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QUALITATIVE, METABOLIC AND NUTRITIONAL ASPECTS OF TRADITIONAL AND INNOVATIVE MINIMALLY PROCESSED FRUIT

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Abstract

Minimally processed fruit (MPF) are products that have to maintain their quality similar to those of fresh ones. Being metabolic active tissues, they show physical and physiological response to minimal processing (wounding), that negatively influence their shelf-life. In the last decades, novel non-thermal processing methods have attracted the interest of food scientists, industries and consumers as technologies useful for shelf-life extension or increasing product functionality, with a minimal impact on the nutritional and sensory properties of foods. The main aim of this PhD thesis was to investigate qualitative, metabolic and nutritional aspects of different MPF, submitted to traditional and innovative non-thermal processes. This issue was addressed considering the product as a dynamic system, both in terms of endogenous physiological activity and porous matrix interacting with the surrounding ambient (during processing and storage), through the application of multi-analytical approach. The most consistent results related to the applied non-thermal techniques confirmed their different potentiality in the optic of processing and product innovation, but the need of their modulation in relation to the different raw material susceptibility to degradation and final product target. Cold plasma treatment effects on fresh-cut fruit, characterized by different kind of stability criticisms, resulted mainly bound to the inactivation of degradative enzymes and microbial cells, without evidencing functional modifications in the final products. The study of osmotic dehydration and vacuum impregnation highlighted as these techniques can be successfully applied for cold formulation/enrichment of MPF, but also the necessity to carefully account for the metabolic and structural modifications induced by the processing on the vegetable tissues. An induction of metabolic stress response was also evidenced as a consequence of pulsed electric fields treatment related to electric field strength. Below the threshold limit of irreversible damages to cell membranes, the treatment promoted only slight and reversible modifications of the metabolic profiles.

List of Papers

This thesis is based on the work contained in the following papers, referred to in the text by their Roman numerals. The papers are attached as appendix at the end of the thesis.

I. Tappi S., Tylewicz U., Cocci E., Romani S., Dalla Rosa M., Rocculi P. (2014) Influence of ripening stage on quality parameters and metabolic behaviour of fresh-cut kiwifruit slices during accelerated storage, *Journal on Processing and Energy in Agriculture*, 17 (4), 149-153

II. Tappi S., Mannozzi C., Tylewicz U., Romani S., Dalla Rosa M., Rocculi P. (2015) Enzymatic activity and metabolism of fresh-cut fruit as a function of ripening degree. *Journal on Processing and Energy in Agriculture*, 19, 5, 219-223

III. Tappi S., Berardinelli A., Ragni L., Dalla Rosa M., Rocculi P. (2014) Atmospheric gas plasma treatment of fresh-cut apple. *Innovative Food Science and Emerging Technologies*, 21, 114-122

IV. Ramazzina I., Berardinelli A., Rizzi F., Tappi S., Ragni L., Sacchetti G., Rocculi P. (2015) Effect of cold plasma treatment on physico-chemical parameters and antioxidant activity of minimally processed kiwifruit. *Postharvest Biology and Technology*, 107, 55-65

V. Tappi S., Gozzi G., Vannini L., Berardinelli L., Romani S., Ragni L., Rocculi P. (2016) Cold plasma treatment for fresh-cut melon stabilization. *Innovative Food Science and Emerging Technology*, 33, 225-233

VI. Ramazzina I., Tappi S., Sacchetti G., Rocculi P., Marseglia A., Berardinelli A., Rizzi F. "Effect of cold plasma treatment on the functional properties of fresh-cut apples". Manuscript

VII. Tappi S., Tylewicz U., Romani S., Siroli L., Patrignani F., Rocculi P. Optimization of vacuum impregnation with calcium lactate of minimally processed melon and shelf-life study in real storage conditions**.** Submitted for publication to *Journal of Food Science*

VIII. Tappi S., Tylewicz U., Romani S., Dalla Rosa M., Rocculi P. Preliminary study on the quality and stability of minimally processed apples impregnated with green tea polyphenols during storage. Submitted for publication to *European Food Research and Technology*

IX. Mauro M.A., Dellarosa N., Tylewicz U., Tappi S., Laghi L., Rocculi P., Dalla Rosa M. (2015) Calcium and ascorbic acid affect cellular structure and water mobility in apple tissue during osmotic dehydration in sucrose solutions. *Food Chemistry*, 195, 19-28

X. Tappi S., Mauro M.A., Tylewicz U., Dellarosa N., Dalla Rosa M., Rocculi P. Effects of calcium and ascorbic acid on osmotic dehydration kinetics and metabolic profile of apples. Submitted for publication to *Food and Bioprocess Technology*

XI. Dellarosa N., Tappi S., Ragni L., Laghi L., Rocculi P. and Dalla Rosa M. Metabolic response of fresh-cut apples induced by pulsed electric fields. Submitted for publication to *Innovative Food Science and Technologies*

Contents

1. Introduction and objectives

Minimally processed fruit (MPF) are products that have to maintain their quality (appearance, texture, flavour and nutritive value) similar to those of fresh products (Alzamora *et al*., 2000).
The key to understand MPF physiology is that they are metabolic active tissues, and as a consequence, show physical and physiological response to minimal processing procedures (*wounding*) (Toivonen & DeEll, 2002) that negatively influence their shelf-life. Quality loss of MPF is principally due to physiological ageing, caused by the loss of cellular compartmentation, due to peeling and cutting, that causes the mixing of enzymes and substrates and an overall increase in metabolic metabolism (Rolle & Chism, 1987).
One of the main aims of the fruit industry is to develop new preservation technologies to efficiently respond to the exigent quality and safety consumer's perception, defining their choices and food economics. Consumers demand high quality and convenient fruit and vegetable products, with natural flavour and taste, and appreciate the freshness of minimally processed food.
In the last decades, novel non-thermal processing methods have attracted the interest of food scientists, industries and consumers as technologies useful for shelf-life extension or increasing product functionality with a minimal impact on the nutritional and sensory properties of foods.
Although some aspects about these technologies have been widely investigated, there are still some aspects that are scarcely known.

Hence, after a preliminary evaluation of the effect of ripening degree on qualitative, metabolic and nutritional aspects of different MPF (**Paper I and II**), the main aim of this PhD thesis was to investigate qualitative, metabolic and nutritional aspects of different minimally processed fruit submitted to traditional and innovative non-thermal processes.
This issue was addressed considering the product as a dynamic system, both in terms of endogenous physiological activity and porous matrix interacting with the surrounding ambient (during processing and storage), through the application of multi-analytical approach based on the combination of different techniques.

In particular the research was focused on the following aspects:

2 Study of the effect of plasma treatment on microbial, qualitative, metabolic and nutritional aspects of different MPF (**Paper III, IV, V and VI**);
3 Study of the effect of vacuum impregnation (VI) as a treatment for technological and nutritional functionality of MPF (**Paper VII and VIII**);

4 Study of the effect of osmotic dehydration on mass transfer, water state and metabolic response of MPF (**Paper IX and X**);

5 Study of the effect of pulsed electric fields (PEF) on the metabolic response of minimally processed apples (**Paper XI**).

2. Minimal processing of fresh fruit

The increasing popularity of minimally processed fruit (MPF) and vegetables has been attributed to the health benefits associated with fresh produce, combined with the ongoing consumer trend toward eating out and consuming ready-to-eat foods with a higher convenience value, but also driven by the trend towards healthier eating. The increasing demand of these products represents a challenge for researchers and processors to make them more stable and safe.

MPF are products that undergo mild processing operations that allow them to maintain their quality attributes similar to those of the fresh ones. The MPF definition has evolved, as the minimal processing concepts have been better understood (Alzamora *et al.,* 2000). According to Bolin & Huxsoll (1989), the definition can include foods in which tissues are not alive but whose freshness should be kept as an important objective of preservation.

The expansion of minimal processing concepts has been reflected in anew, renewed and improved products and processes formulated and designed to produce a greater diversity of MPF. There is also a great interest in the application of new or emerging technologies to obtain MPF using non thermal processes in the framework of the "hurdle" concept (Alzamora *et al.*, 2000).

Key requirements in minimal processing of fresh products are raw material of high quality, strict hygiene and good manufacturing practices, low temperatures during processing, cleaning and/or washing before and after peeling, gentle peeling and cutting, pre-treatment, correct packaging materials and methods and correct temperature and humidity during distribution and retail (Varoquaux & Mazollier, 2002).

2.1 Raw material handling and ripening degree

The quality of the raw material is definitely an essential factors determining the quality of the final product (Varoquaux & Mazollier, 2002).

Vegetables or fruits intended for pre-peeling and cutting must be easily washable and peelable, and must be of top quality (Ahvenainen, 2000). For hygienic reasons, no manure or fertilizer of animal origin should be used (Varoquaux & Mazollier, 2002).

Incoming vegetables or fruits, which are covered with soil, mud and sand, should be carefully cleaned before processing (Ahvenainen, 2000). This first step is really important in order to avoid cross contamination due principally to peeling and cutting operations.

The extent of the physiological response to minimal processing is affected by several factors, both internal and external. Internal factors include species and variety, but also both maturity at harvest and ripeness stage at cutting.

The selection of raw material, and in particular, the correct choice of cultivar is a critical step to obtain final products with long shelf life and high quality attributes and has to take into account various criteria (Varoquaux & Mazollier, 2002) such as: low sensitivity to physiological disorders and microbial diseases; low susceptibility to browning and softening (Hodges & Toivonen, 2008); mechanical resistance of the tissue; resistance to elevated CO_2 concentration (Varoquaux *et al.*, 1996) and/or low O_2; high sugar contents because sugar depletion may be responsible for energy stress (Forney & Austin, 1988); low respiration rate (Varoquaux *et al.*, 1996).

Degree of ripening at harvesting and at processing is an important factor that can influence the intensity of the wounding response (Hodges & Toivonen, 2008). Generally, processing fruits that are unripe or slightly unripe, lead to a better preservation of quality during storage but it could be detrimental for the organoleptic and flavour profile (Beaulieu *et al.*, 2004).

Conversely, it seems that fruit at an advanced stage of ripeness tend to be more susceptible to wounds, hence to minimal processing (Soliva-Fortuny & Martín-Belloso, 2003; Gorny *et al.*, 2000). Minimally processed apples and pears showed to better preserve their visual and textural characteristics during storage if processed while still unripe (Soliva-Fortuny *et al.*, 2002). Furthermore, the fresh-industry prefers to process firmer and less mature fruits because of technological suitability, and a consequent longer shelf-life of the final product (Hodges & Toivonen, 2008). On the other hand, during ripening fruit develop their characteristic organoleptic quality and flavour profile (Gorny *et al.*, 2000; Beaulieu & Lea, 2003; Aguayo *et al.*, 2004b; Beaulieu, 2006)

According to various authors (Beirão-da-Costa *et al.*, 2006; Panarese *et al.*, 2012), maturity stage has an impact also on the effectiveness of pre-treatment such as mild heat treatments and osmotic dehydration (OD) for the production of fresh cut fruits.

Paper I and **II** reported the effect of ripening degree on the evolution of various quality indexes characteristics of fresh-cut fruit; in particular while in **Paper I** the attention is focused on kiwifruit, in **Paper II** a comparison among apples, kiwifruit and melon at different ripening degree during storage after minimal processing was carried out.

Generally, quality characteristics were found to be highly affected by ripening degree in kiwifruit and melon, but only slightly in apple. In particular, kiwifruit and melon processed at low stage of ripeness showed a better ability to retain initial characteristics, both in terms of colour and texture.

As an example, lightness and hue angle of fruit samples during storage are reported in **Table 1**. Tissue lightness (L*) was found to decrease during ripening and during storage, causing surface darkening that was attributed to variation in the internal structure of the tissue and the induction of a translucent water-soaked tissue, while enzymatic browning in these fruits was scarcely influenced

(Agar *et al.*, 1999). In fresh cut melon variations were observed also in hue angle, indicating a variation of hue probably due to changes in the β-carotene concentration (Simandjuntak *et al.*, 1996).

Table 1. Colour of apple, kiwifruit and melon at the two ripening stages selected during accelerated storage in controlled conditions. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit for the same storage time (Table 3 in **Paper II**).

	Fruit	Ripening Stage	Storage time (days)				
			0	0.5	1	2	4
L*	Apple	U	81.45 ^{a} ± 2.05	69.33 ^{a} ± 2.45	65.57 ^{a} ± 1.38	-	-
		R	82.33 ^{a} ± 3.05	71.55 ^{a} ± 2.56	67.67 ± 2.05	-	-
	Kiwifruit	U	48.45 ^{a} ± 2.11	-	47.98 ^{a} ± 3.07	47.2^{1a} ± 3.16	44.08^{a} ± 3.77
		R	38.11^{b} ± 2.45	-	31.93 ^{b} ± 3.61	31.88 ^{b} ± 3.78	31.75^{b} ± 1.81
	Melon	U	57.24 ^{a} ± 2.90	-	45.25 ^{a} ± 1.72	45.26 ^{a} ± 2.12	40.43^{a} ± 4.97
		R	57.85 ^{a} ± 4.50	-	42.92^{a} ± 4.04	44.11^{a} ± 2.48	42.38^{a} ± 4.97
h°	Apple	U	97.57^{a} ± 4.55	93.25^{a} ± 2.56	88.15^{a} ± 2.07	-	-
		R	98.67^{a} ± 3.18	90.03^{a} ± 3.56	84.66 ± 2.28	-	-
	Kiwifruit	U	103.93^{a} ± 2.11	-	103.72^{a} ± 0.76	102.19^{a} ± 2.16	101.17 ^{a} ± 3.11
		R	103.83^{a} ± 2.70	-	104.70^{a} ± 2.80	104.44^{a} ± 2.05	106.32^{b} ± 4.39
	Melon	U	61.81^{a} ± 0.98	-	60.89 ^{a} ± 1.05	59.83 ^{a} ± 1.76	60.08 ^{a} ± 1.03
		R	64.57^{b} ± 0.89	-	62.91^{b} ± 1.16	62.85^{b} ± 1.37	62.11^{b} ± 1.75

On the contrary, surface colour of apple, that is mainly affected by polyphenloxidase activity was apparently not influenced by maturity degree, as no differences were observed in the entity of browning or enzymatic activity in relation to ripening index **(Paper II).**

Similar results were found for textural parameters. Ripening degree influences initial hardness values and texture evolution during storage in kiwifruit and melon. In particular, unripe fruits showed a better ability to retain initial hardness, while riper ones presented very low values of this parameters indicating an advanced stage of internal structures breakdown. The obtained results suggest that with the progressive ripening of the fruit, the internal structure undergoes a breakdown that causes a substantial loss of initial firmness. On the contrary, in fresh-cut apples hardness increased upon ripening in apple samples, both between ripening stages and during 24 h storage.

Moreover, metabolic profiles obtained by isothermal calorimetry have been integrated in order to calculate the total metabolic heat produced by fruit tissues during 24 h. Results are reported in **Figure 1**.

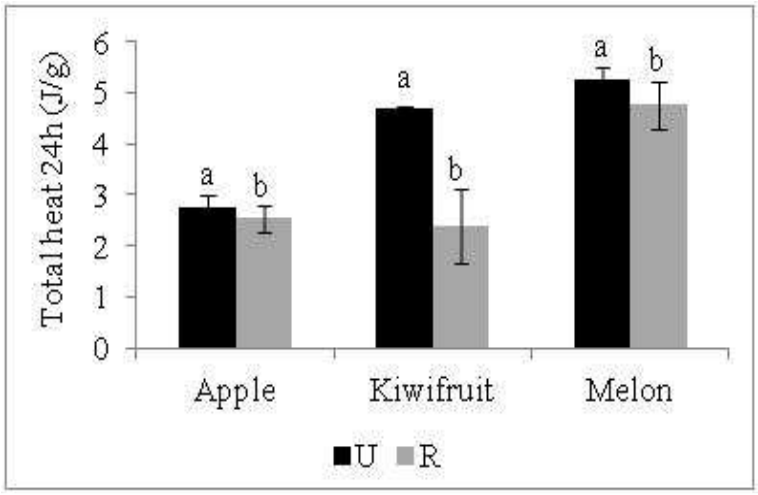

Figure 1. Total metabolic heat production of apple, kiwifruit and melon at the two ripening stages selected during 24 h at 10°C. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit (Fig, 4 in **Paper II**)

These results suggest that fruits at an advanced stage of ripening have a lower metabolism reaction after wounding, probably due to the loss of the ability to repair the damage caused by mechanical stress. While differences were statistically significant for all fruit considered, the highest difference was observed for kiwifruit that showed, at the advanced stage of ripening, a 50% decrease in the metabolic heat production compared to the unripe fruit.

Generally, results described in **Paper I** and **II** highlight how the determination of the optimal ripening degree for fresh-cut production has to be carried out specifically for each type of fruit.

2.2 Peeling, cutting and wounding response

Operations as peeling, cutting and/or slicing play an important role in the product shelf-life. The removal of external barriers, such as the peel, and the tissue disruption due to cutting that cut through cells and leave intact cells of previously internal tissues exposed, cause a complex chain of physiological reaction aimed at repairing the damage caused to the tissue, known as wounding response (Alzamora *et al.*, 2000).

Hence, the key to understand fresh-cut fruit physiology is that they are essentially wounded tissues and present an immediate response to minimal processing, including mechanical stress, removal of epidermal layer and exposure of the internal tissues to air and contaminants (Brecht *et al.*, 2004).

Physiological and biochemical responses induced by wounding regard tissues both adjacent and distant to the wound and are generally deleterious to the quality of the product. Changes can occur immediately after wounding or in the next days.

One of the first response is an increase in ethylene production and in respiration rate that may be related to the induction of phenolic metabolism and the wound healing response of the tissue.

Ethylene in turn can stimulate other processes leading to membrane deterioration, loss of bioactive compounds and development of off-flavours and can make plant tissue more susceptible to microbial spoilage (Brecht *et al.*, 2004). Figure 2 reports the interrelationship among the many effects of wounding on physiological processes in fresh-cut vegetables and fruits.

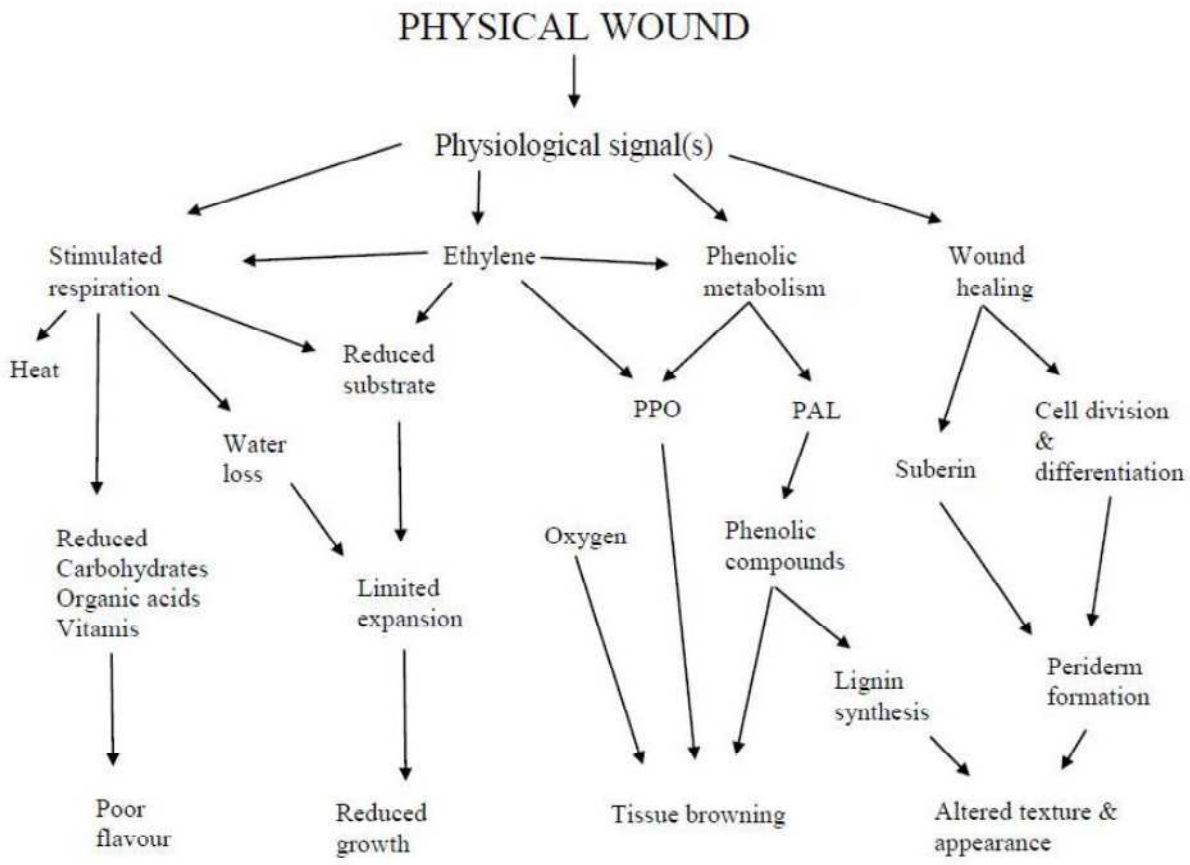

Figure 2. Schematic representation of the main physiological effects of wounding response (adapted from (Saltveit, 2010).

The main physiological responses induced by wounding in minimally processed fruits will be further described in **chapter 3**.

The intensity of the wounding response can be affected by many factors, both internal and external. Internal factors include mainly species, cultivar and ripening degree as described in **paragraph 2.1**. Among external or processing factors, the main are peeling and cutting methods, pre-treatments applied, packaging parameters and processing and storage temperature.

Few studies have been conducted on the effect of cutting methods on the quality of fresh-cut fruit products. In order to be as gentle as possible and minimize injuries, hand peeling with a sharp knife would be the ideal method (Bolin & Huxsoll, 1989; Bolin & Huxsoll, 1991). Nevertheless, for practical reasons, on industrial scale mechanical peeling is generally adopted and, according to

Varoquaux & Mazollier (2002) blades are made of soft stainless steel and generally are extensively used and not sharpened enough.

The use of dull knives and blades leads to more extensive bruising and damage, hence it can significantly and negatively affect product storage life (Garcia & Barrett, 2002).

An example of the effect of blades sharpness is reported by Portela & Cantwell (2001) that found that the use of blunt cutting blades for fresh-cut melon production lead to variation in the respiration rate of the tissue with consequences such as higher ethanol production, off-odour scores and electrolyte leakage.

According to Abe *et al.* (1998), also the cutting direction seems to play a significant role in the extent of the physiological response. In particular, among various cutting modes, 1 cm-thick transverse section banana slices showed the lowest respiration rate and ethylene production during storage.

Cutting shape also seems to be critical for final product quality. Cut cylinders of melon showed to maintain a higher firmness during storage compared to slices trapezoidal cuts, but at the same time showed a higher degree of translucency (Aguayo *et al.*, 2004a).

Finally, considering the extent of wounding, metabolic activity tends to increase by increasing the number of cuts hence the surface/area ratio. Wadsö *et al.* (2004) found that the increase of endogenous metabolic heat produced by different cut vegetables, measured by a calorimeter, was proportional to the surface to volume ratio of the wounded tissue.

2.3 Traditional pre-treatments

There are various chemical and physical preservation strategies that can be applied to maintain fresh-cut quality; they generally focus on reducing microbial growth (Artés *et al.*, 2009), browning (Garcia & Barrett, 2002) and tissue softening after cutting (Gorny *et al.*, 2002). Several methods have been investigated; some of them have a physical approach like lowering or increasing the temperature, while others are based on a chemical approach (Garcia & Barrett, 2002).

Dipping treatment after peeling and/or cutting is the most common chemical approach used in order to delay or control quality degradation in fresh-cut fruit (Oms-Oliu *et al.*, 2010).

Dipping in aqueous solutions of preservatives and/or fortifying agents is very important for the achievement of both high quality products with prolonged shelf-life. The first beneficial effect achieved is due to the rinsing of enzymes and substrates released from damaged cells during peeling and cutting. Furthermore, these treatments allow a partial dehydration of the product and/or the introduction of substances with stabilizing effects (e.g. antifermentative, antioxidant, antimicrobial agents), but also the introduction of substances that have got structuring and functional properties.

Generally, dipping times range between 1 and 5 min. Luna-Guzmán *et al.* (1999) observed that increasing dipping time from 1 to 5 min of fresh-cut melon in $CaCl_2$ solution did not have effect on fresh-cut melon metabolism and quality. Evaluating the effect of calcium lactate on minimally processed melon, in **Paper VII**, we found that the application of vacuum resulted in an increase of hardness and crispness compared to the product dipped at atmospheric conditions. Nevertheless, the traditional dipping allowed a better maintenance of colour and microbial stability during shelf-life.

Treatment temperature seems to impact on the effectiveness of the treatment applied, but results are not always in agreement. For example, while Luna-Guzmán *et al.* (1999) found that increasing temperature improved the firming effect of calcium dips in fresh-cut melon, according to Lamikanra & Watson (2004) low temperatures calcium dips had a positive effect on fresh-cut melon shelf-life.

Traditionally, for sanitizing purposes, chlorinated water is used during industrial processing with dipping in solution containing between 50 and 200 ppm of NaOCl. Recently though, concerns about health issue related to the formation of toxic by-products have been put forwards and alternative sanitizers have been studied (Silveira *et al.*, 2008). Artés *et al.* (2009) reviewed the main antimicrobial compounds investigated including peroxyacetic acid, oxygen peroxide, ozonated and electrolyzed water, organic acids and biological compounds such as essential oils and natural competitive microbiota.

Most dipping treatments aimed at avoiding browning contain acidulants, usually citric acid, in order to lower the product pH and inhibit PPO activity (Brecht *et al.*, 2004). Acidulants are often used in combination with other types of antibrowning agents. One of the most used is surely ascorbic acid, including its various neutral salts, because being a reducing compound, it is able to reduce the o-benzoquinones back to *o*-diphenols preventing enzymatic browning (Dorantes-Alvarez & Chiralt, 2000). Other compounds investigated for browning control or prevention are other reducing agents, for example thiol-containg compounds such as cysteine, chelating agents such as EDTA able to complex the copper present in the active site of the enzyme, complexing agents such as cyclodextrins that are able to entrap or from complexes with the enzyme substrates (Garcia & Barrett, 2002).

Moreover, the postharvest application of aqueous solutions of calcium salts as dips or sprays has long been used to maintain tissue firmness of fresh fruits and vegetables. Calcium is able to form cross-links or bridges between free carboxylic group of the pectin chains, resulting in strengthening of the cell wall and at the same time, preserving the structural and functional integrity of membranes. Application of calcium salts were found to improve texture retention in pears (Gorny *et al.*, 2002), kiwifruit (Agar *et al.*, 1999), nectarines and peaches (Gorny *et al.*, 1999) and melons (Luna-Guzmán *et al.*, 1999).

Although calcium chloride is the most widely used salt, it seems to affect negatively product sensorial quality imparting a bitter taste, so that other calcium salts, in particular lactate, should be preferred (Silveira *et al.*, 2011).

The application of moderate heat treatment in combination with calcium dips have shown positive results for firmness preservation of fresh-cut melon (Luna-Guzmán *et al.*, 1999) and kiwifruit slices (Beirão-da-Costa *et al.*, 2014). The effectiveness of the combined treatments is due to the fact that moderate temperatures promote the activation of PME that brings about the deesterification of pectin, thus increasing the number of calcium binding sites.

In terms of technological and economical aspects, these techniques present some disadvantages related to the necessity of dipping the product in aqueous solution (e.g. plant and consumables costs, disposal of exhausted solutions, labelling of chemical agents, further drying).

2.4 Modified atmosphere packaging

Packaging operations play a crucial role in minimally processed products shelf-life. Surely, the most studied packaging method is the Modified Atmosphere Packaging (MAP), which is based on the alteration of the atmosphere composition within the package. This alteration can be achieved both actively, replacing the internal atmosphere with the desired mixture of gas and passively, exploiting the product respiratory metabolism and the gas diffusion characteristics of the plastic film. In the latter case, the choice of the packing material based on its permeability to gases and water vapour is obviously crucial. The aim of both methods is to reach an optimal atmosphere inside the package in order to delay degradative reaction rates and prolong shelf-life.

Rocculi *et al.* (2006) observed that active MAP reduced the rate of oxygen consumption of fresh-cut apples compared with passive MAP, in particular at the beginning of storage.

Bai *et al.* (2001) compared the application of both methods, flushing the packages with a 4 kPa O_2 plus 10 kPa CO_2 gas mixture or developing the same composition naturally during storage of fresh-cut cantaloupes. The active modification of the atmosphere allowed a better colour retention and reduced translucency, respiration rate, and microbial growth compared to the ones obtained with passive MAP. The authors thought, questioned whether the improved quality of fresh-cut melon was actually worth the additional cost of flushing the package atmosphere.

Traditionally, the main gases used for MAP are O_2, CO_2 and N_2, in various combinations. It is important to recognize though, that while atmosphere modification can improve the storability of some fruits and vegetables, it also has the potential to induce undesirable effects.

Generally, a reduction of O_2 and an increase of CO_2 levels are recommended in order to prolong the fresh-state of fresh product (Sandhya, 2010), but specific levels have to be determined for each

product, particularly because exposition to concentrations outside of the tolerance limits could trigger anaerobic respiration and the production of undesirable compounds (Soliva-Fortuny & Martín-Belloso, 2003).

Shelf-life of apple slices was significantly extended with a 2.5% O_2 and 7 % CO_2 atmosphere because of inhibition of ethylene production (Rojas-Graü *et al.*, 2007). Low O_2 and high CO_2 levels showed a synergistic effect in reducing ethylene production and respiration rates in pears (Gorny *et al.*, 2002) and peaches (Gorny *et al.*, 1999) and in maintaining the initial antioxidant activity of fresh-cut strawberries (Odriozola-Serrano *et al.*, 2010).

Furthermore, although MAP can significantly delay spoilage by most aerobic microorganisms, if anaerobic conditions are reached, the growth of some anaerobic psychotrophic pathogens might be possible or even enhanced (Soliva-Fortuny & Martín-Belloso, 2003).

Anaerobic metabolism can be brought about both by a too low concentration of oxygen (Solomos, 1997) or by a too high concentration of CO_2 that is able to inhibit various enzymes of the Kreb's cycle besides various physiological disorders (Gorny *et al.*, 2002; Oms-Oliu *et al.*, 2008)

High oxygen atmosphere (>70 kPa) has been found to be effective in quality maintenance of MPF, in particular in relation to bacterial spoilage, both aerobic and anaerobic (Kader & Ben-Yehoshua, 2000; Jacxsens *et al.*, 2001) although the effect can vary in different commodities. Moreover, elevated oxygen concentrations have shown to impact respiration rate, metabolism, enzymatic activity and sensory quality but, again, the effect was highly variables among commodities and depends on other aspects, such as temperature, storage time and other gases levels (Kader & Ben-Yehoshua, 2000).

In particular, few works evaluated the effect of high O_2 levels on enzymatic browning. Day *et al.* (1998) hypothesized that exposition to high concentration of this gas caused the substrate inhibition of polyphenol oxidase (PPO) or alternatively, the formation of colourless quinones that in turn cause feedback inhibition of the enzyme. A slower browning rate during storage of apple slices, exposed to 100 kPa O_2 for 12 days before cutting, in comparison with those kept in air, was observed (Lu & Toivonen, 2000).

Recently, in addition to various mixture of N_2, CO_2 and O_2, novel gases have drawn attention for potential benefits in MAP application. Ar, N_2O and He have been admitted for food application in the EU as miscellaneous additives and few studies have been carried out on their influence in MPF shelf-life.

Although noble gases are chemically inert, argon (Ar) is reported to have some biochemical activity, probably due to its high solubility in water compared to nitrogen and it seems to interfere with enzymatic oxygen receptor sites (Spencer, 1995). Some studies have reported an effect on the

growth of certain micro-organisms on the activity of quality-related enzymes and on degradative chemical reactions in selected perishable food products, such as MP fruit (Powrie *et al.*, 1990; Spencer, 1995; Day, 1996; Kader & Ben-Yehoshua, 2000; Jamie & Saltveit, 2002; Mostardini & Piergiovanni, 2002). Rocculi *et al.* (2005) observed a positive effect in the stability of fresh-cut kiwifruit packed in MA with 90% Ar, 5% O_2 and 5% CO_2, limited to firmness and respiration rate and not on colour preservation. Generally, contrasting results have been reported on Ar effect.

The effect of N_2O on fruit metabolism is still not completely clear but seems to be related to the fact that it is characterized by biophysical properties, such as relative stability, high solubility and isoteric linear structure, similar to CO_2. It seems to be able to inhibit respiration (Sowa & Towill, 1991; Sowa *et al.*, 1993) and ethylene action and synthesis in higher plants, delaying ripening (Gouble *et al.*, 1995) and to reduce the incidence of rots in onion bulbs during storage (Benkeblia & Varoquaux, 2003).

Furthermore, an inhibition of postharvest decay and an extension of storage shelf-life was reported upon exposure to N_2O of various fruits such as apples, strawberry, mandarins, tomato persimmon and guava by Qadir & Hashinaga (2001) and banana by Palomer *et al.* (2005).

To this date though, only few researches have been carried out on the use of nitrous oxide in MAP (Rocculi *et al.*, 2004; Rocculi *et al.*, 2005; Rocculi *et al.*, 2006; Cortellino *et al.*, 2015). Results obtained seems to generally indicate that MAP with N_2O alone or in combination with other pre-treatments improves quality maintenance of fresh-cut fruit, delaying softening, reducing respiration rate and ethylene production, inhibiting PPO with positive effect on colour maintenance and delaying microbial spoilage.

