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Original article

An investigation into low-temperature nitrogen plasma environment effect on the content of polyphenols during withering in made Kenyan tea

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Summary Low-temperature nitrogen plasma (LTNP) was used to wither green tea leaf to study its effect on the polyphenol content. Using a dielectric barrier discharge chamber to provide the LTNP environment, green tea leaf was withered at various withering times. Made tea samples indicated that LTNP had an effect on polyphenol content. The highest polyphenol content of 78.56 mg g^{-1} in made tea was attained in 1 h after which it showed a decreasing trend with increasing retention time. For comparison purposes, green tea leaf was also withered in nonplasma environments. Highest polyphenol content of 133.4 mg g^{-1} in made tea was attained in a sample withered anaerobically in nitrogen gas at room temperature and atmospheric pressure for 18 h. In another sample, green tea leaf was directly macerated and dried without withering and fermenting and had polyphenol content of 101.91 mg g^{-1} in made tea. These contents were compared with green tea 4, purple tea and oolong tea that are currently manufactured in Kenya.

Keywords camellia sinensis, green tea leaf, LTNP, polyphenol content, withering time.

Introduction

Rapid advances in technologies in the last two decades have opened up and brought forth new opportunities and challenges in thermal sciences and fluid mechanics. One such area of technological advancement is plasma technology. Plasma technology affects our daily lives in processes and products. The main guiding factors for its increasing use have been due to high demand for product quality, productivity, environmental compatibility, precision and flexibility. Plasma is the fourth state of matter formed by ionisation of elements and gases among others. It comprises electrons, protons, positive and negative ions, neutral molecules and atoms and a variety of other particles all existing in the same environment. The interactions between charged particles and the neutral particles within the same environment are important in determining the behaviour and usefulness of the particular plasma. A broad spectrum of plasma types, characteristics and unique behaviours can be created from different types of atoms, ratios of ions to neutral particles and the particle energies. Due to these factors, their utilisation in biosystems, biochemical and biomedical engineering processes is playing a very vital role where conventional methods could not have been possible.

Among other fields where useful applications have been made are medical treatment especially in sterilisation, surgery, etc. (Heinlin *et al*., 2010), material treatment/surface coating (Kim *et al.* 2010, Boulos, 1996 and Kushner, 2008) and waste treatment, namely decomposition of compounds containing NO_3, NH_3 or CN_x groups as an environmental management technique (Penetrante *et al*., 1999; Osamu, 2008). Others are catalytic reactions in chemical processes, bioprocesses in agriculture and food as a nonchemical gas-phase disinfection agent (Osamu, 2008), nanotechnology and biomaterials (Osamu, 2008). Plasma can be created in thermal and nonthermal states and further created at low and high temperatures, low and high atmospheric pressures, thermal and nonthermal atmospheric plasma, etc. Plasma temperatures and densities range from relatively cool and thin to very hot and dense. Hence, plasma can be created in various types that include low-

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temperature plasma (LTP) and low-pressure plasma (LPP).

In 2008, the American Department of Energy, Office of Fusion Energy Sciences, found that one of the challenges of LTPs was how it interacts with organic materials and living tissue. They had established that the exposure of these materials to LTP environment resulted in chemical and structural changes both on the surface and inside the material. The interaction is known to be complex scientifically and brings about complex scientific changes deep inside the bulk of the material (Kushner, 2008). Investigation into plasma interactions with organic materials and living tissue is said to have been largely empirical (Kushner, 2008). Many unknowns are still unresolved, which range from the interaction mechanisms plasmas modify material structures and tissues, to in-depth higher chemistry and physics of the specific plasma systems used for treating such biological materials (Kushner, 2008). From the literature, due to large number of possible reactions between organic material and the plasma species, the scientific study of such problem is challenging and depends on the specific organic material and plasma species used (Kushner, 2008). While there has been some progress in the interaction of plasmas with organic materials, the study of plasma–living tissue interaction is an almost unexplored field (Kushner, 2008). Two areas where interaction between plasma and living tissue has been exploited are categorised as destructive and nondestructive: destructive sterilisation of medical devices, surgery, etc. and nondestructive treatment of wheat and oat seeds to enhance their germination and early growth (Šerá *et al*. 2010). Research findings have found plasma with the ability to enhance and quicken biochemical reactions (ThBrnblom *et al*., 1992). A report published by the Germany Federal Ministry of Education and Research in November 2001 says plasma is known to offer a new potential for selective application while influencing biochemical processes. When used under careful control, it is possible to intensify certain reactions, while other reactions are suppressed especially for oxygen potential (Chen, 1994). A new area that is beginning to be explored is the use of LTP in modification of cell/tissue surfaces (Kushner, 2008).

