990; Isinguro et al., 2007; Rapisarda et al., 2008); the formation of non-enzymatic browning compounds, such as enediols and reductones, has been retained responsible for this “apparent” TPC increase during storage (Spanos and Wrolstad, 1992) since these compound are able to react with the Folin-Ciocalteu reagent. The method used for ‘TPC’, in fact, is not selective for polyphenols but measures the capacity of an extract to reduce the Folin-Ciocalteu reagent and, thus, it measures the reducing power of the extract itself (Prior et al., 2005).

The higher TPC increase observed on the PD sample could be due to the highest formation of the aforementioned non-enzymatic reactions compounds, and this is in accordance with the results reported by Agcam et al. (2014) and Morales-de la Pena et al. (2010) for orange juice and soy milk beverages. PEF treatment is able, in fact, to promote biochemical reactions and favour the formation of new compounds during aging (Barba et al., 2015).

Additional analysis characterized by a highest selectivity towards phenolic compounds (i.e HPLC) should be, thus, carried out to better evaluate the effect of PEF pre-treatment on phenolic content of saffron during aging.

3.4 Antioxidant activity (AOA)

In Figure 3, the antioxidant activity (AOA) of fresh and differently processed stigmas along with its variation during aging at room temperature, is reported. AOA of dried stigmas was higher than that reported by Pellegrini et al. (2006) on saffron aqueous extract and, in general, in respect of other spices (e.g. rosemary, basil and sage). The high antioxidant activity of saffron is due to the stigma content of flavonoids, included naringenin, kaempferol, and their derivatives (Baba et al., 2015) which play a role as antioxidants (Moon et al., 2006) as well as of the flavonoid taxifolin. In addition, apocarotenoids, crocin and crocetin derivatives, beside their colouring properties, contribute to the stigma antioxidant activity (AOA) (Bolhassani et al., 2014). Apart from their content, the synergistic effect among these bioactive constituents could also be responsible for the *Crocus sativus* and saffron stigmas AOA (Assimopoullou et al., 2005).

PEF treatment influenced the AOA of fresh stigma by determining a TEAC reduction of 24%. The lower antioxidant activity of the P sample compared to that of the F one is probably due to

release of antioxidant compounds in the water treatment due to the formation of pores and cell lysis induced by PEF (Pliquett et al., 2007).

Drying treatment positively affected the antioxidant activity of both fresh and PEF pre-treated stigma. D and PD samples showed, in fact, AOA values respectively 12% and 54% higher than their correspondent control. Despite the lowest AOA value measured on stigma after PEF pre-treatment, PD sample showed an AOA value similar ($p>0.05$) to the conventionally processed sample (sample D). The AOA increase after drying can be related with that of the safranal and crocins content: according to Assimopoulou et al. (2005), the synergistic effects of these compounds can positively affect the AOA of dried saffron.

Aging affected to a similar extent ($p>0.05$) both L and PD samples by decreasing the antioxidant activity of about 7 and 86% after 5 and 10 months, respectively. This result is mostly due to the oxidation of saffron's bioactive compounds such as crocins, which are able to quench free radicals, and other minor antioxidants compounds such as carotenes, lycopene, zeaxanthin, mangicrocin and xanthone-carotenoid glycosidic conjugate (Licon et al., 2012; Maggi et al., 2011; Bathaie and Mousavi, 2010; Hadizedeh et al., 2010; Asai et al., 2005; Li et al., 1999), and polyphenols. The discrepancy between AOA results and TPC results can be related, as already discussed in section 3.3., to the limits of the TPC evaluation that is related to the reducing power of the extract whilst the ABTS radical could undergo to reduction by both single electron transfer (SET) and hydrogen atom transfer (HAT), thus presenting a mixed mechanism of action towards the antioxidants present in the extracts (Prior et al., 2005; Apak et al., 2016).

3.5 Confocal microscope analysis

In this study, confocal laser scanning microscope (CLSM) was used with the aim to evidence the structural modification induced on fresh stigma by processing. In fact, CLSM gives the possibility to examine the surface and the internal structure of biological systems in three dimensions and to avoid artefacts due to sample manipulation. For this reason, this technology represents a powerful tool to investigate animal and plant food structural changes process-induced (Dürrenberger et al., 2001).