3 Physiology and quality deterioration of minimally processes fruit

Minimally processed products deteriorate faster than the correspondent intact product. Being metabolically active tissues, they show physical and physiological reactions to mechanical stress suffered from peeling, slicing, dicing, shredding or chopping known as wounding response. The tissue disruption caused by minimal processing leads to the loss of cellular compartmentalisation and promotes the contact between enzymes and substrates and an overall increase of metabolic activity (Alzamora *et al.*, 2000; Hodges & Toivonen, 2008). Moreover, further processing, packaging and storage conditions may have ulterior consequences on the tissue physiology, product quality and stability.

As shown by **Figure 3**, investigating metabolic, functional and nutritional properties implications of each processing step and of their interactions, it is possible to obtain useful information that can lead to an optimization of the process in order to obtain final products characterized by high qualitative and nutritional parameters and a prolonged shelf-life.

The main factors affected by wounding response are reviewed in the next paragraphs.

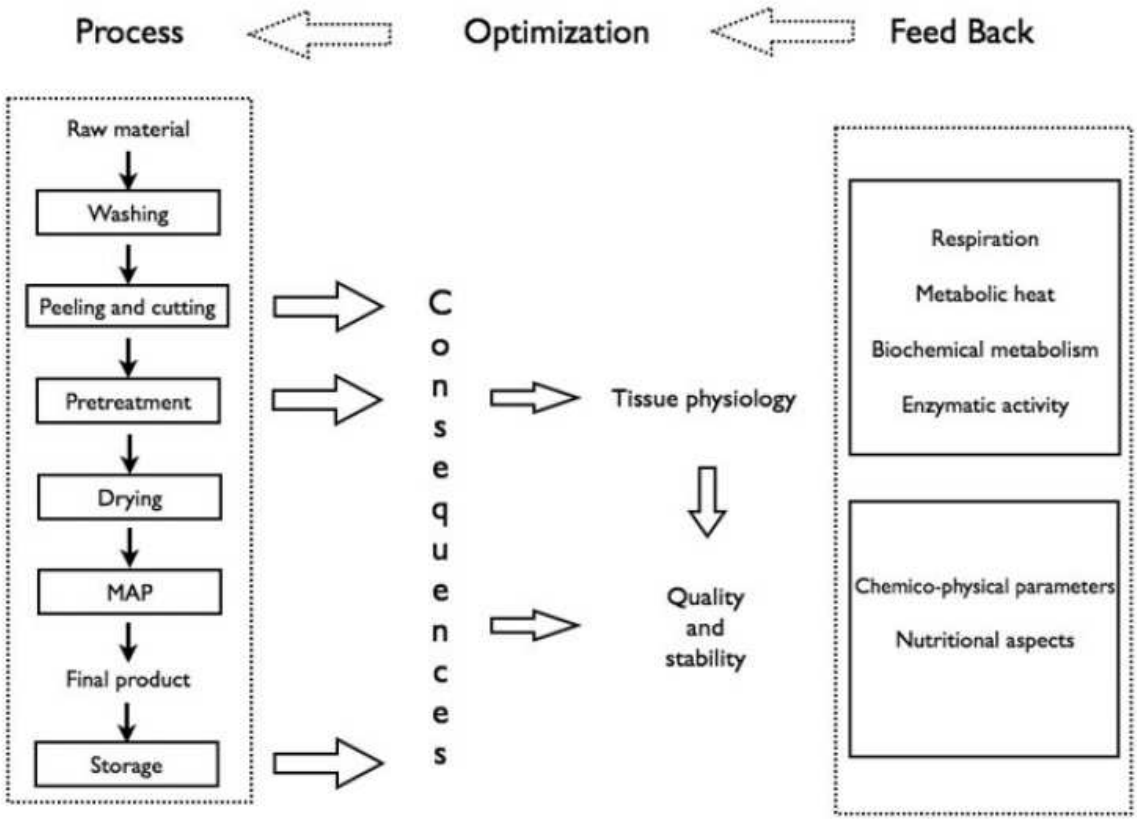

Figure 3. Flow-chart of minimal processing of fruit production and principal consequences on tissue physiology and quality.

3.1 Respiration

The energy required by living organism to carry out the necessary metabolic reactions to maintain cellular organisation, to transport metabolites around the tissue and to maintain membrane permeability is generally supplied by aerobic respiration which involves the oxidative breakdown of organic reserves, generally glucose, to simpler molecules, including CO_2 and water, with release of energy. The process consumes O_2 in a series of enzymatic reactions. Glycolysis, the tricarboxilic acid cycle, and the electron transport system are the metabolic pathways of aerobic respiration. The complete oxidation of glucose through the aerobic pathway produces an equal amount of CO_2 as the O_2 consumed, so that the respiratory quotient (RQ = CO_2 produced (mL) / O_2 consumed (mL)) is 1.

Variations in the RQ may depend on a different substrate used for respiration, such as malate or long chain fatty acids, although generally, an increase in RQ indicates a switch to fermentation reactions (Wills *et al.*, 1999). However, according to Makino (2013), RQ in the range of 0.7 to 1.3 could be considered indicator of aerobic respiration.

In fermentative metabolism, ethanol production involves decarboxylation of pyruvate to CO_2 without O_2 uptake (Fonseca *et al.*, 2002). Anaerobic metabolism can be prompted by either low oxygen or high carbon dioxide concentration in the environment, respectively lower than 2-5 % and higher than 4-5 % (Iversen *et al.*, 1989; Cortellino *et al.*, 2015). However, the O_2 concentration at which anaerobic respiration is triggered, known as the extinction or anaerobic compensation point, varies between tissues and depends on several factors, such as species, cultivar, maturity and temperature (Yearsley *et al.*, 1996).

An increase in the respiration rate of fruits and vegetables is often found as a result of wounding although it seems to depend on the commodity under consideration, since it occurs in kiwifruit but not in banana (Watada *et al.*, 1990). Moreover, the increase in respiration due to wounding appears delayed compared to that found for wound-induced ethylene (Brecht, 1995).

Asahi (1978) observed after wounding variations in mitochondrial structure, number and function and as a consequence, a general enhanced aerobic mitochondrial respiration rate. But the enhancement in aerobic respiration alone seems not to fully explain the increase in the respiration rate due to wounding. Other metabolic pathways, such as α-oxidation of long-chain fatty acids that increased O_2 consumption, were observed by different authors in fresh-cut potatoes (Laties, 1964; Laties *et al.*, 1972).

Because high rates of respiration have been negatively correlated with shelf life potential (Makino, 2013; Kader, 1987), the increases brought about by cutting are expected to result in a shorter shelf life (Rolle & Chism, 1987).

The increase of respiration rate is often proportional to storage temperatures (Watada *et al.*, 1996).

Increases in respiration in response to cutting may be quite substantial in some cases. Slicing of mature green tomatoes results in increased respiration by up to 40% when stored at 8°C, as compared to intact product (Mencarelli *et al.*, 1988).

The increase in respiration rate may also lead to the development of anaerobic conditions inside a package if permeability of the films is not carefully selected, particularly when product is placed in MAP, hence already exposed to high CO_2 and/or low O_2.

3.2 Ethylene production

Ethylene is a vegetable hormone that controls many aspects of growth and development of plants; the rate of its endogenous production can drastically increase following various stress conditions such as mechanical wounding (bruising and cutting), temperature variations, chemicals and pathogenic infection to plant tissues (Hong & Gross, 2000).

On the strength of the role of ethylene in the ripening process, fruit can be divided into two groups (Lelièvre *et al.*, 1997):

1) fruit that could produce large amount of ethylene, which promotes their ripening, defined "climacteric", such as tomato, peach, apple, banana and kiwifruit;

2) fruit that produce only low basal amount of ethylene during ripening and are insensitive to exogenous ethylene, defined "non-climacteric", such as grape, strawberry, watermelon, pineapple and citrus.

Peeling and cutting operations stimulate ethylene production in fresh-cut fruit. This phenomenon can start as soon as few minutes to an hour after wounding, and reach its maximum rate between 6 and 12 hours (Abeles *et al.*, 1992).

The effect can be more evident in climacteric fruit, for which wound-induced ethylene promotes ripening and softening. In climacteric fruit, wound-induced as well as exogenous ethylene may promote the same effect on tissue, leading to an increase in the rate of ripening and softening (Toivonen & Brummell, 2008). Moreover, the entity of the response strongly depends on the type of product considered, the ripening degree and storage temperature (Rocculi *et al.*, 2004).

Generally, the effects of ethylene are negative for fruit quality; therefore, its concentration or activity should be minimized in order to increase product shelf life. Moreover, ethylene can accumulate inside packages and lead to detrimental effects. Abe & Watada (1991) observed that an ethylene concentration of 2 $\mu l\ l^{-1}$ accelerated softening in banana and kiwifruit stored at 20°C and that the loss of firmness was delayed when ethylene absorbents were placed inside the packages.

MAP has been reported to reduce ethylene production and respiration rates for the synergic effect of low oxygen and high carbon dioxide levels (Soliva-Fortuny & Martín-Belloso, 2003). Conversely,

elevated levels of O_2 showed to enhance the ethylene production in fruit and vegetables during storage (Kader & Ben-Yehoshua, 2000).

3.3 Quality deterioration

Quality of MPF can be defined as a combination of attributes, properties or characteristic that determine their value to the consumer. Main qualitative parameters include appearance, texture, flavour and nutritional value.

3.3.1 Colour and visual quality degradation

The appearance of a minimally processed product is the characteristic most immediately perceived and appreciated by the consumer, and it is fundamental in the decision to buy. It can be affected by various unrelated factors, from wound-related effects to drying to microbial colonization.

Colour is one of the main aspects related to visual acceptance. In MPF colour is manly related to the content of some pigments, such as carotenoids chlorophylls, antocians and phenolics, that during ripening, processing and storage can be subjected to degradation (Alzamora *et al.*, 2000).

Enzymatic browning is one of the main factor limiting MPF shelf-life. Browning reactions have generally been considered as the consequence of the reaction of a group of enzymes called polyphenoloxidase (PPO) with polyphenols, made possible by the breakdown of membranes that normally keeps them separated. The loss of the cell compartmentalization can be brought about by various deteriorative processes, such as senescence and wounding response. Cut-edge browning is due to two PPO catalyzed reactions, the hydroxylation of monophenols to diphenols and the oxidation of diphenols to quinones, which in turn involve melanin accumulation, although a partial role not yet fully clarified has been attributed also to the reaction of peroxidase (POD) on polyphenols (Toivonen & Brummell, 2008).

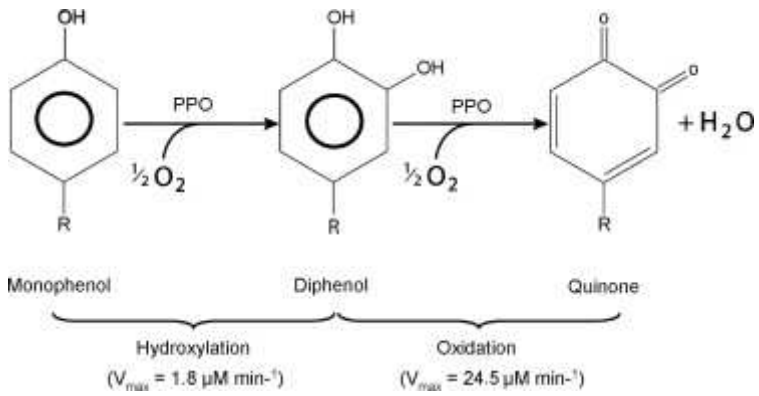

Figure 4. Mechanism for polyphenol-oxidase action on mono and di-phenols. (Adapted from Toivonen & Brummel, 2008)

Figure 4 shows the mechanism for browning that involves the interaction of polyhenolic substrates with PPO in the presence of oxygen. V_{max} values indicate that the hydroxylation process occurs more slowly compared to the oxidation process.

Furthermore, variations from green to yellow or olive/brown colour can be a consequence of chlorophyll degradation. Two main mechanisms have been proposed for its breakdown. The first (Type I) involve the action of enzymes such as Chlorophyllase and Manesium dechelatase that convert chlorophyll into the brown pheophorbide, while the second (Type II) is mediated by oxygen radicals and it is far less controlled (Brown, 1991).

Short exposure to high concentration of CO_2 was observed to have a positive effect on retaining chlorophyll and delaying yellowing in green fruit and vegetables, attributed to the inhibition of ethylene production (Simpson, 1985).

Carotenoids are pigments responsible for colour of many fruits, they are fairly stable but can be oxidised by enzymes such as lypoxygenase in the presence of oxygen. Their oxidation can be accelerated by metal ions, chemical oxidants and by low moisture levels reached during product processing (Dorantes-Alvarez & Chiralt, 2000).

Anthocyanins are a group of water-soluble compounds belonging to the flavonoid family and responsible for bright colours as orange, red and blue. Their stability is highly dependent on pH and oxygen concentration. Also, PPO was shown to play a role in their degradation since they can be involved in oxidation reaction with quinones deriving by the enzyme activity (Dorantes-Alvarez & Chiralt, 2000)

To control and reduce colour modifications in MPF, most successful methods involve combination of factors or the application of hurdles technology.

But appearance of MPF does not depend exclusively on colour, but also on morphological aspects such as size, shape, gloss and absence of defects and decay. Choice of cultivar and correct pre and post-harvest practices and handling are necessary to preserve visual quality of MPF.

3.3.2 Texture loss

Main textural attributes of fruits such as firmness, crispness, juiciness and toughness are important quality factors both for consumers and for shipping ability. Tissue softening and juice leakage can be the main causes of poor quality and un-marketability and are frequently the major problem limiting the shelf-life of MPF.

During ripening, cell wall polysaccharides undergo modifications induced by enzymes such as pectinmethylesterase (PME) and polygalacturonase (PG) released from the symplast into the cell

wall space. Specifically, softening is due to the hydrolysis of protopectins to water soluble pectins, the decrease in cellulose crystallinity, thinning of cell walls, diffusion of sugar to the intercellular spaces and ion movement from the cell wall (Toivonen & Brummel, 2008).

Figure 5 depicts the modifications of major cell wall components that take place during softening of a melting flesh peach variety. It has to be taken into account that the chronological order and extent of most of these events varies between fruit types, with some processes being reduced or absent in other species.

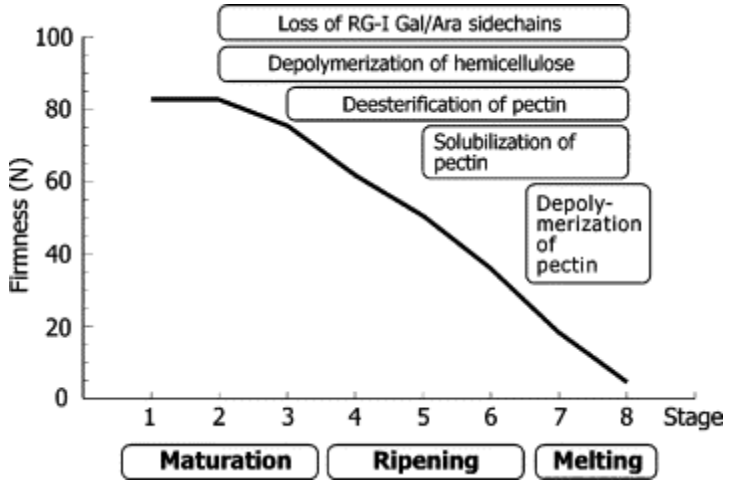

Figure 5. Schematic representation of softening and changes to cell wall components occurring during maturation and ripening of melting-flesh peach (adapted from Toivonen & Brummel, 2008).

Wounding causes the acceleration of the deteriorative phenomena that take place during ripening, so that the rate of softening of cut-fruit is often notably faster if compared to the intact fruit (Toivonen & Brummell, 2008), although there is not always a clear relationship (Aguayo *et al.*, 2004b).

Water loss is another important factor for textural variation in MPF, being strictly related to cell turgor (Toivonen & DeEll, 2002). In whole fruits, water in the intercellular spaces is not in direct contact with the atmosphere. Peeling and cutting operations result in the removal of outer periderm and cuticle and the exposure of internal tissues that together with the increase in surface area to volume ratio, accelerates the rate of water loss leading to turgor and crispness loss. However, proper packaging conditions may reduce water loss by maintaining high level of relative humidity within the package.

Furthermore, membrane deterioration caused by the activity of lypoxygenase and phospholipase and the loss of integrity in damaged cells, can promote leaking of water from the vacuoles diffusing through the tissue, resulting in water movement and turgor loss.

3.3.3 Microbial spoilage

Microbial decay is a very important source of spoilage of fresh-cut product (Artés *et al.*, 2007), since washing and chlorinated water dips only partially remove the microorganisms intrinsic to produce. The presence of damaged cells and the loss of cellular components during processing operations provide optimum conditions for the development of microorganisms.

The microbial type and loads depends on the type of fruit, the cultivation practices and the hygienic conditions during handling and processing, being storage temperature the essential factor. Product pH strongly influences the survival and growth of pathogens. While most vegetables have a pH ≥ 5.0, and consequently support the growth of most foodborne bacteria, the majority of fruits have acidic pH; hence yeasts and moulds represent their main spoilage microorganisms.

Nevertheless, a number of soft fruits/melons have pH values ≥ 5.0 and will support growth of many pathogens. They are therefore considered highly perishable and potentially hazardous foods. The growth of spoilage microflora and various pathogens, including *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, have been observed in melons (Lamikanra *et al.*, 2000; Harris *et al.*, 2003) and have caused various foodborne disease outbreaks over the years (CDC, 2012).

Due to the lack of heat treatments, an efficient temperature control during processing, distribution and retailing is required for maintaining the microbiological quality and the safety of these products. However, potential pre-treatment and temperature control are not able to either eliminate or significantly delay the microbial spoilage of these products entirely, and to ensure the product safety (Soliva-Fortuny & Martín-Belloso, 2003).

Chlorinated water was traditionally used for disinfection, but due to the toxic by-products that can generate in the tissues, the general concern related to health issues has pushed towards the investigation about alternative sanitizers (Silveira *et al.*, 2008).

3.3.4 Flavour changes

Consumer acceptance is often driven by flavour quality of MPF that is mainly related to the perception of sweetness, sourness, astringency, bitterness, aroma and off-flavours.

Nevertheless, establishing shelf-life limits related to flavour quality is difficult because it is affected by various aspects linked to product variability, post-cutting treatments and packaging. As a consequence, uniform flavour quality and consumer acceptance based on flavour remains a challenge for the industry.

Ripening degree at harvest and at processing significantly affects the flavour profile of fresh-cut melons (Beaulieu & Lea, 2007), pears (Dong *et al.*, 2000), nectarines and peaches (Gorny *et al.*,

1999) and mangoes (Beaulieu & Lea, 2003), showing that, generally, increasing maturity lead to improved sensory attributes.

Maturity degree was linearly correlated to total volatile compounds, in particular total esters, non-acetate esters, aromatic (benzyl) compounds, and sulfur compounds, and decreasing levels of acetates and aldehydes in melons (Beaulieu, 2006).

Nevertheless, since an early stage of ripening is desired for better shipping, handling and storage hence for an acceptable visual shelf-life, fruit are generally processed when firmer and immature with a detrimental trade-off between textural and visual quality and acceptable flavour/aroma attributes.

Moreover, unacceptability related to flavour can be due also to the formation of undesired compounds or off-flavours associated with the development of anaerobic conditions (Hodges & Toivonen, 2008) or microbial spoilage (Artés *et al.*, 2007).

3.3.5 Nutritional loss

Fruits are good source of vitamins, minerals, dietary fibres and various compounds that have positive effects on health and on the prevention of various diseases (Martin *et al.*, 2001; Liu *et al.*, 2000).

During post-harvest storage, substantial nutritional losses can occur, particularly in the content of vitamin C, and can be enhanced by physical damages, storage and temperature abuses (Lamikanra, 2002). Furthermore, minimal processing operations can affect the content of those bioactive compounds that are susceptible to degradation when exposed to oxygen or light. Degradation can be promoted also by the activity of oxidatives enzymes such as ascorbate oxidase, polyphenoloxidase, cytochrome oxidase and peroxidase.

Gil *et al.* (2006) studied the antioxidants content of various fresh-cut fruits (kiwifruit, strawberry, melon, papaya, watermelon) during storage compared to the whole product. Vitamin C losses in fresh-cut products, after 6 days of storage at 5°C, ranged from 5% in mango to 25% in cantaloupe pieces, compared to whole fruits, while exposure to light promoted vitamin C degradation in kiwi-fruit slices. Nevertheless, contrary to expectations, minimal processing had little effect on the main antioxidant constituents, while visual quality was the main limit to product shelf-life.

Studies on fresh-cut apples detected a decrease of total phenolic content related to the extent of browning (Rocha & Morais, 2002). The degradation of phenolic compounds was successfully prevented by pre-treatment with ascorbic acid thanks to its reducing activity (Cocci *et al.*, 2006).

On the other hand, it is well known that wounding stress can alter the physiology of fresh produce and, among other consequences, promote the accumulation of phenolic compounds or other

secondary metabolites (Saltveit, 1996). The activation of phenylalanine ammonia lyase (PAL) leads to synthesis of phenolic compounds with the aim of protecting the plant from water loss and pathogen attacks. This may lead to an increase of the antioxidant activity, as it has been observed in carrots (Heredia & Cisneros-Zevallos, 2002) and lettuce (Kang & Saltveit, 2002).

Reyes *et al.* (2007) studied various types of fresh-produce and concluded that the amount and profile of wound-induced soluble phenolics are dependent on the type of tissue, initial levels of reduced ascorbic acid and soluble phenolic compounds.

4 Innovative pre-treatment for MPF production

The term ‘non-thermal processing’ is referred to processing technologies that are effective at ambient or sub-lethal temperatures. In the last decades, the interest of food scientists, industries and consumers has been attracted by the investigation of novel non-thermal processing methods aimed at extending shelf life or increasing product functionality with a minimal impact on the nutritional and sensory properties of foods. Moreover, they may help industries in obtaining added-values products, new market opportunities and added safety margins (Morris *et al.*, 2007).

Although some aspects about these novel technologies have been widely investigated, there are still some aspects that are scarcely known.

In the previous chapters, the implications of the wounding response to the mechanical stress caused by processing operations on the physiology and metabolism of tissues, that in turn affect product quality and shelf-life, have been discussed

Pre-treatments can be considered as further processing steps to the ones normally used for MPF production and the impact on the wounding response of the tissues cannot be ignored.

In this chapter, the influence of innovative non-thermal processing operations such as plasma, vacuum impregnation (VI), osmotic dehydration (OD) and pulsed electric fields (PEF) on some qualitative, metabolic and functional aspects of MPF will be dealt with.

4.1 Cold gas-plasma

Cold plasma is an ionised gas characterized by active particles such as electrons, ions, free radicals and atoms which are both in ground and excited states; the excited species emit a photon (including UV photons) when they get to the ground state (Moreau *et al.*, 2008). It is produced by applying energy to a gas or a gas mixture and it is considered the fourth state of matter.

Non thermal plasma can be generated by microwaves, radio frequency, direct or alternating current; by various set-ups such as dielectric barrier discharge (DBD), atmospheric pressure plasma jet (APPJ) and corona discharges (CD) (Laroussi, 2002; Ragni *et al.*, 2010; Ehlbeck *et al.*, 2011) and by different gas mixtures, including atmospheric gas (oxygen, nitrogen and carbon dioxide) as well as noble gases (e.g. helium and argon). The final composition greatly depends on the kind of gases in the mixture, the selected generator set-up, the operating conditions (flow, gas pressure, power of plasma excitation) and the exposure mode (direct or remote) (Misra *et al.*, 2011), and it includes reactive species such as oxygen and nitrogen species (ROS and RNS), atoms, free radicals and UV radiations.

Because temperature is very close to ambient, the treatment seems promising for heat sensitive food products, but although its effect on microbial inactivation has been quite extensively reviewed (Ehlbeck *et al.*, 2011; Misra *et al.*, 2011; Niemira, 2012), the effect on qualitative, nutritional and metabolic aspects of fresh-products exposed to plasma are still quite scarce.

4.1.1 Effect on microbial inactivation

Cold plasma treatment have been applied to food products mainly for microbial inactivation. For this aim, the most important role seems to be played by reactive species, such as OH and NO radicals, atomic oxygen (O), ozone (O_3) and NO_2, that can cause lipid peroxidation, proteins and DNA oxidation (Laroussi, 2002; Li *et al.*, 2011; Takai *et al.*, 2012).

Once generated, they can be adsorbed onto the microbial cell surface, causing oxidative damages to the microbial membranes, essentially characterized by lipid bi-layers and protein molecules, leading to loss of functionality and exposure of the genetic material.

Various authors have assessed the microbial decontamination of cold plasma on foodstuffs, such as the outer surface of various fruit and vegetables (Baier *et al.*, 2014; Niemira & Sites, 2008; Critzer *et al.*, 2007) and on apple juice (Surowsky *et al.*, 2014; Montenegro *et al.*, 2002).

Generally, results showed that the effect is highly related to several operative parameters chosen to drive the discharge (gas mixture and gas flow, energy level applied and treatment time), characteristics of the microorganism itself (type, load and physiological state), and type of matrix.

Paper V reports the effect of DBD plasma treatment on the microbial quality of minimally processed melon. Samples were exposed to 15+15 min and 30+30 min plasma treatments and different microbial indexes were evaluated during 4 days of controlled storage.

As reported in **Figure 6**, upon plasma exposure, an immediate reductions in cell viability of the indigenous bacteria proportional to the treatment time was observed although inactivation levels were dependent on the type of microorganism considered. During storage, it was observed that microbial shelf-life, calculated through the Gompertz equation as the time necessary to reach the value of 6.0 Log CFU g^{-1}, was improved in the 15+15 min treatment but not in the longer treatment. This result was unexpected, considering that, according to current literature on cold plasma treatments on several raw fruit and vegetables, the inactivation level is generally time-dependent although non-linear inactivation curves are reported (Lee *et al.*, 2015; Baier *et al.*, 2014). Nevertheless, most studies are limited to the immediate effect after the treatment and ignore the effect during further storage.

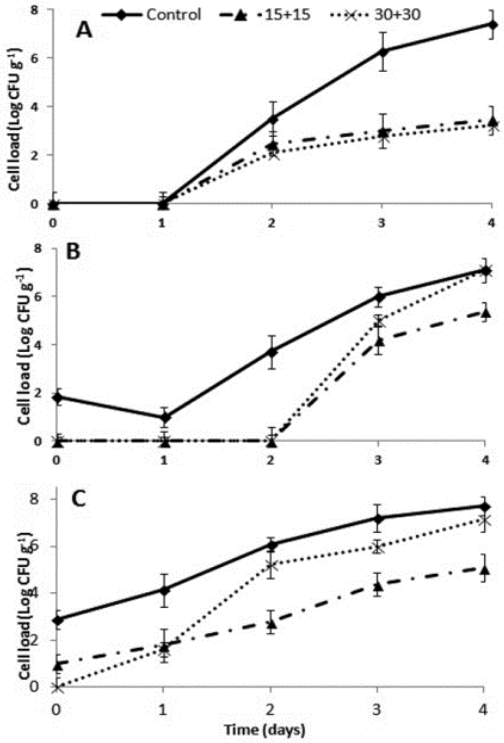

Figure 6. Cell numbers of yeasts (A), lactobacilli (B) and lactococci (C) during storage at 10°C of melon samples (Fig. 5 of **Paper V**)

Results obtained in this study could be attributed to tissue damages (e.g. cell wall weakening) caused by the longer treatment resulting in the higher water loss recorded for the 30 + 30 min treated melons, which probably made fruit more susceptible to microbial spoilage.

These results highlight the necessity of modulating treatment time not only according to its immediate effects, but also on the further shelf-life evaluation.

4.1.2 Effect on quality parameters

As opposed to microbial inactivation power, the scientific literature is quite poor in terms of effects on the product quality that cannot however be overlooked.

Alterations of the surface colour were found only slightly in whole *Granny Smith* apples subjected to a microwave driven plasma torch (Baier *et al.*, 2015), and during storage in fresh pears treated with a dielectric barrier discharge device (Berardinelli *et al.*, 2012).

A negative impact of plasma exposure was observed on surface morphology of lamb's lettuce leaves by scanning electron microscopy (SEM) (Grzegorzewski *et al.*, 2010). Oxidative species generated by the plasma discharge appeared to have promoted some erosion phenomena of the upper epidermis.

Studying the effect of a DBD plasma exposure in MPF, we obtained different results depending on the fruit considered. In particular, superficial browning was reduced in fresh cut apples with a 15+15 min exposure, due to an inhibition of enzymatic activity (**Paper III**), and visual quality of

kiwifruit slices was better preserved during storage, as shown by **Figure 7** (**Paper IV**). Conversely, in fresh-cut melon visual quality was negatively affected, and at the end of the storage slices appeared darker and more translucent (**Paper V**).

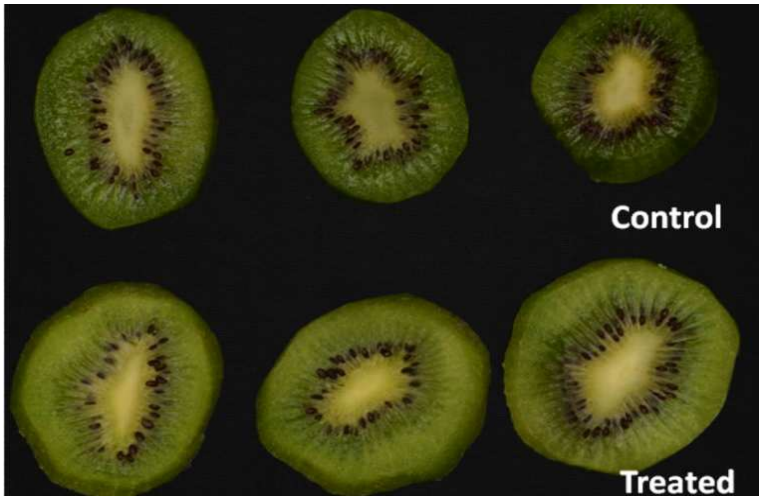

Figure 7. Example of digitalized images of kiwifruit slices subjected to 20 + 20 min DBD gas plasma treatment and control ones acquired after 4 days of storage in controlled conditions (Fig. 3 in **Paper IV**)

Textural parameters seem to be only slightly affected by plasma exposure. In **Paper III**, a slight decrease in the crunchiness of fresh-cut apples subjected to DBD plasma treatment was observed and was attributed to the destruction of the superficial layer of cells, while other authors (Schnabel *et al.*, 2014) did not detect significant differences in textural characteristics of apple flesh.

4.1.3 Effect on enzymatic activity

Recently, the potential effect of cold plasmas on enzymatic activity has been studied and first results showed its potential as innovative treatment for enzymes inactivation and quality preservation in food products.

Various studies on model systems indicted a change in the secondary protein structure and the modification of some amino acids side chains of the enzyme (Deng *et al.*, 2007; Takai *et al.*, 2012); in particular Surowsky *et al.* (2013) found a variation in the relative amounts of alfa- helix structures and β-sheet content upon plasma exposure, that was strongly correlated to the loss of enzymatic activity.

Studying a model system surely allows to obtain an homogeneous effect and a reproducibility in the results in that is often lacking in real food systems, hence to gain a better understanding of the inactivation mechanisms. On the other hand, to reach industrial application, it is necessary to verify the efficacy and the limitations of the treatments. To this date, very few experimental researches

have been conducted to evaluate the effect of cold plasma treatments on the enzymatic activity in real system and in particular in fruit tissues.

Using a DBD generator prototype, in **Paper III**, fresh-cut Pink Lady apples were exposed to cold plasma for different treating times, up to 15+15 min per side. A time-dependent reduction was observed after treatment, up to 45% after 30 min as shown in **Figure 8**.

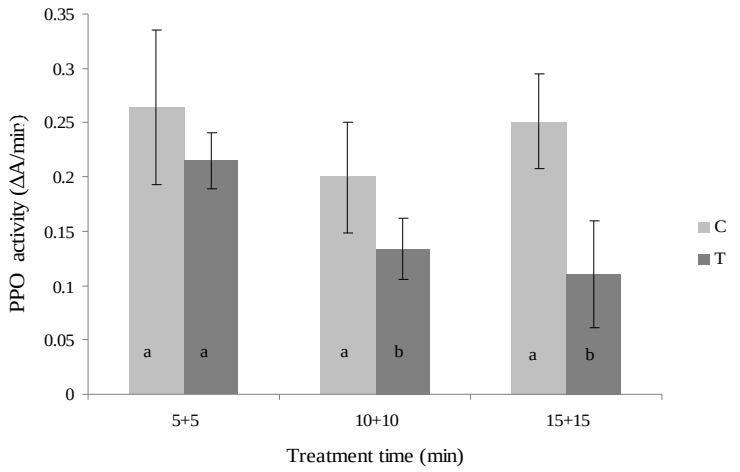

Figure 8. PPO activity (ΔA/min) of apple samples treated with plasma gas compared to the controls. Different letters indicate significant differences between control (C) and treated (T) sample at a p-level<0.05 (Fig. 4 in **Paper III**).

The observed reduction of PPO activity in the treated apple samples was attributed to a modification of the enzyme structure due to OH, NO and other reactive radical species present in the plasma discharge (Ragni *et al.*, 2010).

In **Paper V,** the effect of cold plasma on peroxidase (POD) and pectinmethylesterase (PME) activity was evaluated in fresh-cut melon treated for 15+15 and 30+30 min. As shown in **Figure 9**, POD activity was reduced proportionally to the treatment time, as the residual activity was found 91% (15+15) and 82% (30+30) compared to the control sample. PME activity was not affected by the shorter treatment, but the residual activity was found to be 94% after the longer one.

Comparing results obtained in **Paper III** and **V**, it seems that the effect of plasma is related to the enzyme typology. The different resistance to denaturation by plasma agents could be caused by the different structure and by the presence of isoenzymes. Furthermore, the kind of fruit tissue, the specific microstructure and porosity (e.g. 13.3 ± 0.6 % for melon and 27.3 ± 1.1 % for apple, as found by Muujica-Paz *et al.* (2003)) can be assumed to affect the different treatment response.