Low-temperature plasma is partially ionised plasma with unique features of being almost in a nonequilibrium state. It is chemically active and is strongly affected by the presence of neutral species. It is often characterised by strong nonequilibrium velocity and energy distributions of neutral and charged constituents. The electron temperature is much higher than the temperature of the ions, and the temperature of ions is usually greater than that of the neutral particles. LTP has been used to study its influence on germination and early growth of wheat and oat seeds (Šerá *et al*. 2010), which improved the former meaning that it did not harm the living cells of the seed. LTP has also been used for the treatment of a wool fabric in which the wool characteristics of wettability were changed (Chi-wai *et al*., 2004). Frank Denes, an associate professor of biological systems engineering at the College of Agricultural and Life Sciences University of Wisconsin USA, has previously stated that plasma species interact with inorganic and organic materials and change their structures. LTP is usually free of complicated magnetic fields, and ultraviolet ray emissions are negligible (Chen, 1994); thus, it can be used for food processing (Mastwijk *et al*., 2010). By studying the effect of low-temperature nitrogen plasma (LTNP) on specific organic materials, plasma interaction may be used to target specific elements and compounds in the material and aid in the formation of specific compounds within the material.

One such agricultural produce whose chemical changes during withering can be enhanced with the resultant made tea beneficial to human health is tea. Green tea leaf is a multicellular material consisting of a variety of elements, organic and inorganic compounds. The interaction of these elements and compounds with LTP environment may enhance their concentrations in made tea. With these in mind, the current research investigates the effects of LTP on polyphenol concentration within the green tea leaf during withering stage. Among the methods that can be used for the creation of an LTP environment is the dielectric barrier discharge (DBD) chamber (Conrads *et al*., 2000) (Fig. S1).

Tea withering process

During withering in LTP, oxygen and nitrogen neutral particles by virtue of changes in osmotic and partial pressure within the green leaves diffuse into the intercellular spaces/substomatal cavities of the leaves as governed by the laws of diffusion, that is, by Fick's law and Graham's law. Once inside the leaf, their movements are governed by Henry's law where plasma particles diffuse from the intercellular air spaces to the sites of polyphenol decomposition inside the cells (John *et al*., 1996).

Polyphenol occurrence

Catechins are the building blocks of tannins in plants. Catechins present in green tea leaf are usually referred to as polyphenols (Claudia *et al*., 2008). The polyphenols occurring in the tea plant are derivative of gallic acid ($C_6H_2(OH)_3COOH$) and catechins ($C_{15}H_{14}O_{64}H_2O$). Hence, they are naturally found in plants including tea. The six main polyphenols are catechins (C), epicatechin (EC), epicatechin gallate