CLSM images of fresh stigma (Figure 4) show a compact and linear reticulo-tubular structure consisting in cells closely bonded with each other through extensive cell-to-cell contacts. This cellular organization is consistent with the images reported by Himeno et al. (1988) obtained by Scanning Electron Microscope (SEM) and by Rocchi (2019) by CLSM.

CLSM image of dipped stigma shows a compact structure, but compared to fresh stigma, cells appear more elongated, and red autofluorescence, given by compounds with $\lambda_{em} = 595/50$ nm, can be also observed. As explained by Rocchi (2019), these differences can be related to the observation of a different portion of the stigma, which seems to be composed by cells with different specialization in the production of pigment depending on its section.

As regards the stigma pre-treated by PEF treatment (Figure 4), it is possible to note that the tissue structure is very similar to that one of DIP sample, but in this case, green autofluorescence given by compound located at extracellular level, can be evidenced. This result can be due to the electroporation phenomenon that form pores in the cell membranes and promote the release of intracellular compounds (Pakhomov et al., 2010).

Drying treatment affected the stigma microstructure by determining a reduction of the cell volume and the collapse of some cells, which allowed to evidence intracellular organelles

(Figure 5). However, the cell organization appears still preserved, compact, with large area characterized by linear cells. In D samples autofluorescence was given by compounds with λ_{em} of 595/50 nm, while some green fluorescent compounds are also evidenced at extracellular level. On the contrary, after drying, PEF pre-treated samples showed a structure deeply irregular, characterized by collapsed cells of reduced width, compromised cellular boundaries, and a high content of green fluorescent compounds at extracellular level.

Thus, CLSM images of P and PD samples, confirmed the hypothesis that PEF treatment strongly impaired the stigma's cells, thus enhancing the extractability of intracellular compounds from the saffron matrix.

4. Conclusions

This study investigated the potential use of PEF as drying pre-treatment for the improvement of saffron quality and the enhancement of its colouring, flavouring, aroma and functional properties. Overall, PEF pre-treatment negatively affected the quality indices and functional properties of *Crocus sativus* L. due to the loss of safranal, crocins, and other bioactive secondary metabolites, as an effect of cell membranes electroporation and their consequent leaching into the treatment medium. After drying, PEF pre-treated saffron showed a lower quality compared to saffron obtained by conventional processes despite their similar functional properties. On the other side, aging significantly contributed to enhance the concentration of picrocrocin, crocin and safranal of the PEF saffron samples, and to allow an ISO-like classification (Rocchi et al., 2019). Thus, overall, the use of PEF as drying pre-treatment increases the extractability of intracellular compounds leading, overall, to higher extraction yield in subsequent extraction processes of secondary metabolites and to enhanced coloring and flavoring properties.

For food applications, the results of this study suggest that PEF-pretreated saffron could be proposed as high quality ingredient with enhanced technological functionalities that, thanks to the higher yield of extraction could be used in food preparations and products in smaller quantities, with an economic saving positive effect. The same concepts are valid and applicable also in non-food sectors and products (e.g. colouring abilities in textile; cosmetics, etc.).

Ongoing analysis and investigation are focused on the evaluation of the contribute of Maillard reaction products on the aroma and functional properties of saffron, the optimization of PEF

process parameters with the aim to limit the depletion of fresh stigmas with respect to microcrocins, crocins, safranal and other bioactive compounds.

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Figure Captions

Figure 1. Schematic flow-chart of the production process of saffron samples.

Figure 2. Total polyphenol content of fresh and differently processed stigmas after processing (a) and dried samples upon aging (b); F: fresh stigmas, DIP: dipped stigmas, P: PEF pre-treated stigmas, D: dried stigmas, PD: PEF pre-treated and dried stigmas. Data with different letters and (*) are significantly different at $p \leq 0.05$.