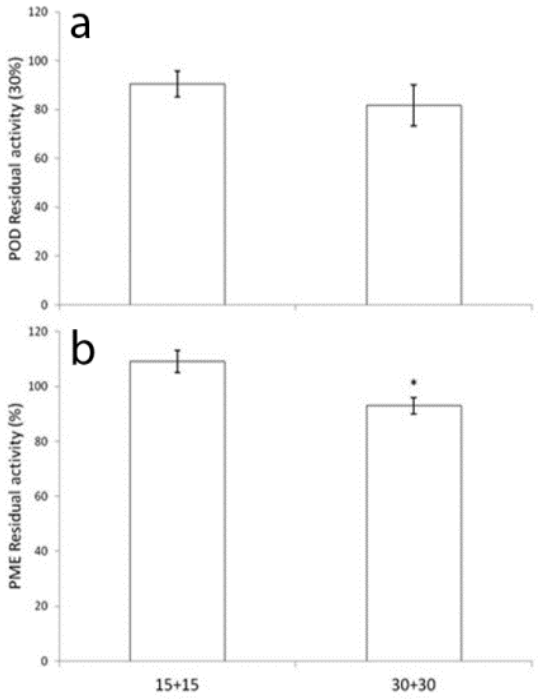

Figure 9. Peroxidase (POD) and (b) Pectinmethylesterase (PME) residual activity of melon samples treated with plasma for 15+15 and 30+30 min. * indicates samples that were significantly different from control sample at a p-level<0.05 (Fig. 2 in **Paper V**).

4.1.4 Effect on tissue metabolism

The impact of plasma exposure on tissue metabolism is, at date, still largely unknown.

Respiration rate during storage of strawberries and cherry tomatoes was found not significantly affected by in-package plasma treatment by Misra *et al.* (2014) and Misra *et al.* (2014).

Papers III and **V** evaluated the production of the tissue metabolic heat assessed by isothermal calorimetry upon plasma exposure on apple and melon samples respectively. For apples, plasma treated samples showed an irregular and higher heat production, but only in the first six hours after treatment. These results may indicate a physiological reaction to the stress induced by the reactive species. In the second part of the analysis, in treated samples a lower heat profile compared to the control sample was observed.

For melons, heat production after plasma treatment was lower compared to the controls for all the duration of the analysis. Differences were more pronounced as the analysis proceeded, as shown by the total metabolic heat produced obtained integrating metabolic heat profiles during the first 12 h and during 24 h at 10°C, as reported by **Figure 10**.

Simultaneously, in both apple and melon tissues, an alteration of the cellular respiration pathway was observed after cold plasma exposure. In particular, plasma exposure seemed to have caused a decrease of O_2 consumption in relation to the CO_2 production. To explain these results, a partial

conversion of the tissue respiratory metabolism from aerobic to anaerobic has been hypothesized as a consequence of a chemical stress of the fruit tissue promoted by the treatment (**Paper III** and **V**). Nevertheless, the mechanisms appeared complex and the relation with treatment time was not clear highlighting the need for further clarifications on the response mechanisms.

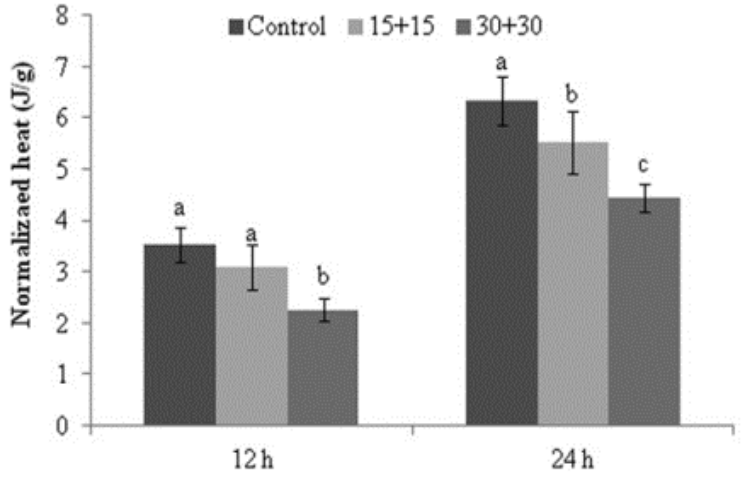

Figure 10. Normalized heat produced by melon samples during 12 and 24 h of analysis at 10°C. Different letters indicates samples that were significantly different at p-level<0.05 (Fig. 4 in **Paper V**).

4.1.5 Effect on nutritional value

Although few researches have been carried out to this date about the impact of plasma treatment on the nutritional value, it is important to take into account the fact that plasma reactive species could promote the oxidation of bioactive compounds such as vitamins C and E, or of polyphenols which are naturally occurring antioxidants in fruit and vegetable products. Moreover, considering that oxidation of components occurs mostly at the product surface, the penetration power of plasma reactive species into the food matrix is not known and it probably depends on the food porosity and microstructure.

Wang *et al.* (2012) observed that loss of vitamin C content in different vegetables exposed to plasma was only slight and probably mostly affected by UV.

Grzegorzewski *et al.* (2011) and Grzegorzewski *et al.* (2010) studied how plasma treatment on lamb's lettuce (*Valerianella locusta*) affected polyphenolic compounds content, comparing the effect on pure substances and within the product matrix. Degradation of compounds was proportional to treatment time and reduced by the effect of the matrix.

Berardinelli *et al.* (2012) and Gozzi *et al.* (2013), using the same plasma generator we used, studied the effect of plasma treatment on whole "Abate Fetel" pear and "Fuji" apples on the antioxidant

activity of both pulp and peel, finding a reduction as a consequence of the longer treatment, but the results were affected by the type of fruit considered.

Generally, the literature review showed that the shielding effect of the matrix greatly influences the effect of plasma reactive species on bioactive compounds, hence the nutritional evaluation should be carried out for each specific product.

Paper IV reports an approach to test the effect of double barrier discharge (DBD) cold plasma treatment on the potential effect on health properties of minimally processed kiwifruit. Content of antioxidant compounds such as chlorophyll, carotenoids, ascorbic acid and polyphenols was evaluated after plasma exposure but also during storage. Furthermore, the *in vitro* antioxidant activity was quantified by different *in vitro* assays: the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay, the 2,2-diphenyl-1-picrilhydrazyl (DPPH·) radical scavenging assay, and the ferric reducing antioxidant power (FRAP).

According to the obtained results, no significant changes in antioxidants (ascorbic acid and polyphenols) content and antioxidant activity were observed among treated samples and control ones.

In **Paper VI** a plasma treatment, that was already proved to be beneficial in terms of enzymatic browning reduction in fresh-cut apples, was tested in order to evaluate the effects on antioxidants content and antioxidant activity of the product. The phenolic composition of treated and control samples, analysed by HPLC-MS (high-performance liquid chromatography-mass spectrometry) showed a different effect on different classes of compounds according to treatment time. In particular, as a consequence of catechin polymerisation products and of the increase of hydroxycynnamic acids and chalcones, phenolic profile of fresh-cut apples was significantly affected by 10 min treatment, both in quantitative (about 20% of increase) and qualitative terms.

After 30 min of treatment, when plasma effect on enzymatic browning inhibition was significant, flavan-3-oils content was lower compared to not treated apples, while dihydrochalcones and flavonols evidenced a slight increase.

As expected, considering its strict connection between polyphenols amount, antioxidant activity evaluated by different *in-vitro* methods followed a similar trend, increasing after 10 min of processing. The DPPH• showed a lower sensibility compared with TEAC assays, while FRAP showed similar values to TPI assay, as reported in **Table 2**.

Moreover, for the longest treatment time investigated (30 min), that was found to have a significant positive effect on browning inhibition, some *ex-vivo* antioxidant test were carried out, in particular the ability of polyphenolic extracts from treated and untreated apples to protect Caco2 cells, which show biochemical characteristic of normal adult enterocytes, against oxidative stress.

Table 2. Total phenolic content (TPI) and antioxidant activity (μmol $kg^{-1}_{f.w.}$) of Pink Lady apples as affected by plasma treatment time (**Table 3** in **Paper VI**)

		time (min)				
	Extract	0	10	20	30	120
TPI[a]	amphiphilic	12633[b]	13702[a]	12460[b]	11383[c]	8207[d]
TPI[a]	hydrophilic	855[b]	917[ab]	981[ab]	1050[a]	623[c]
TPI[a]	hydrophilic + amphiphilic	13484[b]	14622[a]	13357[b]	12242[c]	8790[d]
ABTS[b]	amphiphilic	14265[b]	15076[a]	14734[ab]	13526[b]	10098[c]
ABTS[b]	hydrophilic	855[a]	644[b]	840[ab]	686[b]	803[ab]
ABTS[b]	hydrophilic + amphiphilic	15120[ab]	15720[a]	15574[a]	14212[b]	10900[c]
DPPH[b]	hydrophilic + amphiphilic	22543[a]	23008[a]	21311[a]	21868[a]	16608[b]
FRAP[c]	hydrophilic + amphiphilic	26900[b]	30123[a]	27781[b]	25913[b]	17024[c]

Values followed by different letters within the same row are significantly different at a $p<0.05$ level.
[a](μmol GAE $kg^{-1}_{f.w.}$), [b](μmol TE $kg^{-1}_{f.w.}$), [c](μmol Fe^{2+} $kg^{-1}_{f.w.}$)

To our knowledge, no previous researches have assessed the effect of chemical species generated during gas plasma treatment on human cell line. Results obtained (**Paper VI**) demonstrated that the polyphenolic extract from plasma treated apple does not induce significant changes in cell proliferation in comparison with untreated apple. Furthermore, Caco 2 cells exposed to moderate oxidative stress induced by the polyphenols extracts administration are able to protect themselves through the expression of phase II detoxifying enzymes.

According to the results obtained in this first study, the DBD plasma treatment seems to be a promising tool to preserve the qualitative properties and the phytochemical profile of fresh-cut Pink Lady apples. Further, apple exposure to gas plasma does not seem to generate chemical species harmful to human cells although other studies in cellular models are needed to confirm this preliminary data.

4.2 Vacuum Impregnation

Vacuum Impregnation (VI) is an innovative technology that allows the introduction of desirable ingredients into a porous food structure, exploiting a mass transfer known as hydrodynamic mechanism (Fito *et al.*, 2001). The process involves a two-step pressure change. In the first (vacuum step) the pressure in a solid-liquid system is reduced causing the expansion of the gas in the product pores and its partial outflow until mechanical equilibrium is achieved. In the second step (atmospheric step), the atmospheric pressure is restored, the residual gas in the pores compresses and the external liquid flows into the pores (Tylewicz *et al.*, 2012). Compared to the classical diffusion processes, such as candying, salting, soaking, osmotic dehydration, which are carried out by simple dipping or prolonged immersion of the product in the solution for several hours or days, VI has the advantage of a fast penetration – only few minutes – of the active substances directly into the internal structure of the product (Saurel, 2002).

VI has been recognized as a potential technology for the enrichment of food with nutritional substances, nutraceutical and/or functional compounds, antimicrobial and antioxidant substances, organic acids, structuring substances, etc. (Betoret *et al.*, 2003).

4.2.1 VI for technological functionality

VI treatment can be considered a gentle process with short treatment times at low temperatures that minimize heat damage to plant tissues thus allowing to preserve colour, aroma and heat sensitive components. It has been used as a pre-treatment prior to drying, freezing and frying (Bolin & Huxsoll, 1993). Moreover, according to Alzamora *et al.* (2000), VI can exert a positive effect inhibiting oxidative and enzymatic browning by oxygen removal from the pores.

With the aim of improving product quality and stability, VI has been used in combination with different compounds in order to inhibit browning phenomena (Perez-Cabrera *et al.*, 2011), reduce pH (Derossi *et al.*, 2013, 2010), improve freezing tollerance (Velickova *et al.*, 2013; Phoon *et al.*, 2008) and for aroma enrichment (Comandini *et al.*, 2010).

Few works have been carried out on the impregnation with calcium to improve texture of minimally processed products. Occhino *et al.* (2011) investigated the effect of calcium and other structuring compounds in zucchini slices, while Degraeve *et al.* (2003) and Guillemin *et al.* (2006) combined calcium and pectinesterase for strawberry and apples impregnation finding a remarkable synergistic effect in increasing firmness. Nevertheless, generally published studies are limited to the immediate effect of the treatment and do not take into consideration the evolution of the product quality during storage in real conditions.

In **Paper VII**, the effect of VI with calcium lactate (CaLac) was investigated on qualitative parameters of minimally processed melon during storage.

In the first part of the research, an optimisation of the process parameters, such as vacuum pressure and CaLac concentration was carried out using a 2^3 factorial design and modelling data with second order polynomials that allowed to evaluate the effects of linear, quadratic, and interactive terms of the independent variables. Moreover, in order to better understand the interactive effects of the independent variables, surface plots based on these models were drawn.

An example of the graphical representation of the effect of the selected variables on product firmness is reported in **Figure 11**.

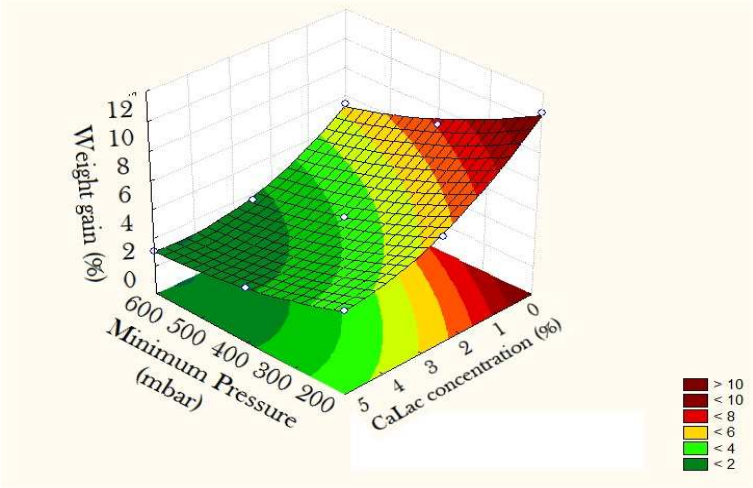

Figure 11. Three-dimensional contour plots showing the effects of the interactions [P] × [C] on weight gain (WG, %) (Fig. 1 in **Paper VII**).

Both pressure and CaLac concentration were found to influence significantly the impregnation level and the qualitative parameters assessed. On the basis of the overall results obtained in this first part of the study, sample impregnated at 600 mbar and 5% CaLac concentration, although it showed the lower impregnation level, was chosen for the further storage study.

In the second part of the study the effect of the selected vacuum treatment (VI), compared to a dipping at atmospheric pressure in the same CaLac solution (D) for the same duration and to the control ones (C) on some quality aspects of fresh-cut melon samples during storage were analysed.

Despite of the high variability of the raw material, results showed that VI allowed a better maintenance of texture during storage (**Figure 12A**). Nevertheless, other quality traits were negatively affected by the application of vacuum. Impregnated products were characterised by a darker and more translucent appearance on the account of the alteration of the structural properties (**Figure 12B**). Moreover microbial shelf-life, calculated as the time necessary to reach a threshold

microbial load of 6 Log CFU g^{-1}, was reduced to four days compared to the seven obtained for control and dipped samples.

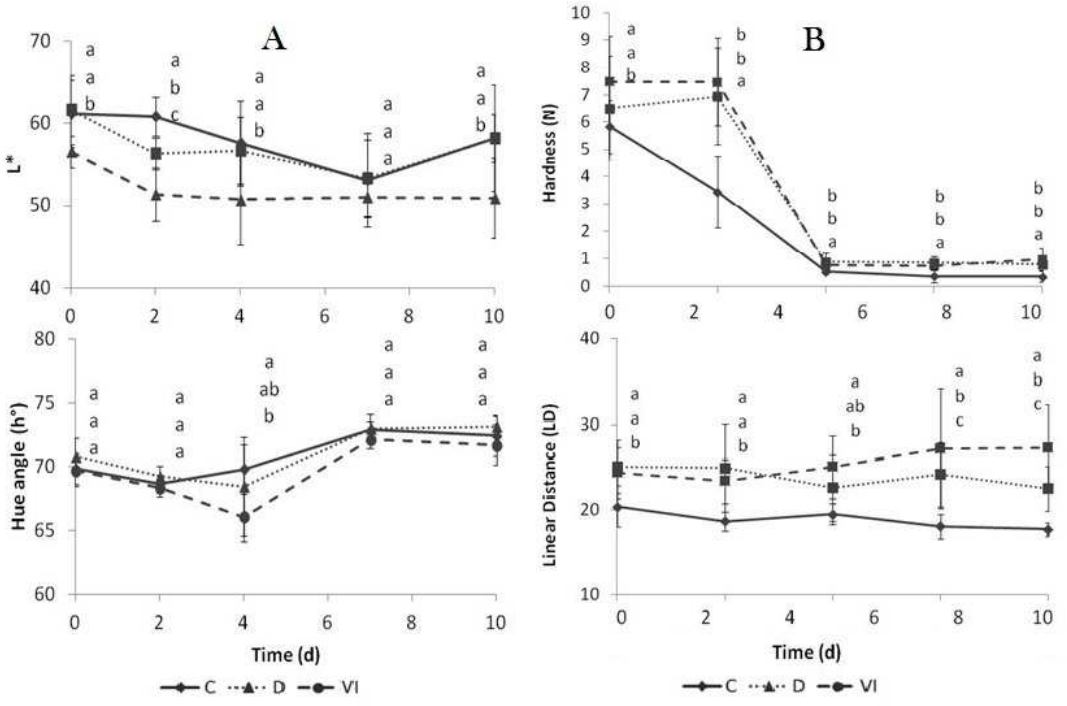

Figure 12. Textural parameters (hardness and linear distance) and colour coordinates (L* and h°) of melon samples during storage. Different letters indicate statistically significant values among samples at the same storage time ($p<0.5$). (Adapted from Fig. 3 and 4 in **Paper VII**)

4.2.2 VI for nutritional functionality

Main studied carried out on the nutritional enrichment of fruit and vegetable products by VI with different bioactive compounds have been reviewed by Alzamora *et al.* (2005) and include mainly fortifications with probiotic microorganisms or minerals such as calcium, iron and zinc in order to increase the daily intake and reach nutritional recommendations. More recently, fruit juices with a high content in bioactive compounds (Castagnini *et al.*, 2015; Diamante *et al.*, 2014; Betoret *et al.*, 2012) or other health-promoting substances such as Aloe vera (Sanzana *et al.*, 2011) or honey (Rößle *et al.*, 2011) have been used for product enrichment.

The enrichment of fruit and vegetable matrices with functional compounds can be interesting from different perspectives, it opens the opportunity of developing novel products for the industry combining the health properties of both components, but can also increase the bioavailability of the active components. Indeed, various authors agree on the increased health benefits of bioactive compounds within a food matrix compared to the consumption of the single compounds (Betoret *et al.*, 2012).

Moreover, VI process itself seems to exert a positive effect on the stability of some compounds such as anthocyanins in strawberries, helping to preserve antioxidant activity during processing (Watanabe *et al.*, 2011).

Surely, in order to develop a functional product it is necessary not only to successfully impregnate the matrix with the bioactive compounds but also to evaluate their stability during storage and their bioavailability.

In **Paper VIII**, VI of apple cylinders has been carried out with an isotonic sucrose solution containing a green tea extract (GTE) with the addition of ascorbic acid and the stability of some qualitative and nutritional characteristics of the obtained product has been evaluated during storage.

Green tea is a high source of catechins, known to have strong antioxidant properties, widely studied both in *in-vitro* and *in-vivo* studies, and its consumption is related to the decrease of various diseases. The amount of GTE was chosen in order to obtain after impregnation a concentration of catechins in the final product equal to the quantity found in a cup of tea (50-110 mg), according to Lavelli *et al.* (2011), considering a 50 g apple portion.

The addition of GTE to the impregnating solution promoted various effects. Firstly, a reduction of the respiration rate, evaluated measuring the gas composition of packages, was observed. Lack of previous reports on the impact of catechins on fruit tissue metabolism makes these results difficult to interpret, and highlights the need for further investigation.

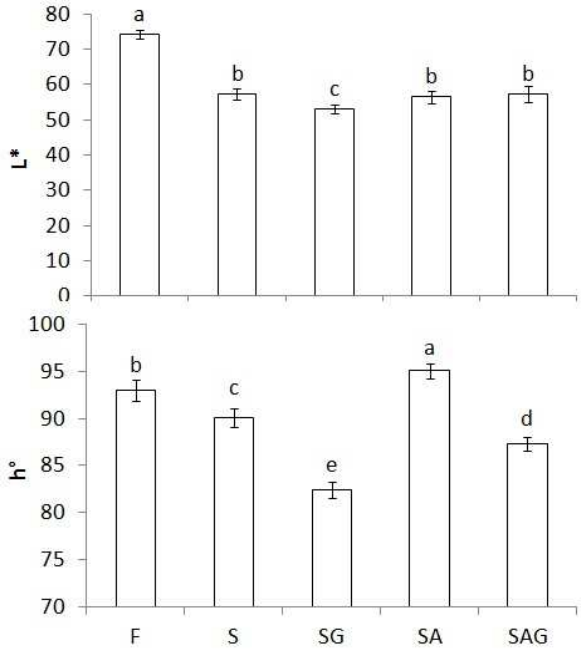

Figure 13. Colorimteric parameters, L* and h°, of of fresh apple (F) and vacuum impregnated samples with sucrose (S), sucrose and GTE (SG), sucrose and AA (SA) and sucrose, AA and GTE (SAG)as affected by VI treatment. Different letters show significant differences among samples (P-level < 0.05). (**Fig.1** in **Paper VIII**)

Main variations though were observed on product colour and antioxidant properties during storage. In particular, the addition of catechins leads to an immediate increase of the yellow/orange colour components of impregnated samples as shown by **Figure 13**, and to a higher degree of browning development. Due to oxidation phenomena, a marked surface browning was observed during storage in samples impregnated with GTE, as shown by **Figure 14**, along with a decrease of total phenolic content (TPC).

Figure 14. Visual examples of fresh, untreated sample (F) and samples impregnated with sucrose and GTE (SG) and sucrose, ascorbic acid and GTE (SAG) solutions at the end of the storage. (**Fig. 3** in **Paper VIII**).

On the other side, antioxidant activity, measured with the *in-vitro* DPPH method, although it showed a good correlation with the TPC, did not always reflected the decrease in antioxidant compounds. To better understand this discrepancy, a further investigation on the quality of the phenolic compounds during storage should be carried out.

The addition of 1% of ascorbic acid allowed to better preserve colour and antioxidant properties during storage, limiting oxidative phenomena. It is known that ascorbic acid is able to inhibit browning reactions, mainly because of its ability to scavenge oxygen and to be oxidised reducing quinones to phenols, before they can participate in further reactions that lead to coloured pigments.

Results obtained in this preliminary study showed that enrichment of apples with GT catechins and ascorbic acid seems to be promising in order to obtain a nutritionally fortified fruit product, nevertheless represented only a first step in this direction. Future researches on the qualitative characterization of the phenolic compounds during storage and their relationship with the antioxidant activity, together with further *in-vitro* and *in-vivo* experiments for the evaluation of the antioxidant properties need to be carried out. Moreover, aspects related to the tissue metabolism and respiration of the fresh apple tissue need further investigations.

In terms of sensorial acceptability, the impact of apple VI with green tea has to be carefully examined, considering its astringency and bitter taste, taking into consideration the possibility to change in quantitative/qualitative terms the formulation of the impregnating solution.

4.3 Osmotic Dehydration

Osmotic dehydration (OD) consists in the partial dewatering of a cellular tissue process by immersion in a hypertonic solution. The concentration gradient between the solution and the intracellular fluid is the driving force of the process that involves dehydration or water removal from tissues and the simultaneous impregnation of the solute (or solutes) present in the solution in contact with the food (Kowalska & Lenart, 2001).

OD has a wide range of applications in the development of minimally processed plant foods or as pre-treatment for other preservation methods such as freezing or drying (Loredo *et al.*, 2013).

The type of solute used in the osmotic solution greatly affects the dehydration kinetics, but also the organoleptic and nutritional properties of the final product and the process cost.

Calcium in OD solution has been used with the aim to increase firmness of plant tissue and enhance the process efficiency, restricting the sugar gain and increasing the water loss (Pereira *et al.*, 2006; Ferrari *et al.*, 2010; Mavroudis *et al.*, 2012), but also as a method for obtaining nutritionally fortified products (Silva *et al.*, 2014b).

The addition of ascorbic acid to the osmotic solution has been used for reducing enzymatic browning by Lenart (1996) and for compensate the loss of ascorbic acid in the fruits during the dehydration process by Ramallo & Mascheroni (2010).

4.3.1 Mass transfer kinetics

OD involves two main mass transfer phenomena: water flowing out of the tissue and the simultaneous counter flow of solutes into the tissue, but there can also be some leaching of native soluble solutes such as organic acids, sugars, minerals and flavours. Kinetics of mass transfer depends on many variables, some in relation to process parameters, such as temperature, treatment time, concentration of the osmotic solution and type of solutes, some in relation to structural parameters of the biological tissue, such as cell maturity and porosity.

The kinetics of mass transfer is usually described through the terms: water loss, solids or solutes gain, and weight reduction. These parameters can be modelled to obtain constant able to describe the dehydration kinetics. The equation proposed by Peleg (1988) and redefined by Palou *et al.* (1994) that has already been successfully applied to describe osmotic dehydration kinetics (Tylewicz *et al.*, 2011; Santagapita *et al.*, 2013), was used in **Paper X**.

Moreover, the efficiency of water removal in relation to sugar impregnation of tissues, can be expressed by the following equation (**Paper IX**):

$$Efficiency = \frac{WL}{\Delta Suc}$$

where WL indicate water loss and ΔSuc indicates the gain in sucrose.

Paper IX and **X** investigated the effect of the addition of calcium lactate (CaLac) and ascorbic acid (AA) to sucrose (Suc) osmotic solutions on mass transfer in apple tissue.

The presence of calcium in solution promoted a higher water loss and a lower sucrose gain, compared to the Suc solution enhancing process efficiency, as already observed in various fruit tissue subjected to osmotic dehydration in the presence of calcium salts (Pereira *et al.*, 2006; Mavroudis *et al.*, 2012; Silva *et al.*, 2014a; Silva *et al.*, 2014b). The effect of restriction of solute transport has been attributed to the reduction in cell wall porosity and to the formation of calcium pectate due to the interaction of the ion with pectic carboxylic groups.

With the combination of both solutes, water content was further reduced probably because the higher osmotic potential due to the lower water activity of the ternary solution, while sucrose gain was favoured.

To better understand the influence of the solutes on the mass transfer kinetics, the effective diffusion coefficients for water, sucrose, calcium and ascorbic acid were calculated based on Diffusion Equation (**Paper X**, Materials and Methods section), considering an infinite cylinder; results are reported in **Table 3**.

Table 3. Effective diffusion coefficients of water, sucrose, ascorbic acid and calcium (Table 3 in **Paper X**)

	$D_{w,m}$ $\times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)	$D_{s,m}$ $\times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)	$D_{Ca,m}$ $\times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)	$D_{AA,m} \times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)
Suc	1.4	0.976	5.8	1.2	0.961	6.8	-	-		-	-	
Suc-CaLac	1.6	0.972	6.3	1.4	0.971	5.7	3.4	0.985	12.8	-	-	
Suc-AA	1.8	0.980	4.9	1.9	0.979	8.1	-	-		2.5	0.984	6.6
Suc-CaLac-AA	1.4	0.973	6.9	0.9	0.951	7.5	2.0	0.984	6.5	1.7	0.982	5.4

When the selective permeability of the membranes is preserved, the transport of larger molecules such as sucrose through the cell tissue is reduced compared to the diffusion of smaller molecules such as water, as indicated by the higher D coefficient for water in Suc samples.

Moreover, for pure sucrose-water solutions, diffusion coefficients decrease as concentration increases. However, diffusivities in multi-component solutions present particular behaviour because of the interference between the fluxes and the presence of other solutes that may affect diffusivities in a different way (Silva *et al.*, 2014b).

In particular, water and sucrose diffusivities were higher and similar after the addition of 2% ascorbic acid, indicating losses of the tissue integrity, making available all cellular spaces for water and solutes diffusion.

Conversely, when CaLac and AA were both present in the solution the diffusion coefficients, for sucrose but also for Ca and AA, were lower as it is expected when the concentration gradient was higher.

4.3.2 Effect on water state and cellular compartments

Water in cells is compartmentalised into several major divisions, i.e. extracellular spaces, cytoplasm and vacuoles. NMR relaxometry is an analytical method that has been successfully applied for the determination of the water content and its mobility in the different cell compartments (Hills & Duce, 1990). By acquiring transverse relaxation time (T_2) weighted curves, it can give quantitative information in relation to water content and water properties in different proton pools within the tissue (Tylewicz *et al.*, 2011; Panarese *et al.*, 2012). LF-NMR allowed to study a number of different physiological conditions in several fruits and vegetables, including changes caused by ripening, bruising, microbial infection, drying, freezing and high pressure processing (Hills & Remigereau, 1997; Hills & Clark, 2003; Marigheto *et al.*, 2004).

Furthermore, this technique has been used in OD of plants to evaluate water mobility and distribution within the cellular tissue (Tylewicz *et al.*, 2011; Panarese *et al.*, 2012; Santagapita *et al.*, 2013).

Paper IX reports LF-NMR analysis to evaluate the changes on water mobility occurring at cellular level on apple tissues during OD with sucrose (Suc), calcium lactate (Ca) and ascorbic acid (AA).

By fitting the T_2-weighted curves to a continuous distribution of exponential curves, three proton pools were observed and ascribed to cell compartments, *i.e.* vacuole, cytoplasm plus extracellular space and cell wall (Cornillon, 2000). The relative intensity of the three populations observed during OD treatment is reported in **Figure 15**.

Upon OD, water was dislocated from vacuoles to cytoplasm+free space. This expected effect was influenced by the presence of other solutes. In particular, the Suc-CaLac-AA solution promoted a higher water released from vacuoles, probably because of a higher osmotic potential.

The presence of calcium in the osmotic solution seemed to have had an impact on the water population related to the cell wall, reducing its intensity compared to other treatments.

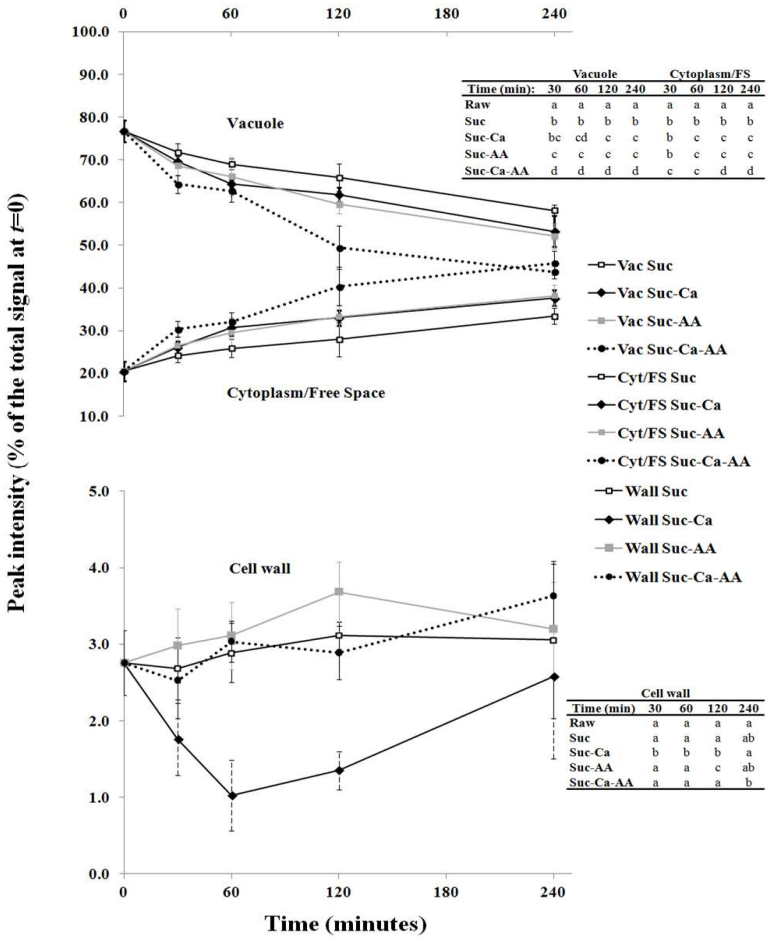

Figure 15. Peak intensity of the proton pools in different cellular compartments as a function of the osmotic dehydration time, in different osmotic solutions. All the intensities were scaled so that the total signal from fresh samples (t=0) equals 100. **(a).** Vacuole and cytoplasm plus free space; **(b).** Cell wall. The same letter on the same column in the auxiliary tables means no significant difference by the Duncan test ($p < 0.05$) (**Fig. 3** in **Paper IX**).

4.3.3 Effect on cell viability and tissue metabolism

Various authors have studied the effect of OD on cell viability and tissue metabolism. Panarese *et al.* (2012) observed a progressive reduction in the metabolic heat production during OD in kiwifruit slices and suggested that the decrease was due to a reduction of cell viability induced by osmotic stress. Mavroudis *et al.* (2004) found that only few layers of cells on the surface are expected to die upon an osmotic treatment of apple tissue in a 50% sucrose solution, while plasmolysis and shrinkage occur in the rest of the tissue. Salvatori & Alzamora (2000) found that a 25% w/w sucrose solution can cause vesciculation and rupture of cell membranes in apple tissue.

Moreover, a reduction in the respiration rate of osmotically dehydrated mangoes (Torres *et al.*, 2008), strawberries (Castelló *et al.*, 2010) and grapefruit (Moraga *et al.*, 2009) has been observed.