(ECG), epigallocatechin gallate (EGCG), gallocatechin (GC) and epigallocatechin (EGC). Green and black teas are processed differently during manufacturing. Fresh green tea leaves that are very rich in catechins are not fermented, but are withered, and catechin oxidation by the enzyme polyphenol oxidase is prevented either by anaerobic environment, by steaming or by panning to produce green tea. The process maintains the polyphenols in their monomeric forms (Claudia *et al*., 2008). Black tea is produced by oxidation during fermentation stage, which reduces the polyphenol concentration. During chemical reactions, the enzymes and polyphenols are the key participants in which the enzymes polyphenol oxidase (PPO) and peroxidase (PO) catalyse the oxidation. Theaflavins and other compounds are formed when polyphenols are oxidised or fermented. The physical parameters that affect the polyphenol quantities are therefore the type of environment of the process reaction (leading to/not oxidation) and withering time among others. To maintain the contents of the polyphenols therefore, depends on the processing methods and environment in which oxidation or non-oxidation takes place.

Polyphenols are abundant micronutrients in our diets, and there is an evidence of their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases (Manach *et al*., 2004). The health effects of polyphenols depend on the amount consumed and on their bioavailability (Manach *et al*., 2004). Their content in foods vary according to numerous genetic, environmental, processing and technologic factors. For example, fruit peeling, dehulling of legume seeds and removal of surface layer result in loss of polyphenols (Manach *et al*., 2004). Break-up of plant tissues such as tea maceration leads to oxidative degradation of polyphenols. In view of these, the current study looks at the use of low-temperature nitrogen plasma (LTNP) environment for purposes of optimising polyphenols during withering within the green tea leaf. In conventional chemistry, less amount of energy is transferred to the reactants at a slower rate, whereas in plasma chemistry, a comparatively larger amount of energy is transferred to the reactants at a faster rate resulting in the formation of intermediate energetic products. From this point of view, the hypothetical question as to what effect low-temperature nitrogen plasma environment has on polyphenols in green tea leaf during withering stage is asked. Factors known for influencing formation of polyphenols within the green tea leaf are the type of gaseous environment used for withering and withering time (Lin *et al*., 2004).

In the current research, a new environment was introduced, that is, nitrogen plasma environment provided at low temperature. The gas was ionised at a temperature of 300 K and atmospheric pressure. It is nonthermal with a very high electron temperature (10^5 K) compared with the temperature of ions and neutral particles which remains at room temperature (i.e. about 300 K). The electrons do not share the energy gained from the electric field because collisions with heavier ions and neutral species are elastic due to their small masses.

Low-temperature nitrogen plasma generates oxygen gas as a result of dissociation, association, ionisation, etc. of the species in plasma. The two gases diffuse into the leaf to create the environment that affects the polyphenols. Hence, studying this phenomenon will improve our knowledge on tea withering and enable us to adapt plasma technology for withering green tea leaf with a view to optimise the content of polyphenols. By the fact that larger amount of energy is transferred to the reactants at a faster rate in LTP environment, the time taken for processing is expected to decrease. The overall objective was to investigate the effect of LTNP environment on polyphenol content in the green tea leaf during withering.

Material and methodology

Low-temperature nitrogen plasma (LTNP) and non-LTNP environments were used for withering green tea leaf to understand and compare their effect on the concentrations of the polyphenols in made tea. Green tea leaf samples were collected from a tea-growing location (Limuru in Kenya) on different days and had the following characteristics:

- Same variety and clone.
- Same maturity (picked only two leaves and a bud, 2 weeks old).
- Picked the same two leaves and a bud all the time.
- Picked from the same location and farm.
- Cultivated using the same agronomical practices.
- Grown in the same climatic and soil conditions.
- Picked on occasions of similar climate, that is, to avoid leaf with feasible moisture (green leaf without any wetness).
- Leaf average moisture content of 68% at this time of the year.

Seven samples of green tea leaf were withered in LTNP, and six samples of similar green tea leaf were withered in non-LTNP environments. Three samples of made tea withered in non-LTNP environment were picked from the local market for analysis. Four main constituent catechin compounds, namely epigallocatechin, epicatechin gallate, epigallocatechin gallate and caffeine, were investigated for their contents in made tea.