Figure 3. Antioxidant activity of fresh and differently processed stigmas after processing (a) and of dried samples upon aging (b); F: fresh stigmas, DIP: dipped stigmas, P: PEF pre-treated stigmas, D: dried stigmas, PD: PEF pre-treated and dried stigmas. Data with different letters and (*) are significantly different at $p \leq 0.05$.

Figure 4. Two-dimensional micrographs of saffron stigmas obtained by confocal laser scanning microscopy; F: fresh stigmas, DIP: dipped stigmas, P: PEF pre-treated stigmas.

Figure 5. Three-dimensional micrographs of dried (D) and PEF dried (PD) saffron stigmas obtained by confocal laser scanning microscopy.

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CRedit author statement

Lilia Neri: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision.

Marianna Giancaterino: Investigation, Formal analysis, Writing - Original Draft, Visualization. **Rachele Rocchi:** Methodology, Investigation, Writing - Review & Editing.

Urszula Tylewicz: Methodology, Investigation, Writing - Review & Editing.

Luca Valbonetti: Methodology, Investigation.

Marco Faieta: Investigation, Visualization.

Paola Pittia: Conceptualization, Methodology, Writing - Review & Editing, Project administration, Funding acquisition

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Object: manuscript submission for publication on the *Innovative Food Science and Emerging Technologies*, Neri et al.

Dear prof. D. Knorr

Editor-in-Chief of the Innovative Food Science and Emerging Technologies Journal

on behalf of all the authors and in agreement with them, I would like to claim the following statement regarding the manuscript titled “Pulsed Electric Fields (PEF) as drying pre-treatment: effect on quality and functional properties of saffron (*Crocus sativus* L.)” (authors: Lilia Neri, Marianna Giancaterino, Rachele Rocchi, Urszula Tylewicz, Luca Valbonetti, Marco Faieta, Paola Pittia)

'Declarations of interest: none'.

My best regards

Paola Pittia

Teramo, 19th August 2020

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Highlights

- PEF was applied as drying pre-treatment to increase saffron quality and functionality
- Flavour, aroma and colouring strength of saffron stigmas was PEF affected the
- PEF decreased the trans/cis crocins ratio in fresh stigmas
- Safranal production during drying and aging was influenced by PEF
- PEF affected the biogenesis and extractability of colouring and flavouring compounds

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Table 1

Quality parameters and assigned ISO-like category of different saffron samples: picrocrocin λ_{\max} =257 nm; safranal λ_{\max} =330 nm and crocins λ_{\max} =440 nm.

Sample	$E_{1cm}^{%}$			ISO-like Category
	Picrocrocin (Flavour strength)	Safranal (Aroma strength)	Crocins Colouring strenght	
	257 nm	330 nm	440 nm	
F	51.7 ^d ± 5.1	8.19 ^e ± 1.39	146 ^d ± 8	-
DIP	45.4 ^e ± 1.4	7.44 ^e ± 1.22	138 ^{de} ± 10	-
P	29.5 ^f ± 2.9	4.80 ^f ± 0.77	87.9 ^f ± 3.1	-
D	82.5 ^c ± 1.8	20.1 ^d ± 0.7	232 ^b ± 27	I
PD	81.8 ^c ± 1.7	4.91 ^f ± 1.8	123 ^e ± 20	n.c.
D	114 ^b ± 7	27.2 ^c ± 1.2	262 ^a ± 13	I
PD	113 ^b ± 4	26.8 ^c ± 2.7	280 ^a ± 21	I
D	116 ^b ± 12	57.6 ^a ± 6.5	172 ^c ± 10	n.c.
PD	133 ^a ± 14	52.0 ^b ± 6.0	182 ^c ± 10	n.c.

F: fresh stigmas, DIP: dipped stigmas, P: PEF treated stigmas, D: dried stigmas, PD: PEF treated and dried stigmas, n.c. not classified.

Data in the same column with different letters are significantly different at $p \leq 0.05$.

Table 2

Total UHPLC area of crocins isomers, relative content of the cis- and trans- isomers (% on the total area) and trans/cis ratio of differently processed and aged saffron samples.