In paper **IX** and **X**, we attempted to evaluate the effect of OD with calcium and ascorbic acid on cell viability and tissue metabolism in apple cylinders.

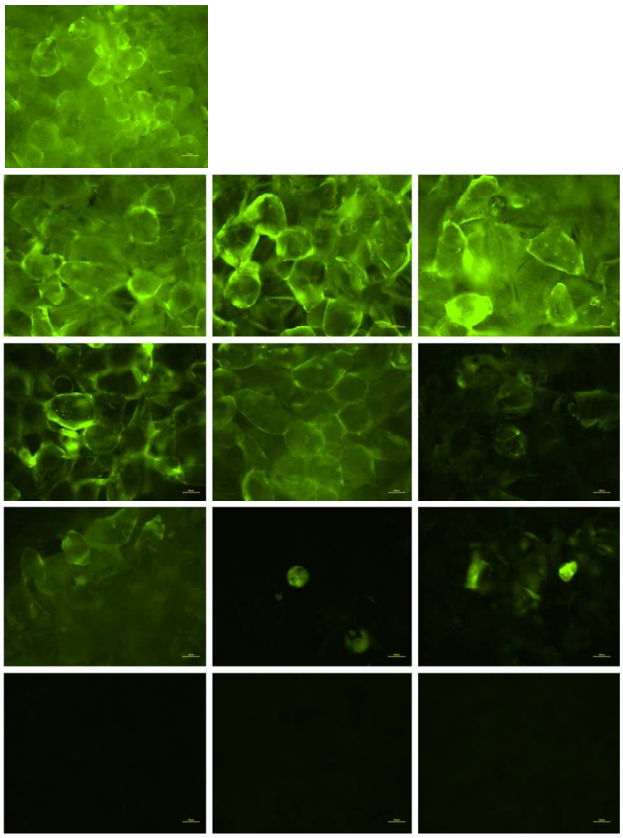

Figure 16. Slides of parenchyma apple tissue stained with FDA after immersion in osmotic solutions for 120 min: **(a).** Control; **(b-d).** Osmotically dehydrated in osmotic solutions; **(b.1).** 20%Suc; **(b.2).** 30%Suc; **(b.3).** 40%Suc; **(c.1).** 20%Suc+2%CaLac; **(c.2).** 30%Suc+3%CaLac; **(c.3):** 40%Suc+4%CaLac; **(d.1).** 20%Suc+1%AA; **(d.2).** 30%Suc+1.5%AA; **(d.3).** 40%Suc+2%AA; **(e.1).** 20%Suc+2%CaLac+1%AA; **(e.2).** 30%Suc+3%CaLac+1.5%AA; **(e.3).** 40%Suc+4%CaLac+2%AA (Fig. 2 in **Paper IX**)

Figures 16 and **17** show apple tissues stained with fluorescein diacetate (FDA) and neutral red (NR) after OD. As it can be observed, while the 40% w/w sucrose treatment generally preserved the viability of apple cells, the presence of other solutes had different effects. In particular, high concentration of calcium (4%) reduced fluorescence intensity and although vacuoles were still visible, the red colour was less spread out highlighting the possibility of some membrane damage.

On the other hand, the presence of AA in the solution, lead to a strong loss of cell viability and no stained vacuoles suggesting that high AA concentrations and/or very low pH affect the membrane integrity and permeability.

Unexpected results were found for treatments in Suc-CaLac-AA solutions that strongly affected the tissue functionality. Although it is possible to visualize vacuoles in **Figure 17e.1** to **17e.3**, the vitality was completely lost in cells that underwent this treatment (**Figure 16e.1** to **16e.3**). This effect may be due to a different pH of the solutions, higher after CaLac addition, that allowed a better preservation of the tonoplast semi permeability although plasmalemma was probably damaged due to the low pH and/or high AA concentration, because no vitality was detected.

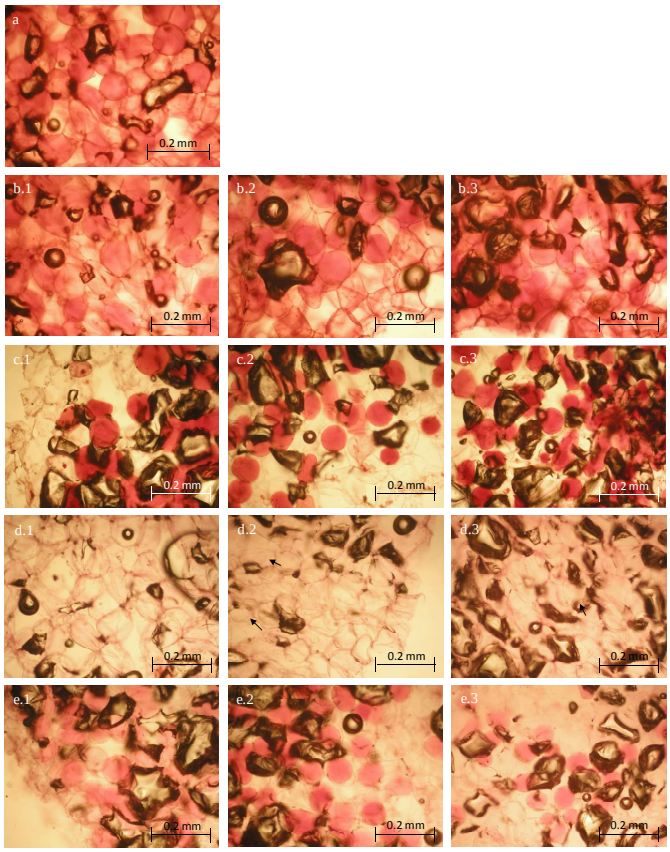

Figure 17. Slides of apple tissue stained with neutral-red before immersion in osmotic solutions for 120 min: **(a).** Control; **(b-d).** Osmotically dehydrated in osmotic solutions; **(b.1).** 20%Suc; **(b.2).** 30%Suc; **(b.3).** 40%Suc; **(c.1).** 20%Suc+2%CaLac; **(c.2).** 30%Suc+3%CaLac; **(c.3):** 40%Suc+4%CaLac; **(d.1).** 20%Suc+1%AA; **(d.2).** 30%Suc+1.5%AA; **(d.3).** 40%Suc+2%AA; **(e.1).** 20%Suc+2%CaLac+1%AA; **(e.2).** 30%Suc+3%CaLac+1.5%AA; **(e.3).** 40%Suc+4%CaLac+2%AA (Fig. 3 in **Paper IX**).

In order to better understand the effect of the treatments, in **Paper X**, metabolic heat production and respiration rate of samples dehydrated in the four different solution for 30, 60 and 120 min, were evaluated up to 24 h after the treatment.

Although fluorescence results indicated that viability was preserved (**Paper IX**), a slight reduction in metabolic heat production proportional to treatment time and a lower respiration rate compared to the fresh samples, both in terms of CO_2 produced and O_2 consumed (**Figure 18**) were observed after Suc treatment.

The presence of calcium in the osmotic solution caused a further decrease of metabolic heat production and respiration rate. In previous studies (Castelló *et al.*, 2010; Luna-Guzmán *et al.*, 1999), the ability of calcium to slow down tissue metabolic activity and thus to enhance its stability has already been observed.

On the other hand, the presence of AA in the osmotic solution promoted a drastic increase of metabolic heat production as treatment time increased, up until 50% compared to the fresh sample.

Paper IX already showed that the presence of AA can cause serious injury to cellular structure, probably due to low pH of the solutions (Limbo & Piergiovanni, 2007; Rocculi *et al.*, 2005).
Furthermore, a change in the respiratory pathway was observed in sample dehydrated with the Suc-AA solutions (**Paper X**), showing a sharp increase in oxygen consumption compared to other samples that in turn promoted a respiratory quotient inferior to one.

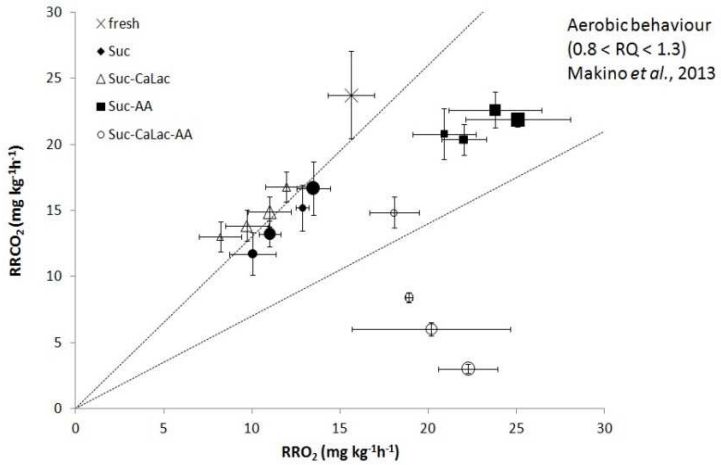

Figure 18: Respiration rates expressed as oxygen consumed (RRO_2) and carbon dioxide produced ($RRCO_2$), for treatment time of 30 min (smaller size symbol), 60 min, 120 min and 240 min (higher size symbol) (**Fig. 3** in **Paper** X).

When combined with Ca, heat production decreased sharply to a level lower than untreated samples, except for those treated for 240 min (higher solid gain), which showed the highest heat production values. These results confirm previous findings, suggesting that AA solution can promote a stress response on specific fresh-cut vegetable tissues, and an increase of their endogenous metabolic activity, confirmed by a higher O_2 consumption observed by head space gas determination.

4.4 Pulsed Electric Field (PEF) treatment

PEF technology is an innovative non-thermal process that involves the application of short duration pulses of high voltage electric fields to a sample between two electrodes. It has been studied primarily as a tool aimed at microbial inactivation (Timmermans *et al*., 2014; González-Arenzana *et al*., 2015), but recently several studied have focused on its application for mass transfer enhancement (Taiwo *et al.*, 2002; Donsì *et al*., 2010; Puértolas *et al*., 2012). The partial cell membranes electroporation induced by PEF treatment that is exploited for the acceleration of mass transfer processes, can cause permanent damage on cell membranes and even induce cell death.

The extent of the process, also known as electroporation, strongly depends on the applied process parameters such as electric field strength, number and shape of pulses, their width and frequency. Indeed, different goals and industrial applications can be achieved by adjusting the treatment conditions (Barba *et al.*, 2015).

Application for MPF, aimed at aiding mass exchange between the tissue and an outer solution, could be limited since PEF, by acting at the level of membranes, can also deeply affect the cell activities. As a consequence, metabolic stress responses of cells can be induced and lead to undesired effects on the quality of the final products.

In **Paper XI**, the evaluation of the metabolic response of apple tissue subjected to PEF treatment was attempted. Treatments were carried out using near-rectangular shaped pulses with fixed 100 µs pulse width and 10 ms repetition time at three different specific voltage (100, 250 and 400 V cm^{-1}) and fixed pulse number series (n=60).

Isothermal calorimetry was used to measure metabolic heat production during 24 h after the PEF treatment. Simultaneously O_2 consumption and CO_2 production were monitored through a static method and compared to a fresh untreated sample. Results showed that the lowest voltage (100 V cm^{-1}) applied did not induce a significant modification in the metabolic heat production but promoted a slight acceleration of the respiration rate, that was attributed to a response to the stress caused by the treatment. Conversely, samples subjected to medium and high voltages (250 and 400 V cm^{-1}) showed a heavy reduction of the metabolic heat and of the respiration rate suggesting irreversible electroporation of apple cells. Hence, it can be affirmed that the metabolic stress response induced by pulsed electric fields was strongly related to the electric field strength.

Moreover, in order to better understand the impact of PEF treatment on tissue metabolism, high resolution ^1H nuclear magnetic resonance (HR-NMR) was employed for the analysis of the metabolic profiling. Among the 38 metabolites identifies, statistical multivariate analysis allowed to select 8 compounds that discriminated the samples. The quantification of these 8 metabolites,

represented in **Figure 19,** allowed to put forward some hypothesis about the different PEF-induced metabolic pathways.

Variations of ethanol, acetaldehyde and iso-propanol confirmed that anaerobic respiration took place and that loss of cell viability occurred at the highest treatment field strengths.

Moreover, the generation of reactive oxygen species (ROS) induced immediately after the formation of pores (Teissie *et al.*, 1999) seems to have caused an oxidative stress to the tissue highlighted by the changes in tartaric acid and epicatechin, while an alteration of the Kreb's cycle was hypothesized on the basis of the variation of γ-Aminobutyric acid, as already observed by Galindo *et al.* (2009) on PEF treated potato tissue.

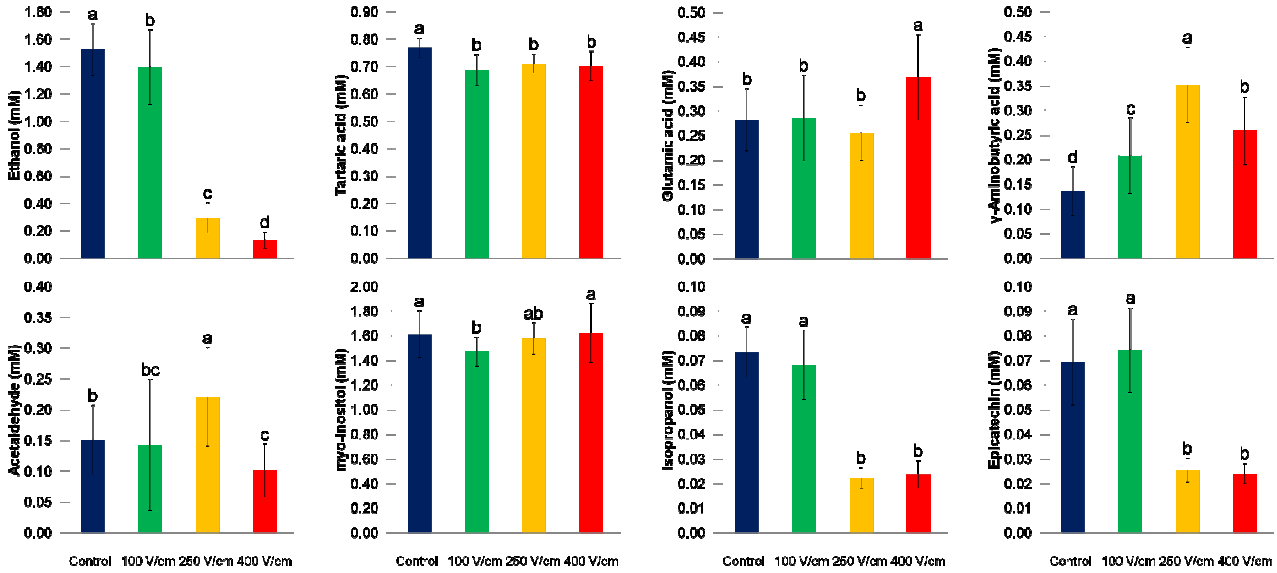

Figure 19. Concentrations of the important metabolites as arisen from metabolomic analysis. Values are means ± standard deviations (n=36) and differences between means with the same letter are not significant at $p<0.05$ (Fig. 4 in **Paper XI**)

Since irreversible damages to membranes are not desirable in MPF, an accurate control of the process parameters is fundamental for the feasible application of PEF to this kind of products. On the other side, the application of electric field strength below the threshold of irreversibility, promoted only slight modifications of the metabolic profiles, indicating the feasibility of the treatment at those conditions.

5 Conclusions

The research findings of this PhD activity increases the understanding of the main phenomena involved on quality aspects of fruit minimal processing. The most consistent results related to the applied non-thermal techniques confirmed their different potentiality in the optic of processing and product innovation, but also the need of their modulation in relation to the different raw material susceptibility to degradation and final product target.

Cold plasma treatment effects on fresh-cut fruit characterized by different kind of stability criticisms, resulted mainly bound to the inactivation of degradative enzymes and microbial cells, without evidencing functional modifications in the final products.
The study of osmotic dehydration and vacuum impregnation highlighted the potentiality of these techniques to be successfully applied for cold formulation/enrichment of minimally processed fruit, but also the necessity of carefully accounting for the metabolic and structural modifications promoted by the processing on the vegetable tissues. An induction of metabolic stress response was also evidenced as a consequence of pulsed electric fields treatment, related to electric field strength. Below the threshold limit of irreversible damages to cell membranes, the treatment promoted only slight and reversible modifications of the metabolic profiles, evaluated through calorimetry and metabolomic analysis on fresh-cut apples.

The applied methodologies, approaching the product as a dynamic system both in terms of endogenous physiological activity and porous matrix interacting with the surrounding ambient (during processing and storage), resulted very promising for the development of innovative vegetable products increasing related knowledge. This can only be carried out through the application of a multianalytical approach based on the combination of different techniques, such as calorimetry, fluorescence/light microscopy, TD-NMR and NMR-based metabolomics, coupled with other physico-chemical and enzymatic assessments.

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Paper I

Influence of ripening stage on quality parameters and metabolic behaviour of fresh-cut kiwifruit during accelerated storage

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INFLUENCE OF RIPENING STAGE ON QUALITY PARAMETERS AND METABOLIC BEHAVIOUR OF FRESH-CUT KIWIFRUIT SLICES DURING ACCELERATED STORAGE

UTICAJ STANJA ZRENJA NA KVALITATIVNE PARAMETRE I METABOLIČKO PONAŠANJE SVEŽE SEČENIH KRIŠKI KIVIJA TOKOM UBRZANOG SKLADIŠTENJA

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ABSTRACT

The aim of this work was to evaluate the influence of the ripening stage on fresh-cut kiwifruit tissues in terms of quality and tissue metabolism, during accelerated storage (AS) in controlled conditions. For this purpose three different ripening stages were selected, corresponding to about 11, 13 and 15 °Brix. For each ripening stage the fruits were peeled and cut into 1 cm thick slices and stored in climatic chambers at 10 °C, 90% RH, for 3 days. During storage, quality indices as soluble solid contents, titrable acidity, colour and texture were monitored. Metabolic assessment was carried out evaluating the endogenous metabolic heat using isothermal calorimetry, and monitoring O_2 consumption and CO_2 production simultaneously. Differences were found in the evolution of the quality indexes during storage; in particular kiwifruit at the lowest ripening stage showed the slowest quality degradation, both in terms of softening and visual quality modifications. Metabolic heat production results were different at the different ripening levels investigated, evidencing a strict connection between the physiological state of the tissue and the entity of its wounding response.

Key words: *kiwifruit, accelerated storage, quality.*

REZIME

Cilj ovog rada je bio da se proceni uticaj stadijuma zrelosti sveže sečenog tkiva kivija u pogledu kvaliteta i metabolizma tkiva, tokom ubrzanog skladištenja (AS) u kontrolisanim uslovima. Za ovu svrhu izabrana su tri različita stadijuma zrelosti, što odgovara sadržaju suve materije od 11 , 13 i 15°Brix. Za svaki stadijum zrelosti plodovi su oljušteni i isečeni na 1 cm debljine i čuvani u klima komorama na 10°C, relativnoj vlažnosti od 90%, tokom 3 dana. Tokom skladištenja, praćeni su kvalitativni pokazatelji: rastvorljivih čvrstog sadržaja, titraciona kiselost, boja i tekstura. Metabolička procena sprovedena je ocenjivanjem endogene metaboličke toplote koristeći izotermni kalorimetar i praćenjem potrošnje O_2 i proizvodnje CO_2 istovremeno. U oceni kvaliteta tokom skladištenja pronađene su razlike; posebno je kivi na najnižem stadijumu zrenja imao najsporiju degradaciju kvaliteta, kako u pogledu omekšavanja tako i vizuelnih promena kvaliteta. Rezultati metabolički proizvedene toplote bili su različiti pri različitim nivoima zrelosti, pokazujući strogu vezu između fiziološkog stanja tkiva i odgovora subjekta na oštećenje.

Ključne reči: *kivi, ubrzano skladištenje, kvalitet.*

INTRODUCTION

Minimally processed fruits are products that have to maintain their quality attributes similar to those of the fresh ones. However, being metabolically active tissues, they show physiological reactions to mechanical stress suffered from peeling, slicing, dicing, shredding or chopping. These minimal processing operations result in a major tissue disruption, consequently the loss of cellular compartmentalisation promotes the contact between enzymes and substrates and an overall increase of metabolic activity (*Alzamora et al.,2000; Hodges e Toivonen, 2008*).

Severity of the wounding response could be affected by several factors, both internal and external. Internal factors include species and variety, but also both maturity at harvest and ripeness stage at cutting. Many studies have shown that the more advanced is the stage of ripeness, the more susceptible the fruit is to wounds, hence to minimal processing (*Brecht, 1995; Gorny et al., 2000; Soliva-Fortuny and Martın-Belloso, 2003*) thus emphasizing the fact that maturity influences stress tolerance (*Hodges and Tovoinen, 2008*). Mature green apple slices exhibited a better preservation of their initial firmness and color during storage compared to partially ripe and full ripe slices (*Soliva-Fortuny et al., 2002*). Slices of slightly under ripe 'Conference' pears underwent minor browning and softening processes compared to slices of riper fruits (*Soliva-Fortuny et al., 2004*), proving the first to be more suitable to minimal processing procedures. Generally the fresh-industry prefers to process firmer and less mature fruits because of technological suitability, and a consequent longer shelf-life of the final product (*Hodges and Toivonen, 2008*), although at this ripening stage the fruit organoleptic quality and flavour profile are lower (*Gorny et al., 2000; Beaulieu and Lea, 2003; Aguayo et al., 2004; Beaulieu 2006a*). Even, generally the flavour is indicated by consumers as the most important quality attribute for fruits and vegetables, textural flaws are more often cause of non-acceptability of a fresh product (*Harker et al., 2003*). Furthermore consumers are often found to be more sensitive to small changes in texture than flavour (*Beaulieu et al., 2004*).

Furthermore several researches have been focused on the influence of maturity stage on the effectiveness of pretreatment for the production of fresh cut fruits. *Beirão-da-Costa et al.* (2006) evaluated the application of mild heat treatments to delay quality

loss in fresh cut kiwifruit, and found that the treatment was effective only if the fruit was at an early maturity stage. *Panarese et al.* (2012) studied the use of osmotic dehydration (OD) on kiwifruit at different ripening stages, finding a higher sensibility of fully ripened fruits to osmotic stress. The higher reduction of metabolic heat production in the ripe fruit was attributed to the loss of membrane integrity and the consequent increase of membrane permeability during ripening.

Kiwifruit is commercially important as fresh-cut fruit (*Antunes et al., 2010*) and it has been the subject of several studies addressed to the increase of its shelf-life through the application of different pre-treatments (*Mao et al., 2007; Villas-Boas and Kader., 2007;* Beirão-da-Costa *et al., 2006, 2008, Dalla Rosa et al., 2011)* and modified atmosphere packaging (*Antunes et al., 2010*). In kiwifruit, minimal processing operations are known to lead to excessive tissue softening (*O'Connor-Shaw et al., 1994*; *Varoquaux, et al, 1990*), to increased CO_2 and ethylene production, to larger mass loss (*Agar, et al, 1999*) and to decreased flavour intensity (*O'Connor-Shaw et al., 1994*), although loss of firmness has been indicated as the most noticeable change in kiwifruit even at low temperatures storage (*Varoquaux et al., 1990; Gil et al., 2006)*. As a consequence, raw kiwifruits used in the fresh-cut industry require high firmness and low soluble content (*Beaulieu, 2010*) in order to perform the mechanical operations, resulting though with an inadequate level of ripening for consumption.

Although several researches have been carried out regarding fresh-cut fruit quality, some basic aspects are still unknown, mainly because only a few studies have been performed about fresh-cut fruit metabolic response to processing stress. The aim of this work was to evaluate the influence of ripening stage on fresh-cut kiwifruit tissues in terms of quality and tissue metabolism, during accelerated storage (AS) in controlled conditions.

MATERIAL AND METHOD

Raw materials

Kiwifruits (*Actinidia deliciosa* var *deliciosa* cv Hayward) were brought on the local market. They were partially ripened at 4 ± 1 °C and 90-95% of relative humidity in air. Along the ripening time, three different stages were selected, corresponding to refractometric index values of 11.6 ± 0.9 (LB-Low Brix); 13.5 ± 0.8 (MB-Medium Brix) and 14.9 ± 0.6 (HB-High Brix). For each ripening stage the fruits were hand peeled and cut into 10 mm thick slices with a sharp knife and stored in climatic chambers at 10 °C, 90% RH, for 3 to 4 days.

Qualitative determinations

Moisture content of kiwifruit samples was determined gravimetrically by difference in weight before and after drying in vacuum oven (pressure≤100 mm Hg) at 70 °C. Drying was performed until a constant weight was achieved (AOAC International, 2002). Triplicate measurements were conducted for each sample. Soluble solids content (SSC) was determined at 20 °C by measuring the refractive index with a digital refractometer (PR1, Atago, Japan) calibrated with distilled water. Titrable acidity (TA) was determined by titration with NaOH 0.1 N until pH 8.1 (AOAC Official Method 942.15) and expressed as mg of citric acid/100 g FW. For each sample, SSC and TA were determined in triplicate on the juice obtained from 10 kiwifruit slices, after filtering through Whatman #1 filter paper. The ratio between SSC and TA has been used as a ripening index (*Sweeney et al., 1970*).

Firmness (N) was evaluated by performing a penetration test on kiwifruit slices outer pericarp tissue using a TA-HDi500 texture analyzer (Stable Micro Systems, Surrey, UK) with a 5 kg load cell. Experiment was run with a metal probe of 6 mm diameter, and a rate and depth of penetration of 1 mm s^{-1} and 6 mm, respectively (*Beirão-da-Costa et al., 2006*). Firmness (N) was evaluated as the first peak force value according to other authors. The mean of two replicates of each kiwifruit slice was averaged (n=30).

Surface colour was measured using a colour-spectrophotometer mod. Colorflex (Hunterlab, USA). Colour was measured using the CIELab scale and Illuminant D65. The instrument was calibrated with a white tile (L*93.47, a* 0.83, b* 1.33) before the measurements. Results were expressed as L* (luminosity), a* (red index) and b* (yellow index).

Metabolic determinations

Six cylinders were sampled using a core borer from the outer pericarp tissue of each kiwifruit slice and placed in sealed 20 ml glass ampoule. Four replicates for each sample were performed. The rate of heat production was continuously measured in a TAM air isothermal calorimeter (Thermometric AB, Järfälla, Sweden), with a sensitivity (precision) of ±10 μW (*Wadsö and Gómez-Galindo, 2009*). This instrument contains eight twin calorimeters. Each calorimeter had its own reference and the measured signal is the difference between the sample signal and the reference signal. The reference is a sample with thermal properties similar to the sample, except that it does not produce any heat; water was chosen as the reference material. By assuming that the heat capacity of kiwifruit dry matter (CST) is 1 J g−1 K−1, the quantity of water in each reference ampoule (Mw) was determined as:

$$M_W = \frac{C_{ST} \cdot M_{ST} + C_W \cdot M_W}{C_W} \quad (1)$$

where M_{ST} is the dry matter content and M_W is the water content of the kiwifruit sample; C_W is the water heat capacity. The measurements were performed at 10 °C for 24 h. Immediately after the ampoules discharging from the calorimeters, the CO_2 percentage was measured in the ampoule headspaces by a gas analyzer (MFA III S/L gas analyzer, Witt-Gasetechnik, Witten, Germany).

Respiration rate was evaluated using a static method. Six cylinders were sampled from the outer pericarp tissue of the slice and sealed in 20 ml glass ampoule. CO_2 percentage of triplicate specimens was measured in the ampoule headspace by a gas analyzer mod. MFA III S/L (Witt-Gasetechnik, Germany) after selected intervals during 24 h at 10 °C from the sampling.

The respiration rate ($RRCO_2$) was calculated as:

$$RRCO_2 = \frac{mm_{CO2} \cdot V_{head} + \frac{\%CO_{2head}}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283} \quad (2)$$

where $mmCO_2$ is the oxygen molar mass (g/mol), V_{head} is the ampoule headspace volume (L), $\%CO_2$, head is the carbon dioxide percentage in the ampoule headspace at time t (h); m is the sample mass (kg); R is the gas constant (L kPa K^{-1} mol^{-1}).

Statistical analysis

Significance of the effects of ripening stage was evaluated by means of t-Test and one-way analysis of variance (ANOVA, 95% significance level) using the software STATISTICA 6.0 (Statsoft Inc., Tulsa, UK).

RESULTS AND DISCUSSION

Physico-chemical parameters

Means values of the soluble solids content (SSC), titrable acidity (TA) maturity index (°Brix/TA) and water content are shown in Table 1.

Table 1. Physico-chemical parameters of kiwifruit samples during storage at 10 °C 90 % RH.

Samples	Days of storage	SSC (°Brix)	TA (mg citric acid/100 g)	Maturity index (°Brix/TA)	Water content (%)
LB	0	11.56[a]	1.45[a]	7.95[a]	84.1[a]
	1	12.07[a]	1.50[a]	8.06[a]	83.9[a]
	3	14.58[b]	1.47[a]	9.93[b]	82.6[b]
MB	0	13.46[a]	1.47[a]	8.92[a]	85.1[a]
	1	14.64[b]	1.57[a]	9.35[a]	81.3[b]
	4	16.27[c]	1.59[a]	10.26[b]	79.6[b]
HB	0	14.88[a]	1.42[a]	10.04[a]	83.7[a]
	1	16.55[b]	1.48[b]	11.26[b]	82.4[b]
	3	17.53[b]	1.56[c]	11.26[b]	80.6[c]

*Values followed by different letters for the same ripening group differ significantly at $p < 0.05$ levels.

Soluble solid content increased of about 3°Brix compare to the initial value in all samples during storage, showing a progressive conversion of starch into soluble sugars as a response induced by minimal processing. TA did not show significant modifications in LB and MB kiwi samples but underwent a slight, but significant increase in High Brix (HB) sample. In general, in all samples an increase of maturity index, calculated as ratio between SSC and TA, was observed.

Initial moisture content was similar for the three different ripening stages groups and decreased in all samples during storage as reported in table 1. Disruption of cell membranes due to wounding can lead to water loss from tissues (*Rolle et al.,1987*). The intermediate ripeness (MB) sample showed a higher water loss, going from 85.1 to 81.3% of water content in the first day and to 79.6 % at the end of the storage. The sample at the lower ripeness degree shows a better retention of water during storage with a loss of less than 2%.

Degree of ripening affected lightness of the flesh, as reported in fig 1a. LB fruit were significantly lighter compared to HB fruits, showing a difference in the L* value of ten units; similar results has been found by *Beirão-da-Costa et al.* (2006). During storage the a* chromatic component of colour reported a progressive decrease in all samples. As far as a* and b* values are concerned, during storage all samples showed modifications that led to the increase of the first and decrease of the second one.

Kiwi fruit samples at LB and MB ripening stages, did not show significant differences, whereas the riper fruit appeared always less green and yellow. The colour of kiwifruit flesh is mainly due to the presence of some pigments, as chlorophyll and carotenoids. Variations from green to yellow are due to the disappearance of chlorophyll that unmask the carotenoids and to the changes in the chemical composition and cell wall structure (*Brady, 1987*). According to *Montefiori et al.* (2009) cultivars of *Actinidia deliciosa* Hayward retain better their green colour during maturation and ripening compared to other cultivars, as a consequence of a low enzymes activity responsible for chlorophyll catabolism and a retention of the chloroplasts structure. The changes of colour observed in this experiment could be caused by a variation in the internal structure of kiwifruit tissue due to ripening. According to *Agar et al.* (1999) surface darkening in kiwifruit slices can be attributed to translucent water soaking of the tissues, while low polyphenoloxidase activity and high ascorbate content inhibit enzymatic colour degradation.

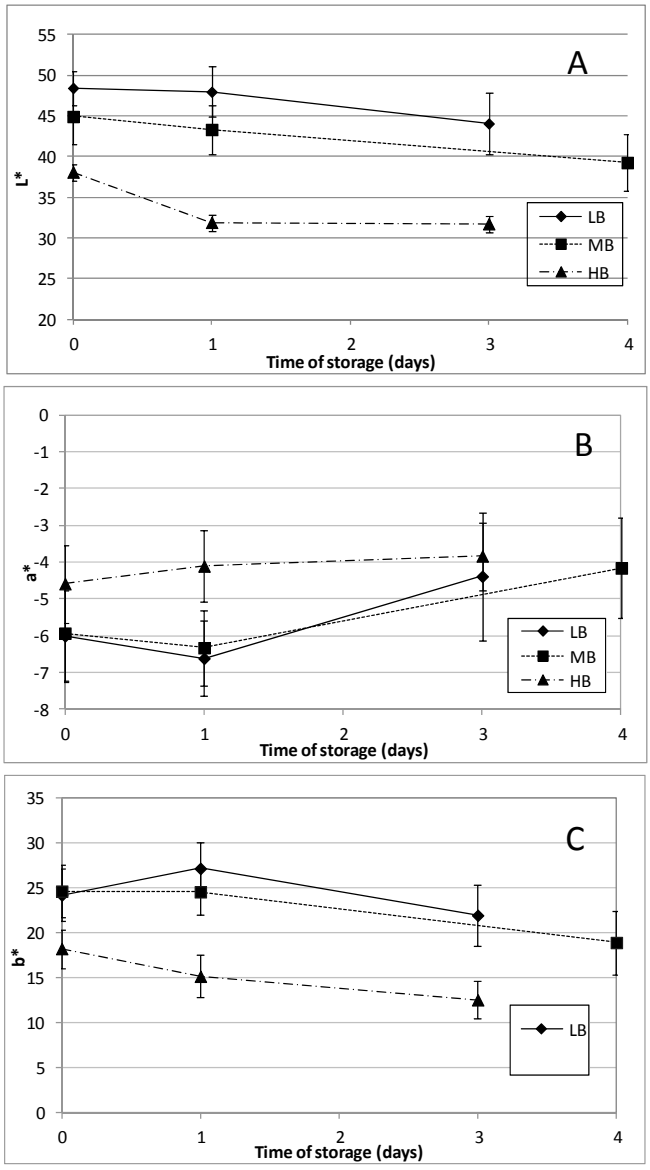

Fig. 1. Changes in colour parameters L, a* and b* of kiwifruit samples (♦Low ■Medium and ▲ High Brix) during storage at 10°C and 90% RH.*

As expected LB fruit showed a higher initial flesh firmness (11.28 N ± 5.22) compared to medium ripe MB (9.33 N ± 3.74) and riper fruits HB (1.66N ± 0.32) indicating a progressive breakdown of internal structure during fruit maturation that it is a consequence of the solubilisation of the protopectin fraction of the cell wall components (*Varoquaux et al., 1990*). Furthermore *Beirão-da-Costa* (2006) found that calcium content was higher in partially mature fruits compared to fully ripe one, resulting in a greater formation of calcium pectates.