Withering in LTNP environment

A dielectric barrier discharge (DBD) chamber method was used to create LTNP environment. During the

experimentation, three external parameters of nitrogen plasma, namely electrical power input, temperature and pressure, were kept constant, and the effect on the content of polyphenols in made tea was studied against withering time. The DBD consisted of a 250-mL glass bottle connected to a high electric power voltage 30-kv source as shown in schematic diagram (Fig. S3).

Sample collection and processing

Fresh green tea leaf samples, 15 years old, were collected from a smallholder farmer on different days from Limuru, a distance of about 50 km from experimental site in a cool box. Samples weighing 200 g each of green tea leaves were withered in LTNP environment.

Withering procedure

Weighted green tea leaf was loaded into the DBD chamber followed by evacuation of air using vacuum pump. The chamber was filled with nitrogen gas to atmospheric pressure and ionised throughout the experiment using 30-kv electric power voltage (field ionisation) (Fig. S3). The samples were withered in the plasma environments as indicated in Table S1 for various withering time intervals. The samples were then macerated and dried.

Withering in non-LTNP environment

In the second set of experiments, 10-kg samples of fresh green leaf were withered anaerobically in nitrogen gas at various withering times. One sample was not withered at all as indicated in Table S1. The samples were then macerated and dried. Three samples of made tea were picked from the local market and included in the analysis.

Sample polyphenol concentration analysis

Made tea samples were analysed for polyphenol content using high-pressure liquid chromatography (HPLC).

HPLC analysis of polyphenols in made tea

Made tea sample preparation

For each of the sample, approximately 200 mg was finely ground using a mini pestle and mortar to fine powder. 100 mg of the sample was weighed into a 50-mL volumetric flask; 20 mL of a mixture of 80% methanol in distilled water was added and ultrasonicated for 30 min while ensuring the water temperature in the ultrasonic bath did not go beyond 30 °C (achieved by placing ice blocks in the water bath). The samples were filtered into a 50-mL volumetric flask using a Whatman filter paper number 42. After completion of filtration, the filter paper was washed with 10 mL of methanol and allowed to drip into the volumetric flask up to complete dryness (done to ensure that no analyte is left on the filter paper). The volumetric flask was toped up to the mark with distilled water. 10 mL of this solution was passed through a microfilter (45 μm) so as to remove further tiny microparticles before analysis by HPLC. 50 μL of the prepared sample was injected into the instrument for subsequent analysis. The above procedures are adopted from the study by Jiang & Zhang (2010), with minor modifications, notably by the addition of the ultrasonication step to ensure the effective extraction of the polyphenols from the tea.

Standard solution preparation

Preparation of stock solution. Of 100 ppm standards were appropriately prepared by dissolving the available standards in a solution of 40% acetonitrile in microfiltered distilled water. The standards available were epigallocatechin, epicatechin gallate, caffeine and epigallocatechin gallate.

Preparation of calibration solutions. From the stock solution, calibration standards were prepared from the range of 2.5, 5, 7.5 and 10 ppm. A multicomponent mixture of the following standards, epigallocatechin, epicatechin gallate, caffeine and epigallocatechin gallate was prepared by mixing the appropriate (calculated) volumes in a 10-mL volumetric flask.

Analysis of standards and calibrations. Prior to analysis of the multicomponent calibration standards, each of the individual standards was injected into the instrument to establish their retention times. After this was achieved, the calibration standards were run, and their peak areas and retention times were noted as shown in Figs 1 and 2.