Aging time (months)	Sample	trans-4	cis-4	trans-3	cis-3	trans-2	trans-2 II	cis-2	cis-2 II	Total Crocin Area (TCA)	Trans/Cis (T/C)
0	F	85.2 ^{cd} ± 0.95	4.64 ^c ± 0.54	2.72 ^{ab} ± 0.12	0.31 ^{de} ± 0.03	2.7 ^{ab} ± 0.36	1.84 ^{bc} ± 0.11	1.71 ^e ± 0.04	0.79 ^{bcd} ± 0.07	6.39E+05 ^{cd} ± 1.33E+05	12.9 ^b ± 1.26
	DIP	87.1 ^{abc} ± 1.06	4.38 ^c ± 0.25	1.17 ^e ± 0.18	0.31 ^{de} ± 0.02	2.89 ^{ab} ± 0.53	1.53 ^{cd} ± 0.07	1.83 ^d ± 0.21	0.76 ^{cde} ± 0.13	7.24E+05 ^{cd} ± 1.52E+05	13.2 ^b ± 1.22
	P	83.3 ^d ± 1.71	7.14 ^b ± 0.93	1.21 ^{de} ± 0.35	0.39 ^d ± 0.05	3.28 ^a ± 0.39	1.60 ^d ± 0.01	2.16 ^c ± 0.04	0.95 ^{ab} ± 0.13	7.27E+05 ^{cd} ± 7.94E+04	8.61 ^c ± 0.87
	D	85.7 ^{bcd} ± 0.74	5.10 ^c ± 0.26	1.09 ^e ± 0.24	0.33 ^{de} ± 0.02	2.92 ^{ab} ± 0.76	2.18 ^c ± 0.09	2.03 ^{cd} ± 0.19	0.68 ^{def} ± 0.08	5.98E+05 ^d ± 1.27E+05	11.7 ^b ± 0.64
	PD	87.4 ^{abc} ± 0.32	5.73 ^{bc} ± 0.17	1.20 ^e ± 0.07	0.27 ^e ± 0.06	2.23 ^b ± 0.02	1.12 ^e ± 0.03	1.52 ^e ± 0.17	0.58 ^{ef} ± 0.10	7.76E+05 ^{bcd} ± 1.51E+04	11.6 ^b ± 0.61
3	D	72.1 ^e ± 0.41	14.6 ^a ± 0.39	2.46 ^{bc} ± 0.17	1.07 ^b ± 0.00	1.50 ^{ab} ± 0.37	1.79 ^{bcd} ± 0.32	4.59 ^a ± 0.00	0.88 ^{bc} ± 0.06	1.38E+06 ^a ± 5.33E+04 ^a	3.74 ^d ± 0.10
	PD	73.3 ^e ± 2.30	14.6 ^a ± 2.02	1.53 ^{de} ± 0.13	1.51 ^b ± 0.02	2.12 ^b ± 0.00	1.93 ^{ab} ± 0.15	3.92 ^b ± 0.21	1.08 ^a ± 0.02	1.30E+06 ^a ± 1.24E+05 ^a	3.78 ^d ± 0.52
10	D	88.5 ^{ab} ± 0.78	5.38 ^{bc} ± 0.53	1.80 ^{cd} ± 0.27	0.54 ^c ± 0.06	2.14 ^b ± 0.00	n.d.	0.76 ^f ± 0.04	0.75 ^{cde} ± 0.01	8.30E+05 ^{bc} ± 6.76E+04	12.8 ^b ± 0.95
	PD	88.0 ^a ± 0.67	4.78 ^c ± 0.37	1.70 ^{cd} ± 0.69	0.48 ^c ± 0.02	2.29 ^b ± 0.33	n.d.	0.57 ^f ± 0.02	0.55 ^f ± 0.02	9.61E+05 ^b ± 3.82E+04	15.1 ^a ± 0.92

F: fresh stigmas, DIP: dipped stigmas, P: PEF treated stigmas, D: dried stigmas, PD: PEF treated and dried stigmas.

Data in the same column with different letters are significantly different at $p \leq 0.05$.