Differences in the ability to maintain initial firmness during storage were also found. As reported in fig 2, at a lowest stage of ripening, kiwifruit slices exhibited a good retention of their tex-

ture after one day of storage at 10°C. Only at the third day, a substantial firmness loss, more than 50% of the initial value, was observed. In MB kiwifruit slices, a 50% loss was observed right after the first day, whether riper fruit slices maintained very low and similar values during the three days of storage.

Texture loss in kiwifruit slices can be considered the most noticeable change during storage (Soda et al., 1986; *Varoquaux et al., 1990; Gil et al., 2006)* and it is related to structural changes *(Muntada et al., 1998)*. According to *Varoquaux et al.* (1990) texture breakdown in kiwifruit slices is due to the enzymatic hydrolysis of cell wall components. These enzymes, activated by operations as peeling and cutting, diffuse through the inner tissue and catalyze the demethoxylation and depolymerisation of pectic compounds. These authors also affirmed that enzymatic activity due to cutting promote the degradation of uronic acid polymers, demethylation of the water soluble fraction and rupture of calcium bridges.

The results of the present study suggest that with the progressive ripening of the fruit, the internal structure undergoes a breakdown that causes a substantial loss of initial firmness. Moreover, mature fruits are more sensible to peeling and cutting, and undergo a faster softening process compared to partially ripe fruits. These results are in agreement with previous findings by *Soliva-Fortuny et al.* (2002; 2004) and Dobrzanski and Rybczynsky (2000).

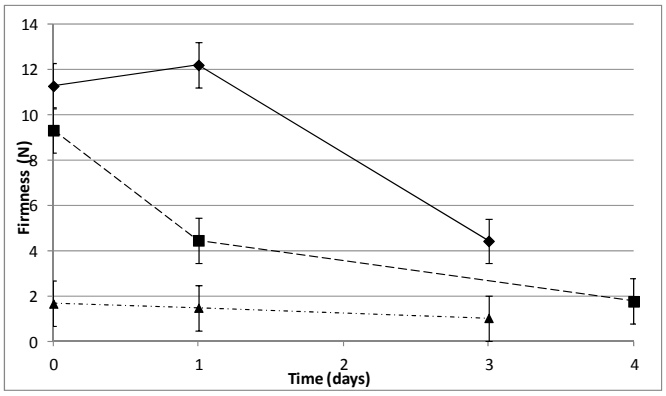

Fig. 2. Evolution of firmness of fresh-cut kiwifruit slices at three ripening stages (◆ Low, ■ Medium and ▲ High Brix) during storage.

Metabolic response

Figure 3 depicts an example of heat production profiles of Low, Medium and High Brix kiwifruit samples during 12 hours at 10°C. The initial disturbance due to ampoule loading into the calorimeter lasted about 1 h and was not taken into consideration. The thermograms evidenced a progressive decrease of the specific thermal power P (mW per gram of sample) by increasing the ripeness level.

Generally, when the wounded tissue 'send the signals', the plant starts a number of protective processes that lead to an increase of the produced metabolic heat (*Wadsö et al, 2004*). As reported by *Gomez et al.* (2004), after wounding, the energy released by the cell is due to the sum of the ''normal'' metabolic activity and that originating from wounding stress produced by the cells near the cut surface. Part of the processes that occur after wounding are design to membrane restoration and strengthening of cell walls by cells close to the site of injury (*Rolle et al, 1987* and *Satoh et al., 1992*). These results suggest that fruits at an advance stage of ripening have a lower metabolism reaction after wounding, probably due to the loss of the ability to repair the damage caused by mechanical stress.

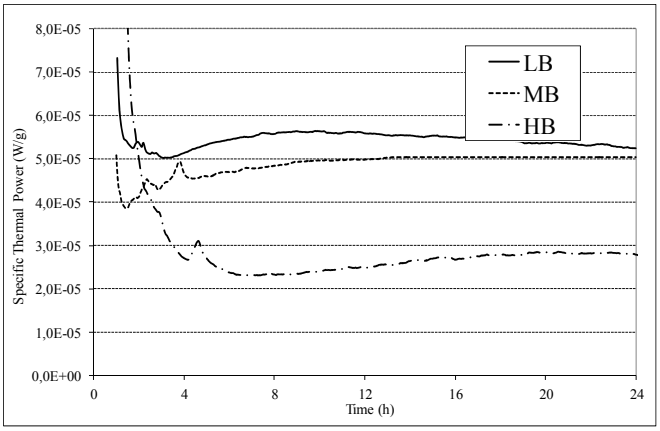

Fig. 3. Specific thermal power profiles of pericarp tissue cylinders or kiwifruit at three ripening stages during 24 h of analysis at 10°C. Each thermogram is an average of four replicates.

Mean values of the respiration rate during storage of 24 h in controlled conditions are reported in Table 2. LB sample showed consistently higher values of CO_2 produced and O_2 consumed compared to the riper samples.

Table 2. Rate of respiration (RCO_2) of pericarp tissue cylinders or kiwifruit at three ripening stages during 24 h of analysis at 10°C.

Time of storage (h)	Sample					
	LB		MB		HB	
2	61.61	± 3.90	36.39	± 4.27	40.82	± 0.59
4	37.9	± 5.60	29.40	± 3.50	32.77	± 2.03
6	33.90	± 9.45	24.23	± 4.41	33.82	± 3.50
8			25.68	± 0.64	26.63	± 2.93
24	33.52	± 2.48	24.51	± 0.44	21.86	± 2.65

CONCLUSION

The present work showed that the ripening stage has an important influence on the entity of the wounding response of kiwifruit tissue. All samples showed an increase of maturity index, while the sample at the lower ripening degree showed a better retention of water during storage. Differences in the ability to maintain initial firmness during storage were also found. At a lower stage of ripening, kiwifruit slices exhibited a good retention of their texture during the first two days of storage at 10°C, while more mature fruits were more sensible to peeling and cutting, showing a faster softening process compared to partially ripe fruits.

Metabolic heat production results were different at the different ripening levels investigated, evidencing a strict connection between the physiological state of the tissue and the entity of its wounding response in terms of heat production

The approach purposed in this research can be very useful to standardize the raw kiwifruit selection from fresh-cut industry, permitting the understanding of kiwifruit metabolic response mechanisms to minimal processing operations.

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Paper II

Enzymatic activity and metabolism of fresh-cut fruit as a function of ripening degree

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ENZYMATIC ACTIVITY AND METABOLISM OF FRESH-CUT FRUIT AS A FUNCTION OF RIPENING DEGREE

ENZIMSKA AKTIVNOST I METABOLIZAM SVEŽE REZANOG VOĆA KAO FUNKCIJA STEPENA ZRELOSTI

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ABSTRACT

The aim of this work was to evaluate the influence of the ripening stage on three fresh-cut fruit (apple, kiwifruit and melon) in terms of quality and tissue metabolism, during accelerated storage (AS) in controlled conditions. For this purpose, two different ripening stages were selected for each fruit. At each ripening stage the fruits were peeled and cut into 10 mm thick slices and stored at 10 °C, RH 90 %, for 24 hour for apples and 4 days for kiwifruit and melon. During storage, samples were monitored for the main quality indexes and the activity of the main degradative enzymes (polyphenoloxidase, peroxidase and pectinesterase). Production of the endogenous metabolic heat was also monitored using isothermal calorimetry.

Results showed that, whether for melon and kiwifruit marked differences were found in the evolution of the parameters considered during storage and in relation to their ripening degree, apple quality and metabolic characteristics proved to be less affected by the ripening degree during storage.

***Key words**: fresh-cut fruit, wounding response, ripening degree, enzymatic activity, tissue metabolism.*

REZIME

Cilj ovoga rada je ocena uticaja stepena zrelosti tri sveže rezane voćne vrste (jabuke, kivija i dinje) u odnosu na kvalitet i metabolizam tkiva, tokom postupka ubrzanog skladištenja u kontrolisanim uslovima. U tu svrhu, dva različita stadijuma zrelosti su odabrana za svaku voćnu vrstu. Svaki uzorak različitih stepena zrelosti je oljušten i izrezan na kolutove debljine 10 mm i potom skladišten na 10°C pri relativnoj vlažnosti vazduha 90%, u trajanju od 24 sata za jabuku i četiri dana za kivi i dinju. Tokom skladištenja, praćeni su glavni indeksi kvaliteta uzoraka i aktivnosti glavnih degradativnim enzima (poliphenolokidaze, peroksidaze i pektinasterase). Proizvodnja endogene metaboličke toplote je takođe praćena korišćenjem metode izotermne kalorimetrije.

Rezultati su pokazali uočljive razlike u ocenjivanju parametara tokom skladištenja kada je reč o dinji i kiviju i njihovom stepenu zrenja, ispostavilo se da na kvalitet jabuke i metaboličke karakteristike, stepen zrenja ima manji uticaj tokom procesa skladištenja.

***Ključne reči**: sveže rezano voće, uticaj sečenja, stepen zrenja, enzimaska aktivnost, metabolizam tkiva.*

INTRODUCTION

Fresh-cut fruit are products that undergo minimal processing operation such as peeling, cutting, dicing, shredding etc. and maintain their quality characteristics similar to those of fresh products. However, their quality degrade faster compared to that of the intact product due to the physiological response to the mechanical stress called 'wounding response' (*Brecht, 1995*).

The loss of cellular compartmentalisation, promoted by processing operations, leads to the contact between enzymes and their substrates and a general increase of enzymatic and metabolic activity that accelerate the quality degradation.

Appearance and texture changes are the main factor determining consumer acceptability of fresh-cut fruit and, being strictly related to tissue deterioration, are often used as measures of product freshness and quality.

The selection of raw material is a fundamental factor for product quality and its shelf-life. Degree of ripening at harvesting and at processing is an important factor that can influence the intensity of the wounding response (*Hodges and Toivonen, 2008*). Generally, processing fruits that are unripe or slightly unripe, lead to a better preservation of quality during storage but it could be detrimental for the organoleptic and flavour profile (*Beaulieu et al., 2004*).

However, few studies have been performed about the influence of ripening degree on the metabolic response to processing stress in fresh-cut fruit. Therefore, the aim of this work was to evaluate the influence of the ripening stage on fresh-cut apple, kiwifruit and melon in terms of quality and tissue metabolism, during accelerated storage (AS) in controlled conditions.

MATERIAL AND METHOD

Raw materials

Kiwifruits (*Actinidia deliciosa* var *deliciosa* cv Hayward) and apples (*Malus domestica* var. *Pink Lady*) were brought on the local market. They were partially ripened at 4 ± 1 °C and 90-95 % of relative humidity in air for about a month, selecting two different ripening stages defined as Unripe (U) and Ripe (R). Melon (*Cucumis melo* var. *Reticulatus*) was harvested at different degrees of development of the abscission layer, also called 'slip', in particular at ½ slip as unripe (U) and full slip as ripe (R). For each ripening stage the fruits were hand peeled and cut into 10 mm thick slices with a sharp knife and stored in climatic chambers at 10 °C, 90 % RH, for different time period depending on the kinetics of quality degradation that were defined in preliminary tests as 24 hours for apples and 4 days for kiwifruit and melon.

Dry matter content, soluble solid content and titrable acidity were determined to characterize the raw material. Dry matter was determined gravimetrically after drying in vacuum oven (AOAC International, 2002). Soluble solids content (SSC) was determined at 20 °C by measuring the refractive index with a digital refractometer (PR1, Atago, Japan) calibrated with distilled water. Titrable acidity (TA) was determined by titration with NaOH 0.1 N until pH 8.1 (AOAC Official Method 942.15) and expressed as mg of citric acid/100 g FW. Triplicate measurements were conducted for each sample. The ratio between SSC and TA has been used as a ripening index. Results are presented in table 1.

Table 1. Physico-chemical parameters of apple, kiwifruit and melon at the two ripening stages selected

Fruit	Ripening Stage	SSC (°Brix)	TA (mg citric acid/100 g)	Maturity index (°Brix/TA)	Dry matter (g/100g)
Apple	U	13.0 ± 0.2	0.36 ± 0.02	35.9 ± 0.4	13.4 ± 0.2
	R	13.4 ± 0.2	0.27 ± 0.01	50.6 ± 1.1	14.5 ± 0.7
Kiwifruit	U	11.6 ± 0.9	1.45 ± 0.02	7.9 ± 0.2	15.9 ± 0.5
	R	14.9 ± 0.6	1.42 ± 0.01	10.0 ± 0.4	16.3 ± 0.5
Melon	U	11.8 ± 0.1	0.7 ± 0.1	16.8 ± 0.3	13.1 ± 0.9
	R	14.3 ± 0.1	0.8 ± 0.1	17.9 ± 0.3	16.2 ± 0.5

Qualitative determinations

Firmness (N) was evaluated by performing a penetration test on fruit pulp using a TA-HDi500 texture analyzer (Stable Micro Systems, Surrey, UK) with a 5 kg load cell. Experiment was run with a metal probe of 6 mm diameter, and a rate and depth of penetration of 1 mm s^{-1} and 6 mm, respectively (*Beirão-da-Costa et al., 2006*). Firmness (N) was evaluated as the first peak force value.

Surface colour was measured with a colour-spectrophotometer mod. Colorflex (Hunterlab, USA) using the CIELab scale and Illuminant D65. A white tile (L* 93.47, a* 0.83, b* 1.33) was used for calibration. Results were expressed as L* (luminosity) and h° (hue angle).

Enzymatic activity

The activity of the enzyme mainly responsible for quality degradation for each fruit was evaluated.

Polyphenoloxidase (PPO) activity was assayed spectrophotometrically according to the methods proposed by *Baritaux et al. (1991)* on fresh-cut apples and after 2, 4, 12 and 24 h of storage for both ripening degrees.

Pectinmethylesterase (PME) was extracted and assayed spectrophotometrically according to the methods proposed by *Hagerman and Austin (1986)* on fresh-cut kiwifruit after 0, 1, 2 and 4 days of storage for both ripening degrees.

Peroxidase (POD) was extracted and assayed spectrophotometrically according to the methods proposed by *Morales Blancas et al. (2002)* on fresh-cut melon after 0, 1, 2 and 4 days of storage for both ripening degrees.

Metabolic determinations

A TAM air isothermal calorimeter (Thermometric AB, Järfälla, Sweden), described in details by *Wadsö and Gómez-Galindo (2009*), was used to assess metabolic heat production. Six cylinders were sampled using a core borer from the fruit tissue and placed in sealed 20 ml glass ampoule. Four replicates for each sample were performed. The rate of heat production was continuously measured at 10 °C for 24 h. Immediately after the end of the analysis, the O_2 and CO_2 percentages were measured in the headspaces by a gas analyzer (MFA III S/L gas analyzer, Witt-Gasetechnik, Witten, Germany).

RESULTS AND DISCUSSION

Qualitative determinations

The values of hardness measured in the fruit samples at different ripening stages during storage are reported in table 2. As it can be observed, hardness increased upon ripening in apple samples, both between ripening stages and during 24 h storage. On the contrary, melon and kiwifruit slices showed a notable loss of hardness between the two ripening degrees selected, almost tenfold for kiwifruit. After 4 d of storage, it can be observed a loss of hardness up to 23 and 60 % compared to the initial values for respectively unripe melon and unripe kiwifruit, while ripe fruit showed always low and similar values.

Textural characteristics are related to ripening degree and variations of such properties are often due to enzymatic hydrolysis of cell wall components. Melon and kiwifruit are particularly subjected to softening during storage *(Silveira et al., 2011; Gil et al., 2006)* and, in agreement with results found by *Beaulieu et al. (2004)*, texture was significantly affected by the maturity stage but the effect of storage time decreased as maturity increased.

Table 2. Hardness (N) of apple, kiwifruit and melon at the two ripening stages selected during accelerated storage in controlled conditions. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit for the same storage time

Fruit	Ripe. Stage	Storage time (days)				
		0	0.5	1	2	4
Apple	U	15.22^{a} ± 3.41	20.12^{a} ± 3.71	19.99^{a} ± 2.84	-	-
	R	19.79^{b} ± 7.80	22.67^{b} ± 4.37	25.35^{b} ± 4.00	-	-
Melon	U	18.42^{a} ± 3.77	-	16.63^{a} ± 3.31	16.08^{a} ± 3.91	14.14^{a} ± 4.12
	R	9.43^{b} ± 4.61	-	10.81^{b} ± 4.40	10.16^{b} ± 4.78	8.98^{b} ± 2.87
Kiwi.	U	11.28^{a} ± 5.22	-	12.21^{a} ± 3.49	17.23^{a} ± 5.26	4.43^{a} ± 0.79
	R	1.66^{b} ± 0.32	-	1.48^{b} ± 0.37	1.26^{b} ± 0.31	1.03^{b} ± 0.18

As it can be observed in table 3, in the first 12 h of storage apple slices underwent a relevant variation of colour parameters in terms of luminosity and hue angle decrease, that indicates a general surface browning that can be attributed to PPO activity. In the second part of the storage, both parameters further decreased but no significant differences were found between ripening stages.

Colour changes in fresh-cut melon and kiwifruit are mainly due to variation in the internal structure of the tissue and the induction of a translucent water-soaked tissue while enzymatic browning is scarcely influent.

Whether in both fruits and in both ripening stages a surface darkening was observed during 4 d of storage, degree of ripening significantly affected lightness in kiwifruit but not in melon. Conversely, hue angle although decreasing during storage, was consistently higher in riper melon slices indicating a variation from yellow to red tonality possibly due to a higher concentration in β-carotene upon ripening *(Simandjuntak et al., 1996)*.

Table 3. Colour of apple, kiwifruit and melon at the two ripening stages selected during accelerated storage in controlled conditions. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit for the same storage time

	Fruit	Ripening Stage	Storage time (days)				
			0	0.5	1	2	4
	Apple	U	81.45 [a] ± 2.05	69.33 [a] ± 2.45	65.57 [a] ± 1.38	-	-
		R	82.33 [a] ± 3.05	71.55 [a] ± 2.56	67.67 ± 2.05	-	-
L*	Kiwifruit	U	48.45 [a] ± 2.11	-	47.98 [a] ± 3.07	47.2[1a] ± 3.16	44.08[a] ± 3.77
		R	38.11[b] ± 2.45	-	31.93 [b] ± 3.61	31.88 [b] ± 3.78	31.75[b] ± 1.81
	Melon	U	57.24 [a] ± 2.90	-	45.25 [a] ± 1.72	45.26 [a] ± 2.12	40.43[a] ± 4.97
		R	57.85 [a] ± 4.50	-	42.92[a] ± 4.04	44.11[a] ± 2.48	42.38[a] ± 4.97
	Apple	U	97.57[a] ± 4.55	93.25[a] ± 2.56	88.15[a] ± 2.07	-	-
		R	98.67[a] ± 3.18	90.03[a] ± 3.56	84.66 ± 2.28	-	-
h°	Kiwifruit	U	103.93[a] ± 2.11	-	103.72[a] ± 0.76	102.19[a] ± 2.16	101.17 [a] ± 3.11
		R	103.83[a] ± 2.70	-	104.70[a] ± 2.80	104.44[a] ± 2.05	106.32[b] ± 4.39
	Melon	U	61.81[a] ± 0.98	-	60.89 [a] ± 1.05	59.83 [a] ± 1.76	60.08 [a] ± 1.03
		R	64.57[b] ± 0.89	-	62.91[b] ± 1.16	62.85[b] ± 1.37	62.11[b] ± 1.75

Enzymatic activity

Figure 1 reports PPO activity measured in fresh cut apples during 24 h of storage. Straight after cutting, riper fruit showed a higher activity compared to unripe one but differences were not statistically significant. After 2 h, it increased sharply until doubling its values, but then decreased again. PPO is rapidly activated by peeling and cutting operations that lead to the contact with its substrate. After 12 and 24 h the activity was very low and did not show any significant differences between samples.

Texture loss is one of the main factor limiting fresh-cut kiwifruit shelf-life (*Gil et al., 2006)* Softening and textural changes are brought about by the actions of a multitude of cell-wall-localized enzymes acting on specific, potentially highly localized substrates. According to *Varoquaux et al. (1990)* texture breakdown in kiwifruit slices is mainly due to the enzymatic breakdown of pectic compounds, due to demethoxylation and depolymerization by pectinolytic enzymes such as PME and polygalacturonase (PG). In particular, PME causes the removal of methylester groups from pectins in the cell wall that are further depolymerised by PG.

Figure 2 shows Pectinmethylesterase (PME) activity in fresh-cut kiwifruit slices during 4 d of storage. Unripe fruits showed an activity tenfold compared to ripe fruit. PME activity is generally higher early in ripening (*Toivonen and Brummel, 2008*) increasing accessibility of PG to its substrate.

Peroxidase (POD) is an ubiquitous enzyme found in most vegetable tissues and comprises many isozymes *(Lamikanra and Watson, 2000)*. Its activity causes the oxidation of mono and di-phenols when even small quantities of hydrogen peroxide act as oxidising agent. It is involved in many degradative reactions that affect colour, aroma, texture and nutritional characteristics of fruit and vegetables. According to *Lamikanra et al. (2005)*, a high POD activity is related to the

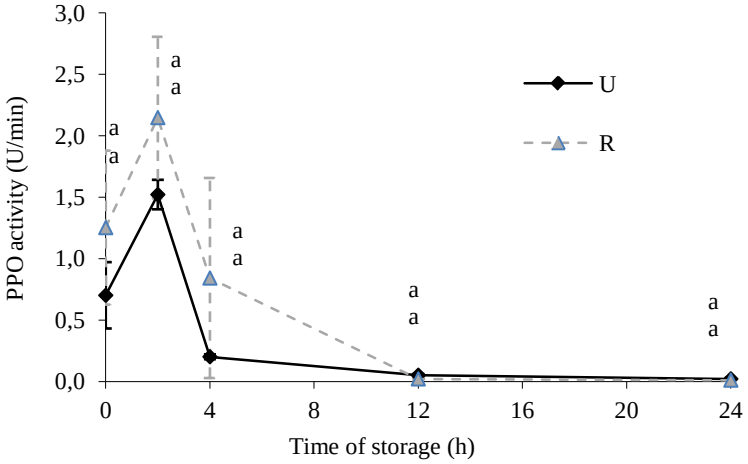

Fig. 1. Polyphenoloxidase (PPO) activity (U/ml) of apple at different ripening degrees during 24 h of storage in controlled conditions. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit for the same storage time

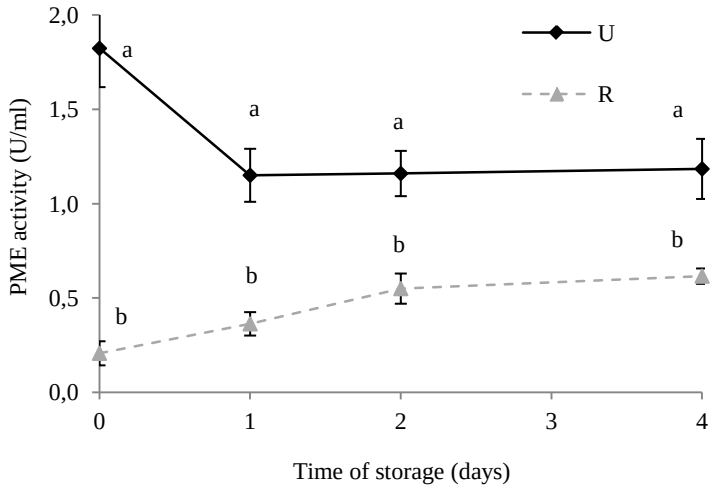

Fig. 2. Pectinmethylesterase (PME) activity (U/ml) of kiwifruit at different ripening degrees during 4 d of storage in controlled conditions. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit for the same storage time

development of off-odour and off-colours in food and *Lamikanra et al. (2000)* suggested that could substantially reduce shelf-life of melons.

As it can be observed in figure 3, POD activity was double in unripe melons compared to ripe ones. In both samples it increased until the second day of storage, probably as a consequence of the physiological response to wounding stress, and then decreased again.

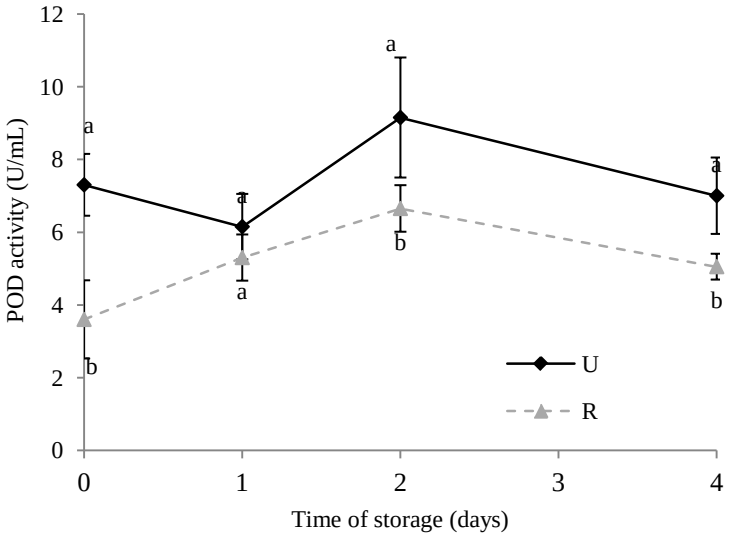

Fig. 3. Peroxidase (POD) activity (U/ml) of melon at different ripening degrees during 4 d of storage in controlled conditions. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit for the same storage time

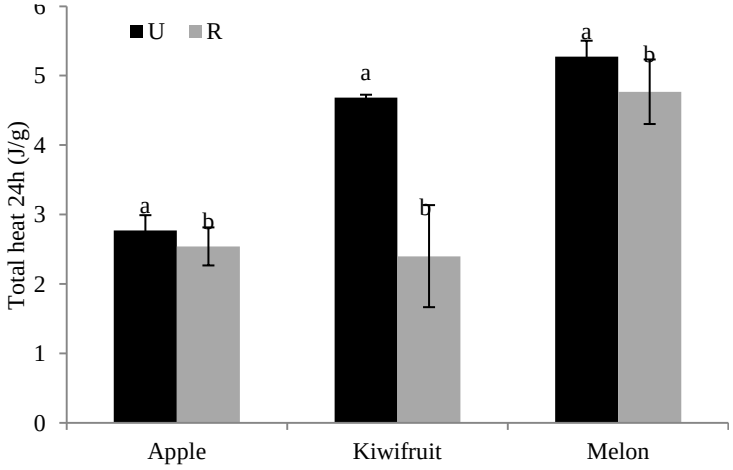

Fig. 4. *Total metabolic heat production of apple, kiwifruit and melon at the two ripening stages selected during 24 h at 10°C. Different letters indicate significant differences (p-level < 0.05) between ripe and unripe fruit*

Metabolic heat production

Metabolic profiles obtained by isothermal calorimetry have been integrated in order to calculate the total metabolic heat produced by fruit tissues during 24 h. Results are reported in figure 4. Operations such as peeling and cutting cause a mechanical stress to tissues that react by starting different metabolic processes aimed at repairing the damages. Therefore , as reported by *Gomez et al. (2004)* and by *Tappi et al. (2013)*, the heat measured after wounding is the sum of the normal metabolic activity and the one due to wounding.

As it can be observed, for all fruits, riper fruits showed a lower metabolic heat production. This may indicate a loss of the ability to repair the damage caused by mechanical stress. While all differences were statistically significant, the highest difference was observed for kiwifruit that showed at the advanced stage of ripening a 50 % decrease in the metabolic heat production compared to the unripe fruit.

CONCLUSION

The results obtained in this research showed how the main qualitative and metabolic characteristics of the three different fruit were affected by ripening degree during storage.

Degree of ripening influenced significantly flesh colour, hardness and enzymatic activity in fresh-cut kiwifruit and melon, but it showed little effect on fresh-cut apple characteristics during the storage time considered.

Metabolic heat decreased upon ripening in all three fruits, showing a strict connection between the physiological state of the tissue and the entity of its wounding response.

Results highlight how the determination of the optimal ripening degree for fresh-cut production has to be carried out specifically for each type of fruit.

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Paper III

Atmospheric gas plasma treatment of fresh-cut apples

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Atmospheric gas plasma treatment of fresh-cut apples

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ABSTRACT

In this study we pioneered the use of gas plasma for the treatment of fresh-cut apples and its potential application in the agri-food precesses. Treatments were conducted on fresh-cut Pink Lady® apples using a Dielectric Barrier Discharge (DBD) generator and considering three different times: 10, 20 and 30 min. Main quality (soluble solid content, titrable acidy, colour by computer vision system and texture) and metabolic parameters (polyphenol oxidase PPO activity, respiration and heat production) were assessed immediately after the treatment and during a storage of 24 h (10 °C, 90% RH). In terms of browned areas, a significant decrease was observed in treated samples compared to the control ones (up to about 65% for 30 min and after 4 h of storage). PPO residual activity decreased linearly by increasing the treatment time (up to about 42%). In general the treatment appeared to slow down the metabolic activity of the tissue. Other qualitative parameters were only slightly affected by the treatment.
Industrial relevance: The potential application on in-packed cold plasma technology and its known effect on microbiological decontamination of foods makes this technique very encouraging for fresh-cut fruit stabilization. However very important aspects have to been clarified in order to deeply understand gas plasma effect on fresh-cut apple quality and on the metabolic response of the tissue.

1. Introduction

Factors controlling the quality maintenance of fresh-cut fruits are a result of a complex process, concerning a number of physico-chemical and biochemical modifications, that mainly affect flavour, colour and texture (Mencarelli & Massantini, 1994). The disruption of the cellular structure due to peeling and cutting puts enzymes and their substrates in direct contact. Several reactions can be promoted, and a sudden increase in the respiration rate and in the metabolism, leading to a faster tissue deterioration, can be observed. These reactions involve negative changes in fundamental characters highly appreciated by consumers, as the visual quality (mainly changes in colour) and the texture (tissue softening) (Toivonen & Brummell, 2008).

For fresh-cut apples the most important phenomenon responsible of its quality degradation is the enzymatic browning (Rocha & Morais, 2003). It is worth noting that apple tissue cutting allows the interaction between the polyphenol oxidase (PPO) with the polyphenolic substrate, in the presence of oxygen (Martinez & Withaker, 1995). Cut-edge browning is due to two PPO catalyzed reactions, the hydroxylation of monophenols to diphenols and the oxidation of diphenols to quinones, which in turn involve melanin accumulation (Toivonen & Brummell, 2008).

At date different chemical and physical techniques were explored to control enzymatic browning of fresh-cut apples. Chemical techniques that act to inactivate the enzyme are based on dipping procedures and on the use of organic acids in combination with calcium salts, carboxylic acids, thiol containing compounds and phenolic acids (Oms-Oliu et al., 2010). Edible coatings, as carriers of the anti-browning chemical agents, were also extensively studied (Baldwin, Níspero-Carriedo, Chen, & Hagenmaier, 1996) and several researches were focused on the contribution of modified atmosphere packaging (MAP) on browning inhibition (Aguayo, Requejo-Jackman, Stanley, & Woolf, 2010; Rocculi, Romani, & Dalla Rosa, 2004).

Recently different innovative treatments were tested to inhibit browning reactions. For fresh-cut apples, UV-C light (200–280 nm) exposure (Manzocco et al., 2011) and short term exposure to nitric oxide (NO) gas (Pristijono, Willis, & Golding, 2006) showed high potentialities.

Among advanced techniques, gas plasma is currently used for biotreatments; it is an ionized gas characterized by active particles such as electrons, ions, free radicals, and atoms which are both in ground and excited states; the excited species emit a photon (including UV photons) when they get to the ground state (Moreau, Orange, & Feuilloley, 2008). The ionization occurs by applying energy to a gas mixture and particularly to electrons which in turn transmit the energy to the heavy species by collisions. Non-thermal or non-equilibrium plasmas are produced at low pressure (e.g. atmospheric), and the behaviour of electrons and ions is in turn influenced by the excitation frequency. When atmospheric air is used as working gas to generate non-equilibrium plasma

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discharges, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed. Ozone (O_3), atomic oxygen (O) and hydroxyl radical (OH), are the main generated ROS active components. OH radicals are produced from the direct dissociation of the water molecules by electronic impact (Moreau et al., 2007). Excited molecules of N_2 and nitric oxide radical (NO) are the main RNS species characterizing a non-equilibrium plasma and having a role in the decontamination. The oxidative species produced during the discharge can cause lipid peroxidation, and protein and DNA oxidation (Montie, Kelly-Wintenberg, & Roth, 2000). Main biological applications of non-thermal plasmas are undoubtedly medicals (Fridman et al., 2008) and regard the microbial decontamination of complex and expensive heat-sensitive medical devices (Weltmann et al., 2008), the sterilization of living tissues and wound healing (Kong et al., 2009). In the medical field, protein destruction (bovine serum albumin) was also observed by treating medical surgical instruments with atmospheric gas plasma (Deng, Shi, Chen, & Kong, 2007; Deng, Shi, & Kong, 2007).