Chromatographic conditions

High-pressure liquid chromatography model: Knauer	
Column type: vertex column	
Column packing material: Eurospher 100–5. C18	
Column length × ID: 250 × 4.6 mm	
Mobile phase: solvent A	Solvent B
Distilled H_2O: 95	Distilled H_2O: 60
Formic acid: 5	Acetonitrile: 40
Flow rate: 1 mL min^{-1}.	
Detector: pulsed diode array	
Wavelength: 280	
Oven temperature: 30 °C	

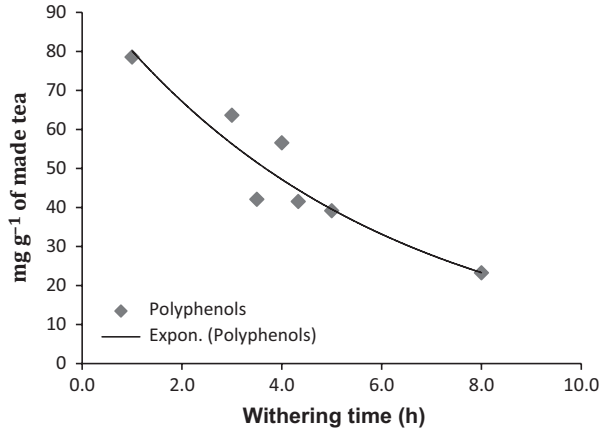

Figure 1 Trend of polyphenol concentration variation in made tea withered in low-temperature nitrogen plasma (LTNP) vs. retention times.

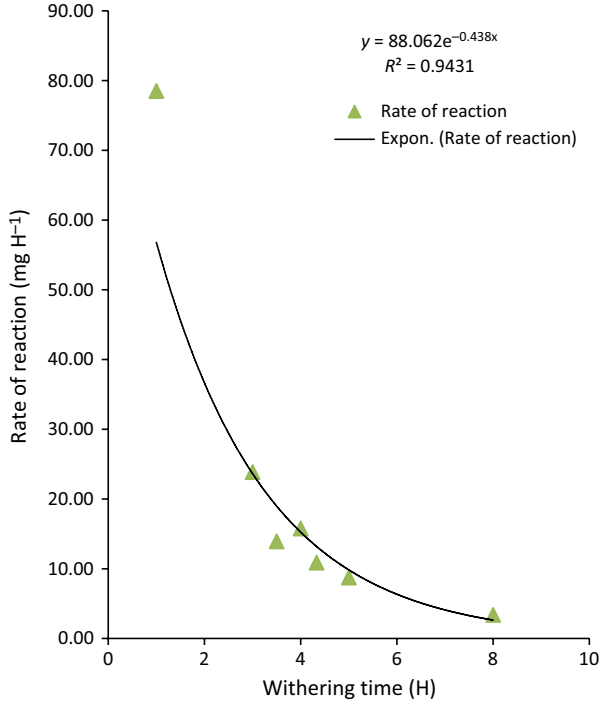

Figure 2 Relationship between the rate of reaction (polyphenol reduction) vs. withering time in low-temperature nitrogen plasma (LTNP) environment.

Statistical analysis

The LTNP samples processed at the various withering times were analysed for polyphenol contents using MS Excel Package. Non-LTNP-withered samples were also analysed for polyphenol content.

Results and discussion

Observation

Laboratory analysis gave the following results for the various conditions and durations under which green tea samples were withered. Table S2 indicates that there was a general decrease in the contents of each of the components with increasing withering time. Epigallocatechin gallate had the highest percentage composition among the components making up the polyphenols in both LTNP- and non-LTNP-withered teas. It was followed by caffeine, while epicatechin gallate and epigallocatechin showed mixed results in terms of quantities over different withering time scales.

Sample green tea 3 withered in non-LTNP environment had the highest amount of epicatechin gallate of 10.58 mg g^{-1} in made tea followed by sample green tea 1 with 9.79 mg g^{-1}. Purple tea had epicatechin gallate concentration of 9.59 mg g^{-1} in made tea, while sample 1 withered in LTNP environment had epicatechin gallate concentration of 8.87 mg g^{-1} in made tea.

Purple tea had the highest content of epigallocatechin of 14.3 mg g^{-1} in made tea followed by sample green tea 4 with 12.43 mg g^{-1} in made tea, and sample green tea 1 had 8.9 mg g^{-1} in made tea. An LTNP-withered sample with the highest amount was sample 3 with 9.3 mg g^{-1} in made tea.