Recently, non-thermal plasma was used for the decontamination of agricultural products and its inactivation power was studied with respect to several microbial species (Shama & Kong, 2012). In terms of microbiological lethal power, main results showed that this technique can be a valuable alternative to the washing procedures with chemicals such as those used for fresh fruits and vegetables such as apples, cantaloupe, lettuce (Critzer, Kelly-Winterberg, South, & Golden, 2007), mango, melons (Perni, Liu, Shama, & Kong, 2008), and pears (Berardinelli, Vannini, Ragni, & Guerzoni, 2012). Other studies regarding the food sector were conducted on the decontamination of shell eggs (Ragni et al., 2010), chilled poultry wash water (Rowan et al., 2007), food packaging materials such as polyethylene terephthalate bottles (Koulik, Begounov, & Goloviatinskii, 1999) and sealed packages (Keener et al., 2012).

Fundamentals of cold plasma technology and its applications to the decontamination of foods have been reviewed by Misra, Tiwari, Raghavarao, and Cullen (2011) and Niemira (2012). Unlike research for food-borne pathogen inactivation, few studies have been conducted to this end to evaluate the effect of cold gas plasma on fresh-cut fruit and vegetable quality aspects.

Grzegorzewski, Rohn, Kroh, Geyer, and Schlüter (2010) and Grzegorzewski, Ehlbeck, Schluter, Kroh and Rohn (2011) studied the effect of non-thermal plasma on lamb's lettuce morphology and chemical composition, particularly on phenolic compounds content. According to their findings, leaf surfaces were significantly affected by the treatment, showing a degradation of epicuticular waxes and an increase in hydrophilicity proportional to plasma exposure time. They also found that generally the treatment caused a reduction of the leaf phenolic content, although the plant matrix acted as a protection against oxidation of bioactive compounds by reactive species generated by plasma.

Baier et al. (2013) tested the antimicrobial efficacy and the physiological effect of non-thermal plasma treatment applied with different power intensities on lamb's lettuce. Results showed that the treatment can cause an inhibition of photosynthetic activity that became more severe and permanent with increased power settings. The impact on tissue physiology was attributed both to the thermal damage, particularly at the higher power, and to the stress brought by charged particles and/or reactive species generated by plasma treatment.

More recently, the effect of cold plasma on tomato peroxidase (Pankaj, Misra, & Cullen, 2013), on polyphenol oxidase and peroxidase in a model food system (Surowsky, Fischer, Schlueter, & Knorr, 2013), was evaluated. Although the results obtained in these studies underline the potential of cold plasma treatments for enzyme inactivation, further researches are needed to assess the effect on more complex systems such as fresh-cut fruit and vegetable.

Since the product temperature during the treatment is very close to the ambient one, this technique could be suitable for fresh-cut product processing, where temperature and pressure must be controlled in order to preserve the product quality.

Given that the application of cold gas plasma treatment on fresh-cut fruit and vegetable is in its infancy, in the present work we have pioneered the study of its effect on fresh-cut Pink Lady® apple quality and endogenous metabolic activity during controlled storage. Particular attention has been given to colour modification and PPO inhibition, considering that enzymatic browning is the most important phenomena limiting the product shelf-life.

2. Materials and methods

2.1. Raw material, handling and storage

Apples (*Malus domestica* cv. 'Pink Lady®') grown in the Emilia–Romagna region of Italy were harvested in November 2012. Fruits were stored at 2 ± 0.5 °C and approximately 100% RH in air, in plastic bins for 2 months. 20 kg of fruits free from defects was selected and transported to our laboratory and placed in a closed refrigerated chamber at 4 °C and saturated atmosphere in darkness for one week. When the experiments were performed, apples had a dry matter content of 15.73 g (± 0.29) 100 g fw^{-1}, a soluble solid content of 14.27 (± 0.35) °Brix and a titrable acidity of 0.39 mg (± 0.03) of malic acid g fw^{-1}.

2.2. The gas plasma generator

A Dielectric Barrier Discharge (DBD) generator was used for the treatment of Pink Lady® apple slices (Fig. 1). Within the low frequency plasma sources, the DBD configuration is one of the most investigated and industrialized non-equilibrium plasma generator. It presents numerous advantages in terms of flexibility of geometrical configurations (planar or cylindrical), operating parameters (medium, frequency and voltage), costs and characteristics of the power supply (Kogelschatz, 2003; Morgan, 2009).

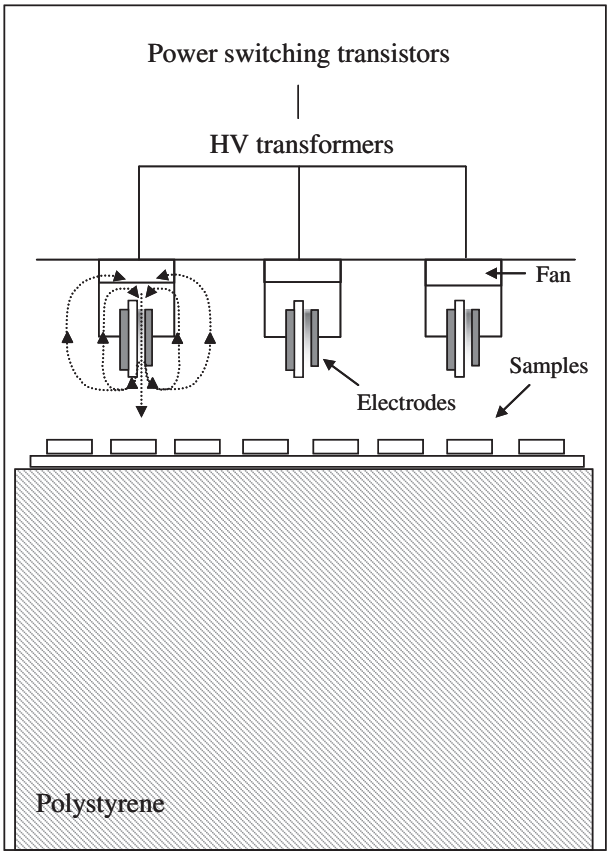

Fig. 1. Layout of the DBD gas plasma generator.

The used generator was characterized by three parallel pairs of electrodes made of brass (supplied by a DC power supply) and a glass (5 mm thick) was used as dielectric material. These electrodes, placed at the top of an hermetic chamber (70 dm^3 of maximum available volume), were powered by high voltage transformers and power switching transistors. A polystyrene parallelepiped was used to reduce the volume chamber (about 29 dm^3). The gas, generated using the atmospheric gas, was directed to the apple slice surface (15 slices for treatment, placed at about 9 cm from the electrodes), by means of three fans mounted over the electrodes. The measured air speed was about 1.5 m/s at the electrodes and 0.8 m/s at the apple surface. The total consumed power for the three electrodes is about 150 W.

The device was electrically and chemically characterized in previous studies (Ragni et al., 2010). According to these studies, a potential difference of 15 kV (peak-to-peak) can be measured at the electrodes with an input voltage of 19 V (fundamental frequency of oscillation: 12.7 kHz). The emission spectroscopy of the atmospheric plasma generated by the device showed the presence of OH and NO radicals and ions promoting plasma chemical reactions.

2.3. Gas plasma treatments and storage

Samples of 30 pieces (40 × 10 × 10 mm) of Pink Lady® apple tissue were prepared starting from about 8 apples. Each sample was randomly divided in two sub-samples of 15 pieces each, one for the treatment and the other for its control.

After preliminary tests aimed at finding treatment conditions that avoid evident damages of the fruit surface, treatments of 10 min (5 min for side), 20 min (10 min for side) and 30 min (15 min for side) were considered.

It was observed in previous studies that the emission of OH radicals can be increased by increasing the humidity of the air (Ragni et al., 2010). Gas plasma treatments were conducted at RH of about 60% (22 °C). This condition was selected on the basis of preliminary experiments, because an excess of water vapour (>80%) can decrease the gas plasma effectiveness (Muranyi, Wunderlich, & Heise, 2008). During treatments, each control sample was stored for the same time and at the same temperature (22 °C) and RH (60%) of the tested treatment.

2.4. Qualitative assessment of fresh-cut apples

2.4.1. Chemical parameters

Soluble solid content (SSC) was determined at 20 °C by measuring the refractive index with a digital refractometer mod. PR1 (Atago Co. Ltd, Tokyo, Japan) calibrated with distilled water.

Dry matter content of apple samples was determined gravimetrically by difference in weight before and after drying at 70 °C, until a constant weight was achieved (AOAC International, 2002).

Titratable acidity (TA) was determined by titration with NaOH 0.1 N until pH 8.1 was reached (AOAC Official Method 942.15, 2000), and expressed as mg of malic acid/100 g fw^{-1}.

For each sample, SSC and TA were determined in triplicate on the juice obtained from 10 apple slices, after filtering through Whatman #1 filter paper. SSC, dry matter and TA were determined immediately after treatment and after 24 h of storage in controlled conditions (10 °C, 90% RH).

2.4.2. Texture

Penetration tests were conducted by means of a Texture Analyser mod. TA-HDi500 (Stable Micro Systems, Surrey, UK) equipped with a 50 N load cell and a 6 mm diameter stainless steel cylinder. Compression test speed of 0.5 mm s^{-1} and a maximum deformation of 90% were respectively used.

For each treatment time 30 apple slices (15 controls and 15 treated) were respectively analysed after 0, 6 and 24 h of storage in controlled conditions (10 °C, 90% RH). From the analysis of the acquired curves, the following parameters were evaluated: Firmness F (N) as the first peak force value representing the limit of the flesh elasticity, the work required to rupture the flesh A_F (Ns) (from 0 to F), the gradient G between 0 and F, and the linear distance (LD) between F and the first 20 s (time required to attain a flesh deformation of about 85%) (Fig. 2). This last parameter was calculated according to the following equation:

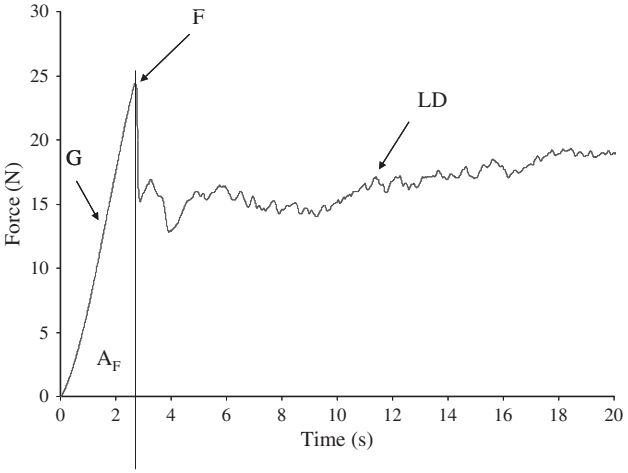

Fig. 2. Force (N) versus time (s) during a penetration test of a Pink Lady® apple slice. F: Firmness (N); A_F: work (Ns) required to rupture the flesh; G: gradient between 0 s and F; LD: linear distance between F and the firsts 20 s.

$$LD = \sum_{x=1}^{x=n} \sqrt{f[(x+1)-f(x)]^2 + [d(x+1)-ds(x)]^2}$$

where f is the force (N) and d is the distance (mm).

The software (Stable Micro Systems, v. 2.61) automatically calculated LD by summing the length of the straight segments connecting each acquired point between selected times. Since for a crunchy product a great fluctuation of the force with a corresponding high value of LD can be observed, this parameter can be considered a useful indicator of the product crunchiness (Gregson & Lee, 2003).

2.4.3. Browning extent using computer vision system (CVS)

Digitalized images of apple pieces were acquired by positioning the samples inside a black box under controlled lighting condition. A digital camera mod. D7000 (Nikon, Shinjuku, Japan) equipped with a 60 mm lens mod. AF-S micro, Nikkor (Nikon, Shinjuku, Japan) was used to acquire the images.

For each treatment time, acquisitions (exposition time 1/2 s; F-stop f/16) were conducted on samples of 20 apple slices each (10 for the treatment and 10 for the control) immediately after the treatment and after 1, 2 and 4 h of storage in controlled conditions (10 °C, 90% RH), in order to understand the treatment effect on the browning kinetic. Time intervals were chosen as the most representative of the browning kinetics of Pink Lady® apple slices, identified by preliminary tests.

Digitalized images were evaluated with an advanced Image Analysis Software (Image Pro-Plus v. 6.2, Media Cybernetics, USA) using RGB scale. Total and browned areas were selected and a colour model was set up according to Rocculi et al. (2004). Two different pixel ranges were identified on the basis of different chromatic characteristics, considered as 'not browned' and 'browned' area. The model was then applied to each digitalized image, and by evaluating all pixels, the percentage of each chromatic area was calculated by the software.

Table 1
Mean values of the measured chemical parameters.

Time of treatment	Time of storage	Sample	Chemical parameters					
			SSC (°Brix)		DM (%)		TA (mg malic acid/100 g)	
			$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
5 + 5 min	0 h	c	14.3[a]	0.11	15.9[a]	0.29	0.37[a]	0.00
		t	14.3[a]	0.15	16.1[a]	0.14	0.39[a]	0.01
	24 h	c	14.8[a]	0.19	18.1[a]	0.29	0.32[a]	0.00
		t	15.2[b]	0.10	17.0[b]	0.05	0.35[b]	0.00
10 + 10 min	0 h	c	14.6[a]	0.18	15.4[a]	0.64	0.42[a]	0.01
		t	15.4[b]	0.05	15.9[a]	0.43	0.42[a]	0.02
	24 h	c	14.7[a]	0.12	17.6[a]	0.78	0.31[a]	0.00
		t	15.4[b]	0.29	17.5[a]	0.65	0.33[a]	0.00
15 + 15 min	0 h	c	13.9[a]	0.12	15.9[a]	0.53	0.39[a]	0.00
		t	14.5[b]	0.29	16.0[a]	0.4	0.41[b]	0.00
	24 h	c	15.5[a]	0.26	17.2[a]	0.62	0.31[a]	0.01
		t	16.0[b]	0.15	16.8[a]	0.19	0.28[a]	0.01

SSC: soluble solid content (SSC). DM: dry matter. TA: titratable acidity. SD: standard deviations. c: control; t: treated. Differences between means with the same letter for the same storage time are not significant at a p-level < 0.05.

2.5. Metabolic determinations

2.5.1. Polyphenol oxidase activity (PPO)

2.5.1.1. Enzyme extraction. Enzyme extraction was carried out according to Baritaux, Amiot, Richard, and Nicolas (1991) with slight modifications. 50 g of sample was homogenised in 100 ml of cold McIlvaine's buffer solution at pH 7.5 containing 0.5% Triton X100, 25 mM ascorbic acid and 0.5% PVPP, using an Ultra-Turrax blender for 30 s. The homogenate was kept under agitation and in the dark at 0 °C for 15 min and then centrifuged for 30 min at 4 °C and 25,000 *g*. The supernatant was filtered and used as extract.

2.5.1.2. Enzyme assay. 4-Methylcatechol 50 mM prepared in McIlvaine's buffer solution at pH 7.5 was used as substrate. PPO activity, measured just after gas-plasma treatment, was determined reading the adsorbance at 420 nm and 25 °C and calculated on the basis of the slope of the linear portion of the curve (ΔA/min).

2.5.2. Respiration rate

Respiration rate was assessed on control and treated apple slices, using a static method. Six cylinders (6 × 10 mm) were sampled from the outer pericarp tissue of each apple piece and subjected to plasma treatments. Controls were obtained by placing cylinders at the same temperature and relative humidity characterising the treatment chamber. Control and treated cylinders were then placed in 20 ml glass ampoules and sealed with a Teflon coated rubber seals and an aluminium crimp cap. O_2 and CO_2 percentage of triplicate specimens was measured in the ampoule headspace by a gas analyzer "check point O_2/CO_2" mod. MFA III S/L (Witt-Gasetechnik, Witten, Germany) after 1, 2, 4, 6 and 24 h at 10 °C from preparation. Respiration rate was calculated as mg of consumed

Table 2
Mean values of the mechanical parameters calculated from penetration tests.

Time of treatment	Time of storage	Sample	F (N)		A_F (Ns)		G (N/s)		LD	
			$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
5 + 5 min	0 h	c	18.9[a]	4.0	22.0[a]	7.8	7.6[a]	1.8	66.1[a]	15.1
		t	21.8[b]	5.6	32.2[b]	10.9	7.0	1.8	61.1[a]	13.2
	6 h	c	21.5[a]	2.4	27.6[a]	6.5	7.7[a]	1.4	57.9[a]	8.1
		t	22.2[a]	5.3	28.9[a]	14.7	8.1[a]	1.5	60.4[a]	8.2
	24 h	c	23.3[a]	4.6	38.6[a]	13.0	6.5[a]	1.3	48.9[a]	7.9
		t	23.9[a]	5.6	45.3[a]	15.0	5.5[b]	1.2	44.1[b]	7.8
10 + 10 min	0 h	c	19.6[a]	3.7	25.4[a]	7.4	7.2[a]	1.7	62.8[a]	11.2
		t	22.4[b]	3.6	36.5[b]	11.2	6.3[b]	1.1	56.0[a]	14.2
	6 h	c	25.7[a]	4.1	38.4[a]	12.6	8.1[a]	1.9	52.6[a]	8.8
		t	27.6[a]	7.6	48.5[b]	19.9	7.3[a]	1.8	51.4[a]	8.1
	24 h	c	24.3[a]	4.8	43.4[a]	14.9	6.3[a]	1.0	49.1[a]	11.4
		t	25.1[a]	4.4	46.3[a]	11.0	6.2[a]	1.4	44.7[a]	9.3
15 + 15 min	0 h	c	19.3[a]	3.6	25.0[a]	11.6	7.3[a]	1.8	64.9[a]	10.3
		t	21.3[a]	6.1	30.3[a]	10.7	6.7[a]	1.7	57.9[a]	9.7
	6 h	c	26.0[a]	4.4	42.0[a]	18.5	7.6[a]	1.2	57.5[a]	7.0
		t	24.5[a]	3.3	41.3[a]	9.5	6.4[b]	1.0	54.0[b]	6.2
	24 h	c	26.4[a]	7.9	49.4[a]	20.5	6.5[a]	2.3	49.1[a]	10.1
		t	25.1[a]	5.3	47.2[a]	15.4	5.9[a]	1.3	45.8[a]	7.9

F (N), A_F (Ns), G, and LD, see Fig. 2. SD: standard deviations. c: control; t: treated. Differences between means with the same letter are not significant at p-level < 0.05.

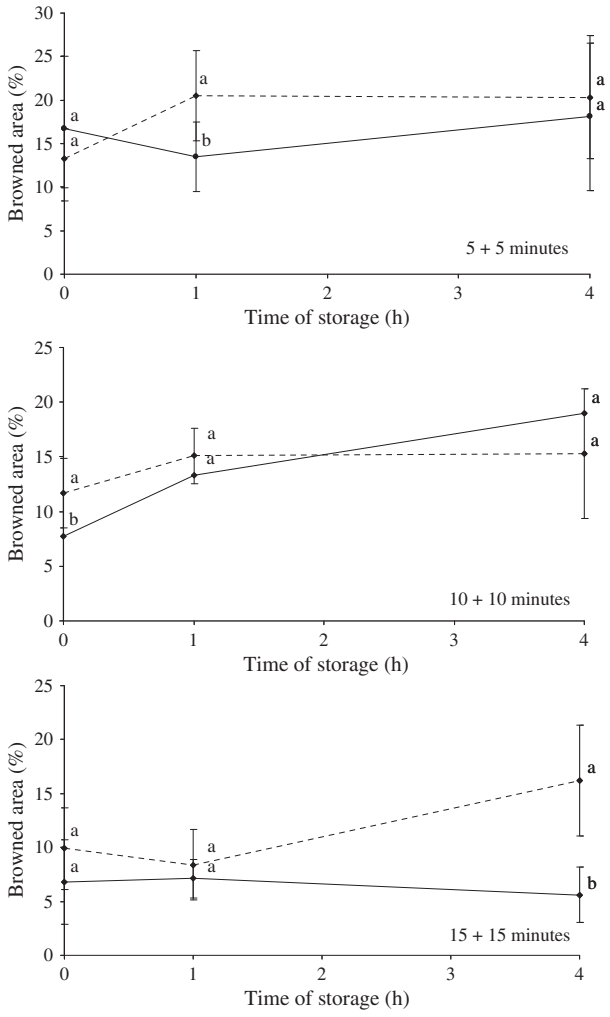

Fig. 3. Mean values of percentage of browning area of apples during 4 h of storage in controlled conditions (10 °C; 90% RH). --- control; __ treated. Differences between means with the same letter are not significant at p-level <0.05 (within the same time of storage).

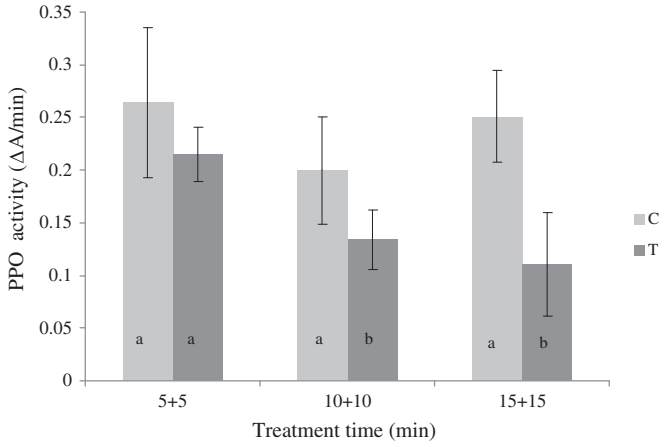

Fig. 4. PPO activity (ΔA/min) of apple samples treated with plasma gas compared to the controls. Differences between means with the same letter are not significant at p-level <0.05.

O_2 (RRO_2) or produced CO_2 ($RRCO_2$) $h^{-1}\,kg\,fw^{-1}$ according to the following equations:

$$RRO_2 = \frac{mm_{O_2} \cdot V_{head} \cdot \frac{\left(20.8 - \%O_{2,head}\right)}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283}$$

$$RRCO_2 = \frac{mm_{CO_2} \cdot V_{head} \cdot \frac{\%CO_{2,head}}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283}$$

where mm_{O2} and mm_{CO2} refer to gas molar mass (g/mol), V_{head} represents the ampoule headspace volume (dm^3), % $O_{2,head}$ and % $CO_{2,head}$ refer to gas percentages in the ampoule headspace at time t (h); m is the sample mass (kg); and R is the gas constant ($8.314472\,dm^3\,kPa\,K^{-1}\,mol^{-1}$).

2.5.3. Metabolic heat by isothermal calorimetry

Rocculi et al. (2012) studied the use of isothermal calorimetry to monitor metabolic responses of different fresh-cut fruit tissues subjected to different processing and storage conditions. The measurements allowed the heat production determination of endogenous (tissue metabolism) biological process. In order to measure the effect of gas plasma treatment on metabolic heat production of apple tissue, the same technique was applied on apple sample cylinders.

From each apple slice, six cylinders were sampled using a core borer and subjected to the different plasma treatments. Controls were obtained by placing cylinders at the same temperature and relative humidity characterising the treatment chamber. Control and treated cylinders were then placed in 20 ml glass ampoule (six cylinders for ampoule, about 2.5 g) and sealed with a teflon coated rubber seals and an aluminium crimp cap. For each control/treatment condition, four replicates were analysed. A TAM-Air isothermal calorimeter (Thermometric AB, Järfälla, Sweden) was used to measure the rate of metabolic heat production. This instrument is characterised by a sensitivity (precision) of ±10 μW (Wadsö & Gómez-Galindo, 2009) and contains eight twin calorimeters in which eight sample ampoules can be inserted, each one with its own reference. The instrument measures the difference between the sample and the reference signal. Reference has to be chosen as a material not producing heat, but with heat capacity similar to the sample analysed. In this case water was chosen as reference material and the amount to be placed in each ampoule (Mw) was determined as:

$$M_W = \frac{C_{ST} \times M_{ST} + C_W \times M_W}{C_W}$$

where C_{ST} is the specific heat of dry matter assumed to be $1\,J\,g^{-1}\,K^{-1}$, M_{ST} is the dry matter content and M_W is the water content of the apple sample; C_W is the water specific heat ($\sim 4.186\,g^{-1}\,K^{-1}$). Isothermal measurements were performed at 10 °C for 24 h. Before and after the measurements, baseline was recorded for 30 min.

2.6. Data analysis

Significant differences ($P < 0.05$) between control and treated mean values were found by using Student's *t*-test and the Analysis of Variance (ANOVA) according to Tukey's HSD. Mann–Whitney test was used in the case of significance of the Levene test (SPSS 13.0 for Windows, IBM SPSS Statistics).

3. Results and discussion

3.1. Qualitative assessment of fresh-cut apples

3.1.1. Chemical parameters

Mean values of the soluble solid content (SSC), dry matter content (DM), and titratable acidity (TA) are shown in Table 1.

Table 3
Mean values of the respiration rate during storage for 24 h in controlled conditions (10 °C; 90% RH).

Time of treatment	Time of storage	Sample	Respiration rate (mg/h kg of sample)			
			$RRO_{2(consumed)}$		$RRCO_{2(produced)}$	
			$\bar{x}$	SD	$\bar{x}$	SD
5 + 5 min	1 h	c	44.37[a]	1.95	43.92[a]	1.59
		t	37.10[b]	1.25	36.03[b]	2.69
	2 h	c	22.13[a]	2.71	22.69[a]	1.20
		t	23.82[a]	1.21	17.15[b]	0.56
	4 h	c	16.41[a]	1.33	13.62[a]	0.32
		t	18.76[a]	1.30	12.26[a]	1.29
	6 h	c	16.52[a]	2.07	12.42[a]	0.67
		t	18.70[a]	0.73	12.80[a]	0.78
	24 h	c	13.70[a]	0.16	12.78[a]	0.62
		t	15.75[b]	0.28	11.38[b]	0.33
10 + 10 min	1 h	c	43.92[a]	1.59	36.24[a]	1.31
		t	36.03[b]	2.69	53.4[a]	4.44
	2 h	c	22.69[a]	1.20	31.2[a]	1.66
		t	17.15[b]	0.56	35.37[b]	1.15
	4 h	c	13.62[a]	0.32	28.09[a]	0.66
		t	12.26[a]	1.29	28.75[a]	1.85
	6 h	c	12.42[a]	0.67	23.24[a]	0.87
		t	12.80[a]	0.78	24.38[a]	0.60
	24 h	c	12.78[a]	0.62	18.61[a]	0.41
		t	11.38[b]	0.33	16.95[a]	0.98
15 + 15 min	1 h	c	50.89[a]	4.69	40.96[a]	4.43
		t	44[a]	1.05	48.39[b]	1.16
	2 h	c	23.92[a]	1.59	35.01[a]	3.24
		t	23.19[a]	2.06	33.85[a]	2.24
	4 h	c	14.24[a]	1.43	30.65[a]	1.77
		t	11.62[a]	1.23	27.98[a]	1.88
	6 h	c	12.94[a]	1.55	24.89[a]	0.68
		t	11.22[a]	0.87	23.22[a]	3.74
	24 h	c	13.47[a]	0.63	19.21[a]	1.25
		t	11.05[b]	0.42	16.30[b]	0.65

RRO_2: mg of consumed $O_2\ h^{-1}\ kg\ fw^{-1}$. $RRCO_2$: mg of produced $CO_2\ h^{-1}\ kg\ fw^{-1}$. SD: standard deviations. c: control; t: treated. Differences between means with the same letter are not significant at p-level < 0.05.

Slight significant differences emerged between control and treated sample means for all the parameters, but not for all the treatments and storage times. In terms of SSC, limited increments in the treated sample values (from 0.4 to 0.8 °Brix) were observed with respect to the control ones, except that immediately after the 5 + 5 treatment. For DM, a slight significant difference was found between control and sample means only after 24 h of storage for 5 + 5 min sample, while in terms of TA the only significant differences were found after 24 h for 5 + 5 min and immediately after 15 + 15 min.

Generally, obtained results evidenced that the response of apple tissue in terms of modification of the measured chemical parameters (often used to describe the ripening evolution of fruit) was very limited, and mainly represented by a slight increase of SSC. In this direction, the physiological stress promoted by the tested plasma treatment could have slightly increased the conversion rate of starch to glucose in the apple tissue, influencing significantly neither the water content nor the titrable acidity value.

3.1.2. Texture

Table 2 summarizes the results of the uniaxial compression tests conducted on apple pieces during 24 h of storage in controlled conditions (20 °C, 90% RH).

In terms of firmness (F) and work required to the flesh rupture (A_F), significant differences emerged between mean values of the control and treated samples for the treatment time 5 + 5 min and 10 + 10 min. Treated sample values were higher than the control ones immediately after the treatment (for F and A_F parameters) and after 6 h of storage (only for A_F). Generally the plasma treatment seemed to trendily increase the sample firmness. On the contrary one of the problems of the traditional anti-browning dipping treatment usually adopted for fresh-cut apple production (based on ascorbic and citric acid or their salts) is the consequent softening of the tissue (Gil, Gorny, & Kader, 1998; Rocculi et al., 2004) and the addition of anti-softening agents such as calcium salts is often unavoidable (Garcia & Barrett, 2002; Rocculi et al., 2004).

Gradient (G) and linear distance (LD) results showed sparse significant lower values for treated samples, compared with the control. Particularly this happened after 24 h of storage following a treatment of 5 + 5 min (for both G and LD), immediately after 10 + 10 min of treatment (only for G) and immediately after 15 + 15 min of treatment (only for LD) and after further 6 h of storage (for both G and LD). Trendily it seems that the gas plasma treatment has reduced the 'crunchiness' of the apple slices (LD and G reduction). From a visual examination, the treated samples seemed covered by a sort of bio-film, probably generated by the destruction of superficial cells promoted by gas-plasma oxidant radicals; this micro-structural alteration could be the cause of the detected LD and G modifications.

3.1.3. Browning extent using computer vision system (CVS)

The results of the image analysis conducted on apple slices are reported in Fig. 3. Mean values of the percentages of not browned and browned areas are shown according to the storage duration for both

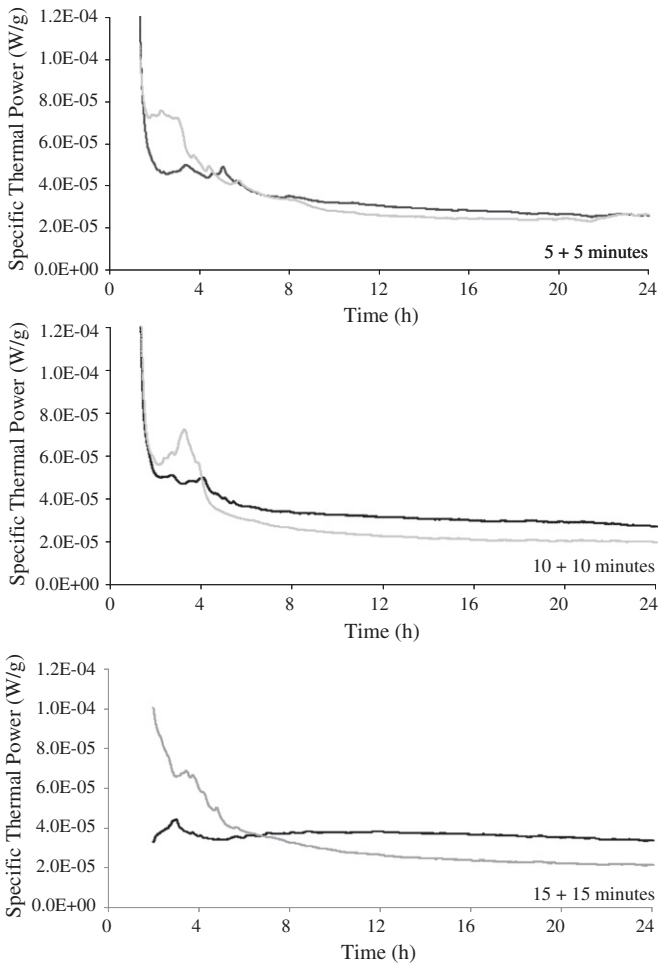

Fig. 5. Specific thermal power profiles of apple tissue cylinders during 24 h of analysis at 10 °C (isothermal calorimetry). Black line: control sample. Grey line: treated sample.

control and treated sample. Significant differences were observed between means for all three times of treatments. In terms of browned area, highest values were measured for the control samples. For the plasma treatment 15 + 15 min and after 4 h of controlled storage (10 °C; 90% RH), control samples showed, with respect to the treated ones, a significant increase in the browned area of about the 65%, while the treated sample seemed to maintain the initial level until the end of the experiment. It is important to underline that Pink Lady® is one the most used apple by industry for fresh-cut production, because of its low susceptibility to browning after minimal processing. However, the gas-plasma treatment seemed to affect apple colour degradation through the inhibition of enzymatic browning, also if the phenomenon was not as intense as for other varieties (Rößle, Gormley, Brunton, & Butler, 2011).

3.2. Metabolic evaluation

3.2.1. PPO activity

The inactivation of fruit and vegetable endogenous enzymes is one of the most important aims of food preservation operations. Recently, for the first time, Pankaj et al. (2013), demonstrated the applicability of in-package cold plasma for the inactivation of vegetable enzymes, particularly of tomato peroxidase that was taken as a model enzyme. In that work, the enzyme activity was found to decrease with both treatment time and voltage, the former variable exhibiting a more pronounced effect. Furthermore, Surowsky et al. (2013) found that treatment time greatly affected inactivation of polyphenol oxidase and peroxidase enzymes in a model food system and observed a two-stage reduction of activity that lead to an irreversible although incomplete inactivation.

Fig. 4 shows the results of the PPO activities (ΔA/min) of treated apple samples, each one compared to its control.

By increasing the treatment time, a significant and roughly linear decrease in sample PPO activities was measured. Taking as 100% the PPO activity of each specific control sample (fresh tissue), treated sample residual activities were about 88, 68 and 42%, respectively for 5 + 5, 10 + 10 and 15 + 15 min treatment times. Takai, Kitano, Kuwabara, and Shiraki (2012) used low frequency helium plasma jet system for the treatment of egg white lysozyme attributing the decrease in the enzyme activity to a change in the secondary protein structure and modification of some amino acid side chains of the enzyme. The study of the effect of atmospheric pressure glow discharge plasma on bovine serum albumin performed by Deng, Shi, Chen, and Kong (2007) confirmed the degradation of protein integrity upon plasma treatment. Surowsky et al. (2013) investigated the relative amounts of secondary structure fractions in PPO and POD enzymes, before and after plasma treatments, through circular dichroism spectroscopy and found a significant decrease in the amount of alpha-helix structures that was strongly correlated to the loss of enzymatic activity. Protein structural modifications were further confirmed by changes in tryptophan emission fluorescence measurements that were affected by plasma exposure times.

Takai et al. (2012) suggested a mechanism of reaction between plasma generated reactive species and proteins to explain the inhibitory effect of cold plasma on tomato peroxidase that was further reported by Pankaj et al. (2013) and in accordance with Surowsky et al. (2013). In particular they hypothesised that OH, O_2^-, HOO and NO radicals induced chemical modifications of chemically reactive side-chain of the amino acids, such as cysteine, aromatic rings of phenylalanine, tyrosine, and tryptophan, that consequently lead to a loss of enzyme activity. A similar mechanism for decomposition of C–H, C–N and N–H bonds of protein was also described by Hayashi, Kawaguchi, and Liu (2009). In this direction the characterization of the atmospheric plasma generated by the device used in this study by using emission spectroscopy revealed the presence of OH, NO and other reactive radical species (Ragni et al., 2010). As just mentioned, the observed decrease of PPO activity in the treated apple samples was probably due to the action on enzyme amino acid structure.

3.2.2. Respiration rate

The oxygen level within the ampoules after 24 h at 10 °C was in the range of 16.7 and 17.6% in the control and in the range of 16.6 and 18.2% in the treated samples.

Mean values of the respiration rate during a storage of 24 h in controlled conditions are reported in Table 3. In general and when significant, lower values in terms of mg of consumed O_2 (RRO_2) were obtained for the treated sample respect to the control one. These differences were generally observed for all the three treatments, particularly after 24 h of storage. The inhibition of respiration in terms of consumed O_2 did not always correspond to a significant decrease of CO_2 production ($RRCO_2$).

Actually, while 5 + 5 min sample showed lower values than the control, CO_2 production of samples 10 + 10 min and 15 + 15 min was higher respectively after 1 and 2 h from experiments. This contrasting behaviour could be caused by an instantaneous response of the vegetable tissue to the chemical stress promoted by the treatment, that macroscopically promoted an increase of CO_2 production. The results obtained in this study suggest that the plasma treatment can cause an alteration of the cellular respiratory pathway; it seems therefore to be a more complex response if compared to traditional anti-browning thermal treatments, such as blanching, that promote a complete inactivation of cell metabolic activity.

3.2.3. Metabolic heat by isothermal calorimetry

Specific thermal power profiles of apple tissue cylinders during 24 h of analysis at 10 °C are reported in Fig. 5.

All the thermograms of treated samples showed during the first 6 h of analysis an irregular and intense heat production profile compared with the control, as there was a physiological response of the tissue to the chemical stress promoted by the gas plasma treatment. After this period, the heat production of the treated samples was lower than the controls for all the 24 h of analysis, proportionally to the treatment time. The decrease of the heat production from vegetable tissue can be attributed to a lower extent of cell vitality promoted by processing operations; this finding has been previously showed as a consequence of blanching for fresh carrot (Gómez, Toledo, Wadsö, Gekas, & Sjöholm, 2004) and of osmo-dehydration for kiwifruit pericarp tissue (Panarese et al., 2012).

4. Concluding remarks and future perspectives

In this study we have pioneered the use of gas plasma for the treatment of fresh-cut apples.

Promising results have been obtained regarding enzymatic browning inhibition and a specific effect on the reduction of polyphenol oxidase activity on the apple slices has been found.

The potential application on in-packed cold plasma technology showed by Pankaj et al. (2013), together with its known effect on microbiological decontamination of foods (Misra et al., 2011; Niemira, 2012) makes this technique very encouraging for fresh-cut apple stabilization.

However several very important aspects have to be clarified in order to deeply understand gas plasma effect on fresh-cut apple quality and on the metabolic response of the tissue.

First of all, considering the high oxidative action of gas plasma treatment, its effect on the bioactive compounds of the fruit tissue has to be deeply studied in order to highlight the nature of gas plasma effect on biochemical tissue response. In addition the sensorial impact of the treatment has to be assessed. Both aspects have to be investigated in terms of direct effect (immediately after the treatments) and further stress response in real storage conditions (e.g. modified atmosphere packaging, refrigeration).

Moreover the metabolic response to the treatment of the fresh apple tissue evaluated by calo-respirometric measurements has to be clarified, eventually with the aid of ultra- and micro-structural techniques to assess the cellular compartment modifications of the apple tissue, verifying whether apple cell wall undergoes degradation during the treatment (Panarese et al., 2012); this could give interesting indications also about the treatment effect on macroscopic textural aspects.

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Paper IV

Effect of cold plasma treatment on physico-chemical parameters and antioxidant activity of minimally processed kiwifruit

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Effect of cold plasma treatment on physico-chemical parameters and antioxidant activity of minimally processed kiwifruit

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ABSTRACT

The efficacy of atmospheric plasma treatment on microbial decontamination of different kinds of food products is already known. Recently, new applications of this innovative technology have been proposed, in order to test the improvement of quality maintenance of minimally processed fruit and vegetables. Nevertheless, the knowledge on the modifications of functional and nutritional properties of minimally processed fruit is still scarce.

The objective of this study was to evaluate the effect of atmospheric double barrier discharge (DBD) plasma treatment on the quality maintenance of fresh-cut kiwifruit. Treatments of 10 and 20 min per side were performed and their consequences were evaluated during four days of storage in controlled conditions by monitoring parameters related to visual quality, texture, chlorophyll, carotenoids and polyphenols. The in vitro antioxidant activity was evaluated through a multimodal approach, combining different assays for the analysis of antiradical activity and reducing activity of antioxidants. According to the obtained results, plasma treatments positively influenced the quality maintenance of the product, by improving colour retention and reducing the darkened area formation during storage, not inducing any textural change compared with the control. Plasma treatments caused an immediate slight loss of pigments, but a better retention during storage. No significant changes in antioxidants content and antioxidant activity were observed among treated samples and control ones.

1. Introduction

Among the known pre-treatments for extending storage life of minimally processed kiwifruit, some of the most used (in order of increasing intensity degree) include: dipping in calcium salt solutions, osmotic-dehydration, edible coating, tissue vacuum impregnation with glucose and hot water dipping (Muntada et al., 1998; Agar et al., 1999; Dalla Rosa et al., 2011; Tylewicz et al., 2011; Benítez et al., 2013). In terms of technological and economical aspects, these techniques present some disadvantages related to the necessity of dipping the product in aqueous solution (e.g. plant and consumables costs, disposal of exhausted solutions, labelling of chemical agents, further drying). In addition, these processing techniques are principally aimed at maintaining colour and texture (Agar et al., 1999) and slowing down deteriorative phenomena induced by tissue wounding response, but not at sanitising the product.

Cold plasma is an ionised gas characterised by active particles such as electrons, ions, free radicals and atoms that is produced by applying energy to a gas or a gas mixture. Operative and configuration conditions of the atmospheric plasma generators and the assessment of the efficacy of the ionised gas on microbial inactivation were extensively reviewed (Moreau et al., 2008). The oxidative species produced during the discharge (reactive oxygen and nitrogen species) can cause peroxidation of lipids and oxidation of proteins and DNA (Montie et al., 2000).

Since the decontamination can be carried out in atmospheric conditions, the treatment was tested in terms of the efficacy of surface decontamination on different kinds of foods such as fruits (Berardinelli et al., 2012; Baier et al., 2014), vegetables (Keener et al., 2012; Baier et al., 2014), almonds (Deng et al., 2005), nuts (Basaran et al., 2008), grains and legumes (Selcuk et al., 2008), shell

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eggs (Ragni et al., 2010), hatching eggs (Pasquali et al., 2010) and meat (Noriega et al., 2011).

Recently, new applications of cold plasma have been proposed in the food processing sector. The oxidative power of plasma was tested in order to preserve the qualitative characteristics of fresh-cut fruit and vegetables. These applications were mainly addressed to the reduction of the activity of oxidative enzymes such as polyphenol oxidase in fresh-cut apples (Tappi et al., 2014; Bußler et al., 2013), peroxidase in tomatoes (Pankaj et al., 2013) and polyphenol oxidase and peroxidase in a model food system (Surowsky et al., 2013).

All the cited works evidenced a significant effect on the activity of degradative enzymes, in some cases with a consequent improvement of the product visual quality, as documented for fresh-cut Pink Lady apples (Tappi et al., 2014).

Other interesting works regard the effect of cold plasma on the total phenolic content of lamb's lettuce (Grzegorzewski et al., 2010, 2011) and on the physiological behaviour of green plant tissue (Baier et al., 2013, 2014). According to the aforementioned authors, plasma exposure leads to a detrimental effect on tissue photosynthetic efficiency, erosion phenomena of the leaves upper epidermis and a time and structure-dependent degradation of phenolic compounds, although the plant matrix seems to protect them from the oxidation caused by interaction with plasma-induced reactive species. In theory, the intense oxidative power of plasma treatment, due to the presence of $OH^{\bullet}$, $NO^{\bullet}$ and other reactive radical species (Ragni et al., 2010), could also promote the oxidation of the bioactive compounds contained in fruit tissue, such as vitamin C and polyphenols, with a consequent decrease of the antioxidant properties of fresh fruit. Wang et al. (2012) found a slight decrease in the vitamin C content of different sliced vegetables subjected to microjet plasma treatment, probably due to oxidation, but within a range considered acceptable. The knowledge of the modifications on the functional and nutritional properties of minimally processed fruit is still scarce, and while there are some reports on the effect of plasma on the antioxidant content and activity of whole fruit (Berardinelli et al., 2012), the effect on fresh-cut fruit is largely unknown.

Among minimally processed fruit, kiwifruit is a very interesting product from a nutritional point of view because it contains high levels of vitamin C, vitamin E, flavonoids, minerals (Du et al., 2009) and significant amounts of pigments, including chlorophyll and carotenoids (Tavarini et al., 2008). Vitamin C is considered the major antioxidant compound in kiwifruit, contributing to about 40% of the total antioxidant activity (Tavarini et al., 2008), but also phenolic compounds have been reported to have a role in the antioxidant properties (Du et al., 2009). The consumption of kiwifruit has been recognised to have a preventative effect against different cancers due to its cytotoxic and antioxidant activities (Collins et al., 2001; Rush et al., 2002).

This study represents an early attempt to test the effect of double barrier discharge (DBD) cold plasma treatment on the quality of minimally processed kiwifruit and on its potential effect on health properties. In particular we monitored physical parameters related to visual quality (colour and darkened area), texture and standard chemical parameters. The chlorophyll, carotenoids, ascorbic acid and polyphenols content was also measured. The in vitro antioxidant activity was quantified by different in vitro assays: the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay, the 2,2-diphenyl-1-picrilhydrazyl ($DPPH^{\bullet}$) radical scavenging assay, and the ferric reducing antioxidant power (FRAP). The same parameters were tested during storage in controlled conditions.

2. Materials and methods

2.1. Raw material, handling and storage

Kiwifruits (Actinidia deliciosa cv. Hayward) were harvested at the beginning of November 2012 (Emilia Romagna region, Italy) and stored in a bin in a refrigerated room for one month (from −1 °C to 1 °C, R.H. 98%). After this period, defect-free fruits of uniform size were selected and transported to the laboratory, where they were stored for a further 15 d at 4 °C and R.H. 98% before being treated. At the time of sample preparation, the raw material had a soluble solid content of 13.5 ± 0.77%, titrable acidity

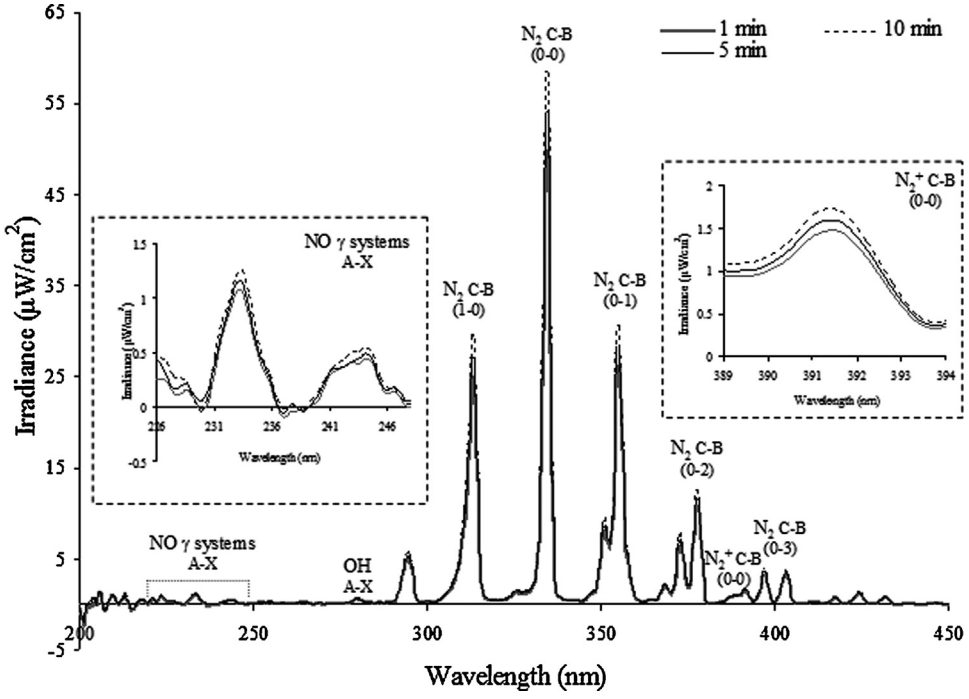

Fig. 1. Emission spectra of the discharge acquired during 10 min of treatment (15 kV peak-to-peak, at about 22 °C and 60% of R.H.).

of 1.47 ± 0.07 g of citric acid kg^{-1} and a dry matter content of $149\,g \pm 5.8\,kg^{-1}$ on a fresh weight basis.

Analytical grade chemicals were purchased from Sigma–Aldrich (Steinheim, Germany). Hydrochloric acid and methanol were purchased from Romil (Feltham, UK).

2.2. DBD gas plasma generator

DBD cold plasma treatments were run at atmospheric conditions inside a cabinet described in previous studies (Ragni et al., 2010; Berardinelli et al., 2012). The ionised gas was produced using atmospheric gas (according to a DBD configuration) between three couples of parallel plate electrodes, made of brass (one electrode of each couple was covered by a 5 mm thick glass sheet as dielectric material). The electrodes were powered by a DC power supply whose voltage can be varied from 2 to 19 V. As described in the cited studies, a potential difference of 15 kV (peak-to-peak) was measured at the electrodes with an input voltage of 19 V (the level chosen for the treatment). The emission in the 200–450 nm wavelength range (at approximately 22 °C and 60% of R.H.) was chemically characterised by an optic fibre probe (Avantes, FC-UV400-2) placed at 20 mm from the discharge and connected to a spectrometer (Avantes, AvaSpec-2048). Preliminary assessments showed that the presence of the fruit does not affect the emission during the duration of the treatments considered in this study.

The emission spectra acquired after 1, 5 and 10 min of treatment are shown in Fig. 1. Probably due to a thermal stabilisation of the system (electrical components, electrodes, etc.), the measured irradiance increases by increasing the min of treatment. After about 10 min, a stabilization of the irradiance values can be observed.

In terms of emission peaks, those related to the neutral nitrogen molecules N_2 (290–440 nm) and to the positive ion N_2^+ (391.4 nm) were dominant, as can be commonly detected for atmospheric air discharge. The emission peaks of OH (305–309 nm) and NO (226–248 nm) radicals were also observed.

2.3. Sample treatment and storage

Kiwifruit slices were placed under the electrodes (at a distance of 70 mm) and the discharge was directed on the surface of the fruit by three fans mounted over the electrodes (fan speed: 1.5 m/s at the base of the electrodes) as shown in Fig. 2.

After preliminary tests aimed at avoiding evident surface damage, two treatment times were chosen: 20 min (10 + 10 min for each side) and 40 min (20 + 20 min for each side). Atmospheric conditions (approximately 22 °C and 60% R.H.) were defined according to previous experiences which showed that OH radicals increase by increasing the air humidity level (Ragni et al., 2010).

Each treatment (10 + 10 and 20 + 20) was repeated in triplicate and for each replication 30 slices from 10 different kiwifruit were used. To minimise differences due to natural variability, kiwifruit slices were randomly divided into two sub-samples of 15 treated and 15 control samples. Control samples were stored at the same temperature and R.H. conditions for the duration of the treatment.

Kiwifruit slices were stored for four days in controlled and constant conditions; in particular, temperature and relative humidity were respectively 10 °C and 95% in order to simulate an accelerated storage. The storage temperature was chosen in order to simulate an accelerated storage that requires the use of a temperature higher than the optimal one, but, at the same time, in order to avoid the change of the typology of degradative reactions, a temperature close to the real storage condition in the supermarket was chosen.

Furthermore, to avoid interaction with packaging variables such as passive modified atmosphere, samples were placed on trays and stored unpacked. Colour, visual quality by image analysis and texture were assessed for each treatment immediately after the plasma exposition and after one and four days of storage.

To analyse chlorophyll and carotenoids content, samples were freeze-dried after treatment and after four days of storage. They were subsequently ground under liquid nitrogen and stored at −18 °C for two weeks.

Antioxidants content and antioxidant activity were determined only on samples subjected to the longest treatment and their relative controls after freeze-drying.

2.4. Qualitative assessment

2.4.1. Visual quality

2.4.1.1. Colour. Surface colour was measured on the kiwifruit pericarp with a Chroma Meter CR-400 reflectance colorimeter (Minolta Italia, Milano, Italy) using the D65 illuminant and the 10° standard observer. For each slice, an average value of three measurements performed in three different points was calculated. The L^*,a^* and b^* parameters of the CIELAB system were measured, a^* and b^* parameters were further used to calculate Hue angle (Eq. (1)) and chroma (Eq. (2)) values (C.I.E., 1987).

$$h^\circ = \left(\frac{\arctan[b^*/a^*]}{2\pi}\right) \times 360 \quad (1)$$

$$\text{Chroma} = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

2.4.1.2. Darkened area by computer vision system (CVS). A digital camera mod. D7000 (Nikon, Shinjuku, Japan) equipped with a 60 mm lens AF-S micro, Nikkor (Nikon, Shinjuku, Japan) was used to acquire digitalised images of kiwifruit slices (exposition time 0.5 s; F-stop f/16) placed inside a black box under controlled lighting condition.

Images (RGB scale) were processed with Photoshop (Adobe Photoshop, 8.0) in order to separate the pericarp area from the core tissue. These two portions were separately analysed with an

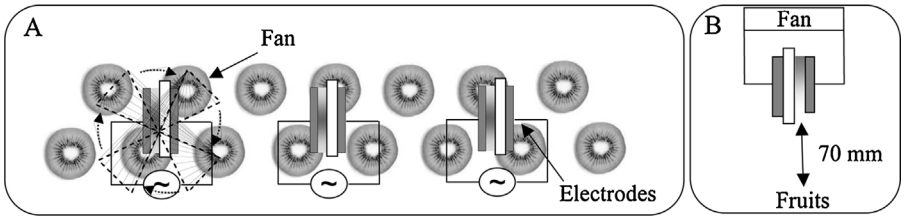

Fig. 2. Top view of the electrodes and of the kiwifruit slices placed under the discharge.

advanced Image Analysis Software (Image Pro-Plus v. 6.2, Media Cybernetics, USA), according to a chromatic model set up by Rocculi et al. (2005). After conversion in grey scale (8 BPP), on the basis of the chromatic characteristics, two different pixel ranges were defined corresponding to "darkened" (0-177 BPP for pericarp and 0-215 BPP for the core) and "not darkened" (178-253 BPP 216-153 BPP for the core) areas. All pixels were then evaluated by the model in terms of percentage of darkened area on the total.

2.4.2. Texture

Penetration tests were run using a Texture Analyser TA-HDi500 (Stable Micro Systems, Surrey, UK) equipped with a 50 N load cell and a 6 mm diameter stainless steel cylinder by setting the test speed at 0.5 mm s^{-1} and the maximum deformation at 90%.

For each slice, penetration tests were carried out in two different points of the pericarp. The acquired curves (Force, N, versus time, s) were analysed and the following mechanical parameters were extracted: firmness, the first peak force (N) value representing the limit of the flesh elasticity (F), work (mJ) required to rupture the flesh from 0 s to F (AF), and gradient (N mm^{-1}) between 0 s and F (G).

2.4.3. Chemical parameters

Soluble solids content (SSC) was determined at 20 °C by measuring the refractive index with a digital refractometer mod. PR1 (Atago Co., Ltd., Tokyo, Japan) calibrated with distilled water.

Dry matter content was determined gravimetrically by difference in weight before and after drying at 70 °C, until a constant weight was achieved (AOAC International, 2002).

Titratable acidity (TA) was determined by titration with NaOH 0.1 N until pH 8.1 was reached (AOAC International, 2000), and expressed as mg of citric acid kg^{-1} on a fresh weight basis.

For each sample, SSC and TA were determined in triplicate on the juice obtained by crushing 10 kiwifruit slices with a food processor, after filtering through Whatman #1 filter paper. SSC, dry matter and TA were determined immediately after treatment and after 24 h of storage in controlled conditions (10 °C, 90% R.H.).

Electrolyte leakage (EL) was measured as described by Rolny et al. (2011) with small modifications. Briefly, one slice of kiwifruit (approx 10 g) was floated on 100 mL of deionised water. The electrolyte content in the solution was measured immediately (C_0) and after 3 h (C_3) of incubation under continuous shaking at room temperature using a conductometer (Crison Instrument, Barcelona, Spain). The flasks were then put in an oven at 100 °C for 1 h. After cooling to room temperature, total conductivity (TC) was then measured again. Results were expressed as percentage of electrolyte leakage according to Eq. (3):

$$\%\mathrm{EL} = 100 \times \frac{(C_3 - C_0)}{\mathrm{TC}} \quad (3)$$

Electrolyte leakage was measured in order to assess a possible cell membrane damage, as an increased value of this parameter indicates disruption of the plasma membrane.

2.4.4. Chlorophyll and carotenoids content

0.5 g of freeze-dried sample were extracted for 2 min with 5 mL of 80% acetone and centrifuged (3273 × *g* for 10 min at 10 °C). The supernatant was directly assayed spectrophotometrically at three different wavelengths (662, 645 and 470 nm). Quantifications were obtained according to Lichtenthaler and Wellburn (1983) using the Eqs. (4)–(6):

$$C_a = 11.75 \times A_{662} - 2.350 \times A_{645} \quad (4)$$

$$C_b = 18.61 \times A_{645} - 3.960 \times A_{662} \quad (5)$$

$$C_s = \frac{1000 \times A_{470} - 2.270C_a - 81.4C_b}{227} \quad (6)$$

where C_a, C_b and C_s are respectively chlorophyll a and b and carotenoids concentrations (mg L^{-1}), and A is the absorbance values at the different wavelengths. Results were expressed as mg kg^{-1} of dry weight.

2.4.5. Antioxidants and antioxidant activity

2.4.5.1. Sample preparation and solid phase extraction for antioxidants determination. Hydrophilic fraction extraction: 0.5 g of freeze-dried sample were added to 10 mL of 0.1 N sulphuric acid in a 50 mL polypropylene tube, wrapped in an aluminium sheet. The mixture was vortexed for 2 min, then was centrifuged for 10 min at 1500 × *g* in an Allegra™ X-22 R centrifuge (Beckman Coulter, Inc., Brea, CA) set at 4 °C. The supernatant was filtered through a Whatman 541 filter paper (GE Healthcare, Buckinghamshire, UK). The solid residue was stored at 4 °C and then used for the extraction of amphiphilic fraction. 4 mL of the sample was loaded onto a Strata C18-U cartridge connected to a vacuum manifolds system (Phenomenex Inc., Torrance, CA, USA) and previously conditioned with 10 mL of methanol 100% followed by 25 mL of water. After the complete absorption of the sample, the hydrophilic compounds were eluted with 2 × 4 mL of 0.1 N sulphuric acid, collected in a 15 mL polypropylene tube (final volume 12 mL) and used for antioxidants and antioxidant activity determinations.

Amphiphilic fraction extraction: 1 mL of methanol 100% was added to the solid residue obtained after hydrophilic compounds extraction and it was dried under a gentle nitrogen stream. Sample was then added to 10 mL of methanol 60% and vigorously shaken for 10 min at room temperature. The mixture was centrifuged for 10 min at 1500 × *g* in an Allegra™ X-22 R centrifuge (Beckman Coulter, Inc., Brea, CA) set at 4 °C. 4 mL of the supernatant were loaded onto the same C-18 cartridge previously used for the separation of the hydrophilic fraction. After the complete absorption of the sample, the amphiphilic compounds were eluted with 2 × 4 mL of methanol 60%, collected in a 15 mL polypropylene tube (final volume 12 mL) and used for antioxidants and antioxidant activity determinations.

2.4.5.2. Ascorbic acid determination. Ascorbic acid was determined on the hydrophilic extract by HPLC analysis carried out according to Odriozola-Serrano et al. (2007). The HPLC system LC-1500 (Jasco, Carpi, MO, Italy) was equipped with a diode array UV/vis detector. A reverse-phase C18 Kinetex (Phenomenex Inc., Torrance, CA, USA) stainless steel column (4.6 mm × 150 mm) was used as stationary phase. Samples were introduced in the column through an autosampler (Jasco AS-2055 Plus). The mobile phase was a 0.01% solution of sulphuric acid adjusted to pH 2.6. Flow rate was fixed at 1.0 mL/min at room temperature. Data were processed by the software ChromNAV (ver. 1.16.02) from Jasco. Ascorbic acid content was quantified at 245 nm through a standard calibration curve that was linear in the range 0–284 μM ascorbic acid.

2.4.5.3. Total phenolic content (TPC) determination. The content of total polyphenols was quantified by the Folin–Ciocalteu phenol reagent method, according to Singleton and Rossi (1965), modified to fit a 96-wells plate. The TPC depends on the specific phenolic profile, in particular the type of phenolics present and their relative amounts or proportions (Naczk and Shahidi, 2004). The analysis was carried

out after solid phase extraction on C-18 cartridges, which has been reported as a suitable technique of separation for phenolic compounds (Antolovich et al., 2000; Nackzk and Shaidi, 2004).

The fresh working solution was prepared daily by diluting Folin–Ciocalteu phenol reagent to a final concentration of 0.6 N in water.

250 μL of Folin–Ciocalteu phenol reagent were aliquoted in a 96-well microplate and the reaction was started by adding 60 μL of hydrophilic or amphiphilic extracts. Six different dilutions for each extract were analysed. After 6 min in the darkness the reaction was neutralised by the addition of 22 μL of sodium carbonate 0.3 g mL^{-1}. After an incubation of 30 min at room temperature in the darkness, the absorbance was measured at 765 nm using the EnSpire™ Multimode Plate Reader (PerkinElmer, Waltham, MA). 60 μL of 0.1 N sulphuric acid or methanol 60% were used for the blank sample, for hydrophilic or amphiphilic extracts respectively.

Gallic acid was used as standard for the calculation of total phenolic content of hydrophilic and amphiphilic extracts. Gallic acid was dissolved in water for hydrophilic extract and in methanol 60% for the amphiphilic extract. 60 μL of water or methanol 60% were used for the blank sample, for hydrophilic or amphiphilic extracts respectively. The standard curve was linear in the range 0–39 μM gallic acid.

2.4.5.4. Radical scavenging capacity by ABTS•+. The ABTS free radical scavenging activity was determined according to the method described by Re et al. (1999) and modified to fit a 96-wells plate. Briefly, ABTS was dissolved in deionised water to a final concentration of 7 mM and ABTS•+ was produced by reacting ABTS solution with potassium persulphate 2.45 mM and allowing the mixture to stand in the dark at room temperature for 12–16 h. ABTS•+ was then aliquoted into small vials for storage at −80 °C until used. For the study, the ABTS•+ solution was diluted in deionised water to an absorbance of 1.829 ± 0.028 at 734 nm. Fresh diluted ABTS•+ solution was prepared daily.

250 μL of ABTS•+ were aliquoted in a 96-wells microplate and the reaction was started by adding 30 μL or 60 μL of hydrophilic or amphiphilic extracts, respectively. Six different dilutions for each extract were analysed. After 5 min of incubation at room temperature in the darkness, ABTS• blanching was measured at 734 nm using the EnSpire™ Multimode Plate Reader (PerkinElmer, Waltham, MA). 30 μL or 60 μL of 0.1 N sulphuric acid or methanol 60% were used for the blank solution, for hydrophilic or amphiphilic extracts respectively.

Trolox was used as standard for the calculation of the radical scavenging activity (RSA) of hydrophilic or amphiphilic extracts. Trolox was dissolved in PBS buffer (pH 7.3) for hydrophilic extract and in methanol 60% for the amphiphilic extract. 30 μL of PBS or 60 μL of methanol 60% were used for the blank sample for hydrophilic or amphiphilic extracts, respectively. The standard curve was linear in the range 0–58 μM Trolox.

2.4.5.5. Radical scavenging capacity by DPPH•. The DPPH free radical scavenging activity was determined according to the method described by Brand-Williams et al. (1995) modified to fit a 96-wells plate. The DPPH• stock solution 0.913 mM was prepared in methanol 100% and was stored at −20 °C until used. The working solution was obtained diluting DPPH• stock solution in methanol 60% to a final concentration of 0.290 mM. Fresh diluted solution was prepared daily. 250 μL of DPPH• were aliquoted in a 96-wells microplate and the reaction was started by adding 60 μL of amphiphilic extract. Six different dilutions of amphiphilic extract were analysed. DPPH• reduction was measured at 515 nm using the EnSpire™ Multimode Plate Reader (PerkinElmer, Waltham, MA) at 25 °C, after 10 min of reaction at room temperature in the darkness. 60 μL of methanol 60% were used for the blank sample. Trolox was used as standard for the calculation of the RSA of amphiphilic extract. Trolox was dissolved in methanol 60% and the standard curve was linear in the range 0–58 μM Trolox. 60 μL of methanol 60% were used for the blank solution.

2.4.5.6. FRAP determination. The FRAP assay was carried out according to the method described by Benzie and Strain (1996), modified to fit a 96-wells plate. The FRAP reagent (1.67 mM ferric chloride and 0.83 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) in 250 mM acetate buffer, pH 3.6) was prepared daily.

250 μL of FRAP reagent was aliquoted in a 96-wells microplate and the reaction was started by adding 60 μL of amphiphilic extract. Six different dilutions of amphiphilic extract were analysed. After 6 min of reaction at room temperature in the darkness, the absorbance was measured at 593 nm using the

Table 1
Pericarp colour and factorial ANOVA results of control (C) and treated (T) for 10 + 10 and 20 + 20 min fresh-cut kiwifruit immediately after the treatment and after 1 and 4 days of storage.

Sample	Treatment time (min)	Storage time (d)	Lightness		Chroma		Hue angle (°)	
			Mean	s.d	Mean	s.d.	Mean	s.d.
C	10 + 10	0	49.3^{a}	5.5	30.1^{a}	6.8	120.4^{a}	2.3
T	10 + 10	0	47.6^{ab}	6.2	27.1^{ab}	8.1	120.2^{a}	2.4
C	20 + 20	0	47.5^{ab}	5.7	29.0^{ab}	7.0	119.9^{a}	2.6
T	20 + 20	0	46.4^{abc}	4.8	26.2^{b}	6.3	120.7^{a}	3.5
C	10 + 10	1	44.7^{abce}	8.4	20.1^{cd}	4.5	111.5^{b}	7.1
T	10 + 10	1	46.2^{abc}	7.4	18.6^{cd}	3.8	111.6^{b}	3.7
C	20 + 20	1	43.0^{cde}	6.9	19.0^{cd}	4.6	112.5^{b}	3.3
T	20 + 20	1	48.1^{a}	9.3	19.7^{c}	5.1	111.4^{b}	2.9
C	10 + 10	4	39.9^{df}	6.4	18.4^{ab}	4.4	112.5^{b}	5.5
T	10 + 10	4	43.2^{bcde}	6.4	21.1^{c}	4.6	112.1^{b}	6.3
C	20 + 20	4	37.3^{f}	5.4	16.7^{d}	3.5	112.4^{b}	4.3
T	20 + 20	4	41.1^{def}	6.5	17.7^{ab}	4.7	111.1^{b}	3.3
F	Sample		10.1	**	1.11	n.s.	0.01	n.s.
F	S.t.		57.1	***	171	***	237	***
F	Sample × S.t.		8.00	***	8.32	***	0.75	n.s.
F	T.t.		4.57	**	6.83	***	0.99	n.s
F	T.t. × S.t.		1.48	n.s.	2.44	n.s.	0.55	n.s.

C: control; T: treated; F: *F* value; S.t.: storage time; T.t.: treatment time, s.d.: standard deviation; n.s.: not significant.
Data marked with the same letter within each column are not significantly different at a $p < 0.05$ level.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

EnSpire™ Multimode Plate Reader (PerkinElmer, Hamburg, Germany). 60 μL of methanol 60% were used for the blank sample.

$FeSO_4$ dissolved in water was used as standard for the calculation of the reduction capacity of amphiphilic extract. The standard curve was linear in the range 0–174 μM $FeSO_4$. 60 μL of water were used for the blank sample.

Results of TPC, $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP are expressed in mmol of standard kg^{-1} on a dry weight basis of freeze-dried sample and were calculated by the ratio of the correlation coefficient of the dose–response curve of sample, and the correlation coefficient of the dose–response curve of the standard.

2.5. Statistical analysis

All the analyses were carried out at least in triplicate on 3 independent samples and results were reported as mean and standard deviation.