Sample 8 had the highest amount of epigallocatechin gallate of 133.4 mg g^{-1} in made tea as compared to sample green tea 1 with 83.3 mg g^{-1} in made tea, while sample 1 withered in LTNP environment had 53.7 mg g^{-1} in made tea.

Sample 3 had the highest caffeine content of 47.1 mg g^{-1} in made tea followed by sample green tea 1 that had 39.5 mg g^{-1} in made tea.

Overall, polyphenol content decreased with increasing withering time. This was observed in both LTNP- and non-LTNP-withered teas. However, the scale of decrease was faster in LTNP-withered tea than in non-LTNP-withered tea. The reason for the observation is the presence of oxygen generated from plasma environment.

Effect of LTNP on polyphenol content

Sample 1 withered under LTNP environment for 1 h had the highest average polyphenol content of 78.56 mg g^{-1} in made tea. Sample 2 had polyphenol content of 71.59 mg g^{-1} in made tea. In general, results given in Table S2 indicate a decreasing trend of the polyphenol content in made tea (as shown by samples 1–7) with increasing withering times (Fig. 2).

Effect of non-LTNP on polyphenol content

Whereas green tea leaf withered in non-LTNP environments gave an average polyphenol content of

133.4 mg g^{-1} in made tea indicated by sample 8, green tea 1, green tea 2 and green tea 3 leaves withered in non-LTNP environments as shown in Table S1 gave polyphenol concentrations of 111.7, 87.51 and 100.59 mg g^{-1} in made tea, respectively.

From the results of Table S2, LTNP-processed tea samples show lower values of polyphenol concentrations compared with the non-LTNP-processed tea.

All the individual samples were analysed in triplicates, and quantification was carried out through external standard calibration. The average concentrations, standard deviations (SD) and percentage relative standard deviation (%RSD) were calculated using Microsoft Excel Package as indicated in Table S2.

The results given in Table S2 were statistically analysed using Microsoft Excel Package and presented in line graphs. The statistical analysis indicated that the relationship between the rate of reduction in polyphenols in green tea leaf and withering time follows an exponential function (Fig. 2). The equation of the trend line has a negative sign, which was an indicator of an inverse relationship.

The rate of change in polyphenols with withering time was calculated and plotted on a line graph against remaining content of polyphenol in the green tea leaf. The relationship between the rates of polyphenol reduction and remaining content of the polyphenols indicated also an inverse proportionality. The coefficient of determination indicated that the relationship follows also an exponential function; that is, as the content of polyphenols in the green tea leaf decreased, its rate of reduction decreased. The reaction rate approaches a limiting minimum rate as the substrate (polyphenol) concentration decreases (Fig. 2).

Discussion

The decrease in polyphenol content in made tea is a result of oxidation caused by the presence of oxygen within LTNP environment in the withering chamber. The presence of moisture is a result of moisture released from green tea leaf due to metabolic processes which underwent partial ionisation. Some of the oxygen ions eventually combined to form neutral oxygen gas in the process. This is shown by the following ionisation reactions occurring within the LTNP chamber.

$$H_2O \xrightarrow{k1} H^+ + OH^-$$

The combination of nitrogen gas and moisture ions gave the following particles in the LTNP plasma chamber.

Negatively charged particles: Electrons (e), O−, O_2−, O_3−, O_4−, H−, OH−, NO−, N_2O−, NO_2−, NO_3−.

Positively charged particles: N^+, N_2^+, N_3^+, N_4^+, O^+, O_2^+, O_4^+, NO^+, N_2O^+, NO_2^+, H^+, H_2^+, H_3^+, OH^+, H_2O^+, H_3O+.

Neutrals/radicals: N, N*, N_2, N_2*, N_2**, O, O*, O_2, O_2*, O_3, NO, N_2O, NO_2, NO_3, $2O_5$, H, H_2, OH, H_2O, HO_2, H_2O_2.

These chemically reactive species instituted and controlled the distribution velocities and energies of electrons, ions and neutral particles including oxygen. As a result, oxidation of polyphenols occurred, leading to the reduction in their concentration in the green tea leaf. This inference can be seen in samples oolong and black teas (Table S2). Oolong and black tea samples show lower polyphenol concentrations compared with other samples. The reason was fermentation during their manufacturing process. Oolong tea is normally rolled up leaf, partially fermented and dried. Black tea gives the lowest concentration due to the fact that it is macerated by cut, tear and crush processes exposing the cells to complete fermentation, hence providing more surface area for oxidation reactions during the fermentation process. Purple tea as given in Table S2 was manufactured using the oolong tea method and as a result also underwent some form of fermentation process. It can also be seen that prolonged storage of green leaf before processing drastically reduces the natural polyphenol content. The reason to this observation is fermentation (oxidation) of the tea leaf as indicated by sample 9 in Table S2. Diffusion of the oxygen particles to the polyphenol reaction sites is governed by the laws of diffusion. From the ionisation reactions above, oxygen ions and particles are the most dominant. This may have led to increased formation of neutral oxygen particles, leading to diffusion into the leaf thereby occasioning oxidation of the polyphenols.

From Table S2, content of the constituent compounds of polyphenols in LTNP-withered tea shows a decreasing trend with increasing withering retention time. This observation is a result of their conversion into other compounds due to oxidation, thereby reducing polyphenols in made tea. Withering time for both LTNP and non-LTNP indicates an inversely proportional relationship with polyphenol content in made tea. However, LTNP-environment-withered teas shows shorter withering times in relation to the polyphenol content in made tea. The explanation to this observation is due to the fact that plasma environment provides energy for a reaction at a faster rate (Kushner, 2008) compared with the nonplasma environments. Other reasons are the presence of charged particles (electrons and protons) needed for molecular bonding also enhanced the reaction (Kushner, 2008). LTPs are in nonequilibrium, with extensive range of positive and negative ions of varying mass and transport coefficients. In this nature, they provide a rich possibility of

waves and instabilities, which increase the reaction potential of the species within the system (Kushner, 2008). In addition, the presence of particles in ionised form within the environment as can be seen from the ionisation reactions above enhances the rate of reaction.

Conclusion

The study showed that LTNP green tea leaf withering has an effect on polyphenol content in made tea. The concentrations of polyphenols in made tea withered in LTNP were less than those of tea withered in non-LTNP environment. Both LTNP- and non-LTNP-withered teas gave higher contents of polyphenols than the existing locally manufactured teas. Compared with existing locally manufactured teas, LTNP-withered tea gives higher polyphenol concentration in a shorter withering time. Also observed in the investigation was that a sample that was directly macerated and dried gave a higher polyphenol concentration of 111.70 mg g^{-1} in made tea than the existing locally manufactured and LTNP-withered teas.

Ionisation density is governed by the strength of the electrical power voltage and the concentration of the gas (nitrogen) inside the DBD chamber. Ionisation density in LTNP environment can be controlled and used in withering green tea leaf for purposes of controlling polyphenol degradation during withering stage. These can be achieved by varying and controlling the electrical power voltage, nitrogen gas concentration within the DBD chamber and the withering retention time.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic diagram of experimental set-up.

Figure S2. Dielectric barrier discharge (DBD).

Figure S3. Overlayed chromatogram showing sample peaks obtained from LTNP withered tea against four standards (EGCG, ECG, EGC and caffeine).

Table S1. Green tea leaf withered in various low-temperature nitrogen plasma (LTNP) and non-LTNP environments at different withering times.

Table S2. High-pressure liquid chromatography (HPLC) peak areas of epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and Caffeine-Concentrations in made tea samples processed in low-temperature nitrogen plasma (LTNP) and non-LTNP environments.