Factorial analysis of variance (ANOVA) was carried out to test the significance of the effects of treatment (sample), storage time (S.t.), and their interaction (sample × S.t.); the effect of treatment time (T.t.) and its interaction with storage time (T.t. × S.t.) was further studied within treated samples.

Significant differences ($p < 0.05$) between mean values were tested by the Tukey's HSD test. In the case of significance of the Levene test, non parametric Mann–Whitney test was used.

Correlations among the results of different antioxidant activity assays were calculated by Pearson's correlation analysis. Statistical analyses were carried out using the software STATISTICA for Windows 7 (StatsoftTM, Tulsa, OK).

3. Results and discussion

3.1. Visual quality

3.1.1. Colour

Mean values and standard deviations of the colorimetric parameters obtained from the measurements conducted on the pericarp of the kiwifruit slices are shown in Table 1.

Samples lightness significantly decreased during storage but, although no significant differences among control and treated samples were observed immediately after the treatment, plasma showed a positive effect on colour lightness maintenance during storage, finally resulting in a less darkened product.

According to Agar et al. (1999), surface darkening of kiwifruit slices can be attributed to translucent water soaking from the tissue, since low polyphenol oxidase activity and high ascorbate content limit enzymatic browning.

Plasma treatments did not affect colour Hue angle and saturation (chroma), whilst the latter was affected by treatment time. Storage time after treatment significantly decreased both parameters.

3.1.2. Darkened area by CVS

In Table 2, mean values and standard deviations of the darkened area (%) calculated for the pericarp and the core areas of the kiwifruit slices are reported.

Storage time significantly increased the extent of darkened areas, and, even though no significant differences were observed immediately after the treatment, plasma treated samples showed a more limited darkening during storage compared to control samples due to the interactive effect between sample and storage time.

Furthermore, a significant interactive effect between treatment time and storage time on the extension of darkened areas of the pericarp was observed, with sample treated for long time presenting a faster darkening than those treated for the shortest time, but resulting in similar final values of darkened area at the end of the storage. Visual examples of control samples and treated samples (20 + 20 min) after 4 d of storage are given in Fig. 3.

Table 2
Pericarp and core darkened area and factorial ANOVA results of control (C) and treated (T) for 10 + 10 and 20 + 20 min fresh-cut kiwifruit immediately after the treatment and after 1 and 4 days of storage.

Sample	Treatment time (min)	Storage time (d)	Darkened area			
			Pericarp (%)		Core (%)	
			Mean	s.d	Mean	s.d.
C	10 + 10	0	5.73^{c}	4.60	8.70^{c}	9.09
T	10 + 10	0	13.2^{c}	18.5	13.5^{c}	9.36
C	20 + 20	0	13.4^{c}	11.6	15.4^{c}	16.3
T	20 + 20	0	19.7^{c}	13.0	21.4^{c}	25.8
C	10 + 10	1	16.9^{c}	7.97	28.0^{c}	17.0
T	10 + 10	1	8.00^{c}	6.34	23.2^{c}	15.3
C	20 + 20	1	55.8^{b}	19.4	61.5^{b}	19.3
T	20 + 20	1	21.7^{c}	8.84	25.0^{c}	15.5
C	10 + 10	4	95.8^{a}	4.93	70.7^{ab}	27.8
T	10 + 10	4	82.3^{a}	24.0	63.3^{ab}	22.5
C	20 + 20	4	94.0^{a}	10.1	87.5^{a}	23.8
T	20 + 20	4	79.2^{a}	23.7	60.8^{ab}	27.6
F	Sample		18.61	***	12.39	***
F	S.t.		435.7	***	108.7	***
F	Sample × S.t.		14.41	***	7.40	***
F	T.t.		21.60	***	12.23	***
F	T.t. × S.t.		14.91	***	1.35	n.s.

C: control; T: treated; F: *F* value; S.t.: storage time; T.t.: treatment time, s.d.: standard deviation; n.s.: not significant.
Data marked with the same letter within each column are not significantly different at a $p < 0.05$ level.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$

3.2. Texture

Results of the penetration test are reported in Table 3. DBD cold plasma treatment did not affect the texture of kiwifruit slices in terms of hardness, work necessary to rupture the flesh and gradient. Storage time determined a dramatic decrease of all the texture parameters after 4 days of storage which were neither influenced by the treatment nor by the treatment time.

According to Varoquaux et al. (1990) and Rocculi et al. (2005), texture breakdown in kiwifruit slices is due to physiological events that include enzymatic mediated degradation of hemicellulose,

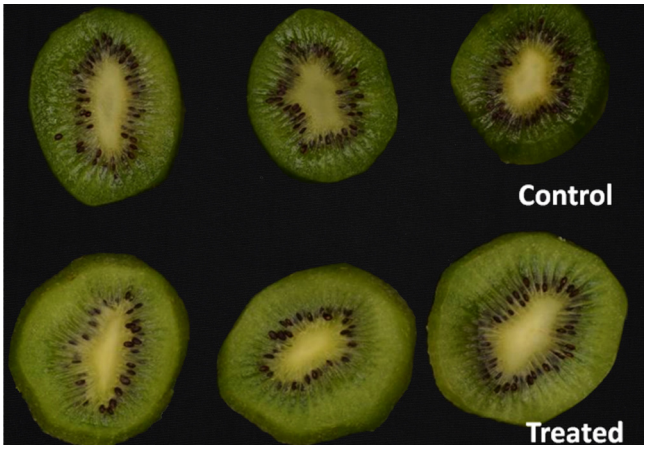

Fig. 3. Example of digitalised images of kiwifruit slices subjected to 20 + 20 min DBD gas plasma treatment and control ones acquired after 4 days of storage in controlled conditions.

Table 3
Pericarp mechanical parameters and ANOVA results of control (C) and treated (T) for 10 + 10 and 20 + 20 min fresh-cut kiwifruit immediately after the treatment and after 1 and 4 days of storage.

Sample	Treatment time (min)	Storage time (d)	Hardness (N)		Energy to rupture (mJ)		Gradient (N mm^{-1})	
			Mean	s.d	Mean	s.d	Mean	s.d
C	10 + 10	0	6.3^{ab}	2.3	4.60^{a}	1.86	9.38^{ab}	2.37
T	10 + 10	0	6.8^{a}	1.8	4.81^{a}	1.63	9.62^{ab}	3.26
C	20 + 20	0	6.0^{ab}	2.1	4.57^{a}	2.09	9.16^{ab}	3.65
T	20 + 20	0	5.3^{b}	2.3	4.20^{a}	2.29	8.12^{b}	3.24
C	10 + 10	1	5.2^{b}	1.8	4.81^{a}	1.77	8.85^{ab}	2.84
T	10 + 10	1	6.3^{a}	1.9	4.47^{a}	1.53	10.93^{a}	3.05
C	20 + 20	1	5.0^{b}	1.8	4.27^{a}	1.42	9.37^{ab}	3.54
T	20 + 20	1	5.4^{ab}	1.9	4.69^{a}	1.82	9.15^{ab}	2.94
C	10 + 10	4	2.0^{c}	0.5	1.84^{a}	1.17	3.67^{c}	2.34
T	10 + 10	4	2.0^{c}	0.6	1.68^{a}	0.79	3.35^{c}	1.58
C	20 + 20	4	1.8^{c}	0.8	1.54^{a}	0.89	3.08^{c}	1.78
T	20 + 20	4	1.7^{c}	0.7	1.47^{a}	0.89	2.94^{c}	1.78
F	Sample		1.01	n.s.	0.63	n.s.	0.26	n.s.
F	S.t.		202	***	350	***	1364	***
F	Sample × S.t.		2.11	n.s.	1.79	n.s.	2.38	n.s.
F	T.t.		8.09	**	5.13	*	4.07	*
F	T.t. × S.t.		0.93	n.s.	0.37	n.s.	0.17	n.s.

C: control; T: treated; F: *F* value; S.t.: Storage time; T.t.: treatment time, s.d.: standard deviation; n.s.: not significant.
Data marked with the same letter within each column are not significantly different at a $p < 0.05$ level.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

solubilization of polyuronide and release of galactose from pectic polymers, cell wall swelling and a decrease in water and osmotic potential. Although in previous studies it has been reported that plasma treatment can promote enzyme inactivation (Pankaj et al., 2013; Surowsky et al., 2013; Tappi et al., 2014), in this research, the activity of enzymes responsible for structure breakdown, generally activated by operations such as peeling and cutting, do not seem to be influenced by the applied treatments. To our knowledge, no researches have yet been carried out on the effect of plasma reactive species nor on this type of enzymes nor in this matrix (kiwifruit), hence, at the moment, to hypothesise a mechanism to explain the different results would be too speculative.

3.3. Chemical parameters

Mean values and standard deviations of soluble solid content (SSC), titrable acidity (TA), electrolyte leakage (EL) and dry matter (DM) are reported in Table 4.

SSC increased with storage time probably due to starch conversion into sugars but was not affected by the treatment. Nevertheless, a significant interactive effect was observed between treatment time and storage time as samples treated for longer time showed a more pronounced increased of SSC.

TA was significantly affected by storage time, treatment time and their interactions, although considering treated and control

Table 4
Chemical parameters and ANOVA results of control (C) and treated (T) for 10 + 10 and 20 + 20 min fresh-cut kiwifruit immediately after the treatment and after 1 and 4 days of storage.

Sample	Treatment time (min)	Storage time (d)	SSC (%)		TA (mg citric acid kg^{-1})		EL (%)		DM (%)	
			Mean	s.d	Mean	s.d	Mean	s.d	Mean	s.d
C	10 + 10	0	15.3^{b}	0.6	13.1^{a}	1.0	67.3^{a}	2.6	10.9^{d}	0.6
T	10 + 10	0	15.4 ^{b}	1.1	13.6^{a}	0.7	61.6^{a}	6.7	11.7^{cd}	0.3
C	20 + 20	0	14.1^{bc}	0.4	11.2^{a}	1.0	59.9^{a}	0.3	12.2^{d}	0.7
T	20 + 20	0	14.9^{bc}	0.5	11.7^{a}	0.5	61.9^{a}	0.5	13.0^{c}	0.3
C	10 + 10	1	16.2^{d}	0.3	14.2^{b}	0.7	55.6^{a}	4.6	11.9^{cd}	0.7
T	10 + 10	1	16.9^{d}	0.3	16.7^{b}	3.1	63.1^{a}	7.7	13.2^{c}	0.2
C	20 + 20	1	18.2^{d}	1.5	15.4^{b}	0.9	60.9^{a}	3.0	13.1^{c}	0.2
T	20 + 20	1	17.2^{d}	0.7	16.7^{b}	0.5	55.2^{a}	3.0	14.3^{e}	0.2
C	10 + 10	4	19.0^{a}	0.8	17.3^{c}	0.4	73.7^{a}	5.7	22.3^{e}	1.0
T	10 + 10	4	17.7^{d}	0.2	14.0^{a}	0.7	66.7^{a}	4.8	20.1^{b}	0.1
C	20 + 20	4	17.6^{d}	0.2	13.7^{a}	1.0	66.4^{a}	2.2	21.8^{a}	1.0
T	20 + 20	4	19.4^{a}	0.3	13.6^{a}	0.7	66.7^{a}	1.8	22.4^{e}	1.2
F	Sample		0.64	n.s.	0.65	n.s.	0.26	n.s.	3.42	n.s.
F	S.t.		79.13	***	47.94	***	7.25	**	793.56	***
F	Sample × S.t.		0.52	n.s.	13.21	***	1.42	n.s.	8.01	**
F	T.t.		0.43	n.s.	14.76	***	0.52	n.s.	26.64	***
F	T.t. × S.t.		6.80	**	8.71	**	0.25	n.s.	0.33	n.s.

C: control; T: treated; F: *F* value; S.t.: storage time; T.t.: treatment time, s.d.: standard deviation; n.s.: not significant. SSC: soluble solid content, TA: titrable acidity, EL: electrolyte leakage, DM: dry matter.
Data marked with the same letter within each column are not significantly different at a $p < 0.05$ level.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

samples at each storage day, the only significant differences observed by Tukey's HSD mean comparison test was after 4 d of storage for the 10 + 10 sample.

EL is a measurement of membrane integrity and it is often used to assess the effect of stress or senescence on plant tissue (Rolny et al., 2011). As it can be observe in Table 4, this parameter was affected only by storage time, although no significant differences were found according to the Tukey's HSD mean comparison test. Hence, it can be assumed that the treatment did not affect cell membrane integrity.

Dry matter content underwent an increase of about 7% compared to the control immediately after the treatment for both samples, probably due to the effect of the fan, similarly to the result found by Wang et al. (2012). During storage, a progressive increase was observed in all samples but, as the interaction between sample and storage time shows, plasma treatment seems to have induced a faster dehydration of the tissue exposed to the air. After the longer treatment, differences in dry matter compared to controls were more pronounced as it can be observed in Table 4.

Generally, the obtained results showed that the response of kiwifruit tissue to plasma treatment in terms of physico-chemical parameters was limited and mainly represented by an increased of dry matter content.

3.4. Chlorophyll and carotenoids analysis

Mean values and standard deviations of the concentration of the chlorophyll a and b and of the carotenoids are reported in Table 5.

A significant decrease in chlorophyll a (about 15%) was observed soon after treatment, even though no significant differences among control and treated samples were observed after 4 d of storage. Storage time significantly decreased the chlorophyll and carotenoids content, but plasma treated samples showed a more limited pigments loss than control samples.

The chlorophyll degradation upon plasma treatment could be associated to the Type II breakdown mechanism (Brown et al., 1991), which is mediated by the presence of oxygen radicals produced during reactions related to tissue metabolism or, as in this case, by plasma. Their presence is favoured by membrane breakdown as it normally occurs in minimally processed fruit and vegetable products. Hence, it can be hypothesised that the free radicals produced during the treatments caused the first pigments oxidation. At the same time, plasma is known to produce a partial protein denaturation (Pankaj et al., 2013; Surowsky et al., 2013; Tappi et al., 2014), which may have caused a slowdown in chlorophyll catabolism operated by enzymes such as chlorophyllase and magnesium dechelatase (Type I breakdown) during storage.

3.5. Ascorbic acid and total phenolic content

Ascorbic acid and TPC of treated and control kiwifruit samples were evaluated and the results are summarised in Table 6.

The minimally processed kiwifruit samples showed an average ascorbic acid content of 33 mmol kg^{-1} on a dry weight basis, higher than values reported by other authors (Agar et al., 1999; Gil et al., 2006; Tavarini et al., 2008) ranging between 20 and 25 mmol kg^{-1} on a dry weight basis. However, Tavarini et al. (2008) showed that the ascorbic acid content of kiwifruit could vary more than two-fold due to harvest time and post-harvest storage.

The DBD plasma treatment did not affect the ascorbic acid content (Table 6) immediately after the treatment, but a significant reduction of ascorbic acid of about 7% after 4 d of storage was highlighted by ANOVA, even though the Tukey's HSD mean comparison test did not evidence significant difference among the samples.

Agar et al. (1999) found a 20% reduction of ascorbic acid in kiwifruit slices after 6 d of storage at 10 °C due to oxidation, while Gil et al. (2006) found a 10% reduction of ascorbic acid after 4 d of storage at 5 °C and a 13% reduction after 6 d.

The TPC of the hydrophilic extracts resulted higher than that of the amphiphilic extracts, even though C-18 cartridges retain most of the phenolic compounds. This happens because the ascorbic acid in the hydrophilic extract, whose recovery was 95% as determined by the standard addition method, could react with the Folin–Ciocalteu reagent (Antolovich et al., 2000; Vinson et al., 2001). This fact is often overwhelmed when discussing total polyphenols data. The analysis of TPC without separation of the hydrophilic fraction (i.e. by SPE) could determine a dramatic overestimation of kiwifruit TPC. For example Tavarini et al. (2008) detected about 16 mmol kg^{-1} on a dry weight basis of total phenolics in kiwifruit using the colorimetric assay with the Folin–Ciocalteu reagent without SPE separation, whilst other authors (Gil et al., 2006; Dawes and Keene, 1999) detected about 1.6 mmol kg^{-1} of total phenolics by HPLC analysis. In this study, a total phenolic

Table 5
Chlorophyll and carotenoids content and factorial ANOVA results of control (C) and treated (T) for 10 + 10 and 20 + 20 min fresh-cut kiwifruit immediately after the treatment and after 4 days of storage. Data are expressed on dry weight basis.

Sample	Treatment time (min)	Storage time (d)	Chlorophyll a (mg kg^{-1})		Chlorophyll b (mg kg^{-1})		Total carotenoids (mg kg^{-1})	
			Mean	s.d	Mean	s.d.	Mean	s.d.
C	10 + 10	0	100.5^{a}	8.04	43.97^{ab}	4.41	42.02^{ab}	3.31
T	10 + 10	0	87.15^{b}	2.08	38.83^{abc}	3.38	40.69^{abc}	0.50
C	20 + 20	0	100.0^{a}	3.45	44.19^{a}	4.00	43.35^{a}	3.49
T	20 + 20	0	86.44^{b}	3.12	41.73^{abc}	2.93	40.21^{abc}	2.31
C	10 + 10	4	88.16^{b}	3.29	37.32^{c}	3.29	37.52^{cd}	0.21
T	10 + 10	4	90.53^{b}	4.19	42.65^{abc}	2.75	38.34^{bcd}	3.44
C	20 + 20	4	88.16^{b}	4.29	37.32^{c}	3.29	37.52^{cd}	0.21
T	20 + 20	4	89.59^{b}	4.65	37.78^{bc}	4.24	35.20^{d}	4.55
F	Sample		26.79	***	0.21	n.s.	4.84	*
F	S.t.		15.61	***	11.34	**	42.44	***
F	Sample × S.t.		47.28	***	10.89	**	1.19	n.s.
F	T.t.		0.24	n.s.	0.18	n.s.	0.19	n.s.
F	T.t. × S.t.		0.00	n.s.	3.89	n.s.	2.16	n.s.

C: control; T: treated; F: *F* value; S.t.: storage time; T.t.: treatment time, s.d.: standard deviation; n.s.: not significant.
Data marked with the same letter within each column are not significantly different at a $p < 0.05$ level.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 6
Ascorbic acid and total phenolic content and factorial ANOVA results of control (C) and treated (T) for 20+20 min fresh-cut kiwifruit immediately after the treatment and after 4 d of storage. Data are expressed on dry weight basis.

Sample	Storage time (d)	Ascorbic acid content (mmol kg^{-1})		Total phenolic content (mmol kg^{-1})			
		Hydrophilic extract		Hydrophilic extract		Amphiphilic extract	
		Mean	s.d.	Mean	s.d.	Mean	s.d.
C	0	34.86^{a}	1.65	7.54^{a}	0.38	4.48^{a}	0.62
T	0	36.69^{a}	0.81	7.91^{a}	0.57	4.21^{ab}	0.62
C	4	31.52^{a}	2.41	7.25^{a}	0.33	3.21^{bc}	0.10
T	4	35.15^{a}	1.83	7.56^{a}	0.36	2.88^{c}	0.35
F	Sample	0.59	n.s.	1.51	n.s.	0.14	n.s.
F	S.t.	5.67	*	1.40	n.s.	23.2	**
F	Sample × S.t.	0.02	n.s.	0.01	n.s.	0.06	n.s.

C: control; T: treated; F: *F* value; S.t.: storage time; T.t.: treatment time, s.d.: standard deviation; n.s.: not significant.
Data marked with the same letter within each column are not significantly different at a $p < 0.05$ level.
* $p < 0.05$.
** $p < 0.001$.

content of about 4 mmol kg^{-1} was measured in the amphiphilic fraction after SPE separation; this amount can be considered comparable with previously reported results, since the TPC of kiwifruit can show up to three-fold variations due to harvest time and post-harvest storage (Tavarini et al., 2008).

The DBD plasma treatment did not affect the response to the Folin–Ciocalteu reagent in both the hydrophilic and the amphiphilic extract (Table 6). A significant reduction of TPC in the amphiphilic extract of about 30% after 4 d of storage was highlighted by ANOVA analysis.

In fresh-cut fruit tissues, phenols could undergo chemical or enzymatic oxidation mediated by polyphenol oxidase (PPO) or peroxidase (POD); however, as a consequence to wounding, new phenolic compounds can also be synthesised through an increase of the activity of the enzyme phenylalanine ammonia lyase (PAL) as a defence mechanism (Heredia and Cisneros-Zevallos, 2009). In this study, the TPC of the kiwifruit amphiphilic extract, accounting for polyphenols, decreased after 4 d of storage whilst Gil et al. (2006) did not find any TPC variation during storage of fresh-cut kiwifruits at 5 °C for 9 d; these differences could be due to the different storage conditions adopted.

3.6. Antioxidant activity (AOA) determination

In the present study, the antioxidant activity of kiwifruit samples was investigated with a variety of methods aimed to measure their RSA and reducing power (Table 7).

The ABTS$^{\bullet+}$ assay was conducted on both the hydrophilic and amphiphilic extracts; the former showed a radical scavenging activity much higher (about 5-fold) than the latter probably because kiwifruit is very rich in ascorbic acid and less in polyphenols. Since the amount of ascorbic acid in kiwifruits was measured and the relative radical scavenging activity of this molecule is known (Re et al., 1999) it is possible to assume that all the antioxidant activity of the hydrophilic extract is due to ascorbic acid.

Even though the TPC in kiwifruit is about 4 mmol kg^{-1} on a dry weight basis (taking into account only the amphiphilic fraction) and ascorbic acid is about 33 mmol kg^{-1}, the former accounts for about the 40% of total TEAC (Trolox equivalent antioxidant capacity) and the latter for the remaining 60%. This data are in complete accordance with those found by Tavarini et al., (2008) using different analytical assays.

DBD plasma treatment did not affect the TEAC of both the hydrophilic and the amphiphilic extract; similar results were also found using the other antioxidant activity assays (DPPH and FRAP), which were carried out on the amphiphilic extracts. The DBD plasma treatment adopted in this study did not affected the antioxidant activity and antioxidants content of kiwifruit. Even though the DBD plasma-induced reactive species could have caused the oxidation of single phenolic compounds responsible for the antioxidant activity of minimally processed kiwifruit, this effect was counteracted by tissue response mechanisms.

Table 7
Antioxidant activity and factorial ANOVA results of control (C) and treated (T) for 20 + 20 min fresh-cut kiwifruit immediately after the treatment and after 4 days of storage. Data are expressed on dry matter basis.

Sample	Storage time (d)	RSA_{ABTS} (mmol TE kg^{-1})				RSA_{DPPH} (mmol TE kg^{-1})		FRAP (mmol Fe^{2+} kg^{-1})	
		Hydrophilic extract		Amphiphilic extract		Amphiphilic extract		Amphiphilic extract	
		Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
C	0	22.46^{a}	0.19	4.89^{a}	0.19	8.89^{a}	0.42	9.49^{a}	0.42
T	0	23.35^{a}	0.55	4.75^{ab}	0.44	8.66^{a}	0.66	8.56^{ab}	0.66
C	4	20.77^{b}	0.45	4.22^{b}	0.45	8.74^{a}	0.64	8.13^{b}	0.64
T	4	21.37^{b}	0.79	4.23^{ab}	0.57	9.28^{a}	0.59	8.18^{b}	0.59
F	Sample	6.97	*	0.09	n.s.	0.02	n.s.	1.64	n.s.
F	S.t.	56.8	**	7.16	*	0.54	n.s.	6.30	*
F	Sample × S.t.	0.72	n.s.	0.15	n.s.	0.02	n.s.	2.01	n.s.

C: control; T: treated; F: *F* value; S.t.: storage time; T.t.: treatment time, s.d.: standard deviation; n.s.: not significant; RSA: radical scavenging activity; GAE: gallic acid equivalents; TE: Trolox equivalents; $FeSO_4$ equivalents.
Data marked with the same letter within each column are not significantly different at a $p < 0.05$ level.
* $p < 0.05$.
** $p < 0.001$.

Table 8
Pearson correlation coefficients between the different assays used to evaluate the total phenolic content and antioxidant capacity of control and plasma treated fresh-cut kiwifruit.

	Total phenolic content, TPC (mmol GAE kg^{-1})		RSA_{ABTS} (mmol TE kg^{-1})		RSA_{DPPH} (mmol TE kg^{-1})	FRAP (mmol Fe^{2+} kg^{-1})
	Hydrophilic extract	Amphiphilic extract	Hydrophilic extract	Amphiphilic extract	Amphiphilic extract	Amphiphilic extract
TPC hydrophylic extract	1.000	0.412	0.365	0.543	0.291	0.279
TPC amphiphilic extract	0.412	1.000	0.682	0.782**	−0.082	0.804**
RSA_{ABTS} hydrophilic extract	0.365	0.682	1.000	0.511	0.156	0.370
RSA_{ABTS} amphiphilic extract	0.543	0.782**	0.511	1.000	0.034	0.843**
RSA_{DPPH} amphiphilic extract	0.291	−0.082	0.156	0.034	1.000	−0.215
FRAP amphiphilic extract	0.279	0.804**	0.370	0.843**	−0.215	1.000

RSA: radical scavenging activity; GAE: gallic acid equivalents; TE: Trolox equivalents; Fe^{2+}:$FeSO_4$ equivalents.
* $p < 0.05$; *** $p < 0.001$.
** $p < 0.01$.

The antioxidant activity of both the hydrophilic and amphiphilic extracts decreased with storage time (Table 7), except for the DPPH• assay which did not evidence any difference in AOA after storage, thus showing a lower sensibility than the other assays. Antioxidant constituents of vegetable tissues are susceptible to degradation when exposed to oxygen or light, or upon interaction with enzymes, such as ascorbate oxidase, polyphenol oxidase, cytochrome oxidase and peroxidase as a consequence of wounding (Gil et al., 2006). Several studies have been performed regarding the effect of wounding response on antioxidant activity (Kang and Saltveit, 2002; Reyes and Cisneros-Zevallos, 2003; Reyes et al., 2007; Heredia and Cisneros-Zevallos, 2009). Results generally indicate that changes in the antioxidant activity as a consequence of wounding depend on the type of tissue, the initial content of ascorbic acid and phenolic compounds, and the specific phenolic profile.

The Pearson correlation analysis was performed to correlate results obtained with different methods (Table 8). A significant correlation between the $ABTS^{•+}$ and the FRAP applied to the amphiphilic fraction was found despite the different reaction mechanisms implied in the two assays. Noteworthy, both these assays were strongly correlated with the TPC when they were carried out on the amphiphilic fraction of kiwifruit extract, which contains only polyphenols. The TPC is based on the capacity of phenolic compounds to react with the Folin–Ciocalteu reagent under basic conditions and thus has been extensively used as a method for the estimation of total phenolics; nonetheless, taking into account that polyphenols show different reactivity with the Folin–Ciocalteu reagent (Nazck and Shahidi, 2004) and that the mechanism is based on a oxidation/reduction reaction, TPC can be also considered an antioxidant method (Prior et al., 2005). The results of the DPPH• assay a less sensible method than the others in evidencing changes in the AOA of the amphiphilic fraction, carried out in methanolic medium, were neither correlated with those of other antioxidant methods nor with the TPC.

4. Conclusions

According to the obtained results, the DBD cold plasma treatments promoted an immediate loss of pigment and visual quality on minimally processed kiwifruit but positively influenced the quality maintenance of the product, by improving colour retention and reducing the darkened area formation over storage time in controlled conditions. In addition, although DBD cold plasma treatment had an effect in increasing dry matter content over storage, it did not induced any textural changes compared with the control.

In general, no significant changes in antioxidants (ascorbic acid and polyphenols) content and antioxidant activity were observed among treated samples and control ones.

In this direction the purposed DBD plasma treatment is a very promising tool to preserve the quality of minimally processed kiwifruit.

Further studies will be necessary to confirm the sanitising effect of DBD plasma already showed for different commodities on minimally processed kiwifruit and the eventual sensorial properties modification induced by the treatment.

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Paper V

Cold plasma treatment for fresh-cut melon stabilization

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Cold plasma treatment for fresh-cut melon stabilization

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ABSTRACT

The aim of this study was to evaluate the effect of gas plasma treatment on fresh-cut melon stability during controlled storage. Plasma treatments of 15 + 15 and 30 + 30 min were conducted on fresh cut melon using a dielectric barrier discharge (DBD) generator. Samples were packed and stored for 4 days at 10 °C and evaluated for qualitative, metabolic and microbiological indexes. Qualitative parameters of fresh-cut melon (titratable acidity, soluble solid content, dry matter, colour, texture) were only weakly affected by plasma treatment. Peroxidase (POD) and pectin methylesterase (PME) activities were slightly inhibited by the treatment up to respectively about 17 and 7%. Tissue metabolic heat production decreased proportionally to the treatment duration, while a partial conversion to anaerobic metabolism was observed. Microbial results showed that a significant increase in microbial shelf-life was achieved following the 15 + 15 min plasma treatment due to a delayed growth of spoilage mesophilic and psychrotrophic microflora.

Industrial relevance: The demand for fresh-cut products characterized by high qualitative and nutritional values and an acceptable shelf-life has promoted the research for non-thermal treatments.

Fresh-cut melon is considered to be highly perishable and potentially hazardous food because it can support the growth of spoilage microflora and several pathogens.

Cold plasma has shown its potentiality as an antimicrobial treatment and has been tested on different food products, but the impact on product quality and metabolism is still scarcely known.

The results obtained in this study contributed to deepen the knowledge on the effect of plasma treatment on microbial, qualitative and metabolic aspects of fresh-cut melon.

1. Introduction

Fresh-cut fruit and vegetables are products subjected to minimal processing operations, maintaining the fresh-like quality, with a high convenience value (Ragaert, Devlieghere, & Debevere, 2007). Minimal processing operations such as slicing, peeling and/or other mechanical injuries cause physical damages to the product, resulting in a number of physiological disorders called 'wounding response' (e.g. increased rate of respiration and ethylene production, enzymatic activity, quality degradation and dehydration), which favour also the growth of the spoilage microflora leading to a very limited shelf-life (Soliva-Fortuny & Martín-Belloso, 2003).

Furthermore, fresh-cut products such as melon, characterised by quite high pH (5.2–6.7) and water activity (0.97–0.99) values, are considered to be highly perishable and potentially hazardous foods because they can support the growth of spoilage microflora and several pathogens, including *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes*, particularly if they are not subjected to adequate preservative treatments and to cold storage (Harris et al., 2003; Lamikanra, Chen, Banks, & Hunter, 2000), or if their surface has been damaged prior to consumption (EFSA, 2014). Melon surface, characterized by a complex netting skin (peel), provides an environment on which bacteria can strongly attach (Ukuku, Olanya, Geveke, & Sommers, 2012; Vadlamudi, Taylor, Blankenburg, & Castillo, 2012) and from which they can be easily transferred onto melon flesh during cutting operations.

The US Centres for Disease and Control and Prevention (CDC) identified 34 foodborne disease outbreaks related to the consumption of melons in the US between 1973 and 2011 (CDC, 2011). Moreover, among the 16 outbreaks reported in 2012 due to contaminated fruits, which caused a total of 858 illnesses, four were associated to melons (Centers for Disease Control and Prevention, CDC, 2011). The majority of these outbreaks were caused by *Salmonella*. Nevertheless, one of the most widespread outbreaks of *L. monocytogenes* food poisoning in the US, which caused 146 illnesses in 28 states and led to 32 deaths, resulted from contaminated cantaloupes (Centers for Disease Control and Prevention, CDC, 2011).

Studies on innovative physico-chemical processes to improve the shelf-life of whole and fresh-cut melons mainly refer to the use of irradiation (Palekar, Taylor, Maxim, & Castillo, 2015), X-ray (Mahmoud,

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2012), UV-C light (Manzocco, Rumignani, & Lagazio, 2013), gaseous ozone (Selma, Ibáñez, Cantwell, & Suslow, 2008), edible coatings (Martiñon, Moreira, Castell-Perez, & Gomes, 2014) or modified atmosphere packaging (MAP) (Zhang, Samapundo, Pothakos, Sürengil, & Devlieghere, 2013). On the other hand, most of the literature data are focused on sanitizers alternative to chlorine washing (Silveira, Conesa, Aguayo, & Artés, 2008; Ukuku, Huang, & Sommers, 2015), which is the procedure currently used at industrial level despite the widely diffused concerns of potential formation of harmful by-products.

The growing demand for fresh-cut products has pushed the researchers to develop new non-thermal treatments able to keep a desirable shelf-life, preserving the original fresh-like attributes of the raw material.

Recently, cold plasma, which in the past has been mainly used in the medical field, particularly for microbial decontamination of surfaces and living tissues (Emmert et al., 2013; Juwarkar, 2013; Weltmann et al., 2012), has drawn considerable attention as a novel non-thermal treatment for food product decontamination (Niemira, 2012). Plasma is considered the "fourth state of matter" and it is generated by applying energy to a gas mixture, causing the ionisation of the gas and the formation of active components, such as radicals, charged particles and UV radiations.

Nonthermal plasma can be generated by microwaves, radio frequency, direct or alternating current; various set-ups such as dielectric barrier discharge (DBD), atmospheric pressure plasma jet (APPJ) and corona discharges (CD) (Ehlbeck et al., 2011; Laroussi, 2002; Ragni et al., 2010); and different gas mixtures, including atmospheric gas (oxygen, nitrogen and carbon dioxide) as well as noble gases (e.g. helium and argon) can be used. The plasma composition greatly depends on the kind of gases in the mixture, the selected generator set-up, the operating conditions (flow, gas pressure, power of plasma excitation) and the exposure mode (direct or remote) (Misra, Tiwari, Raghavarao, & Cullen, 2011), and it includes reactive species such as oxygen and nitrogen species (ROS and RNS), atoms, free radicals and UV radiations.

Among all the different plasma constituents, the most important role in microbial inactivation and protein denaturation seems to be played by reactive species, such as free radicals (Laroussi, 2002; Li et al., 2011; Takai, Kitano, Kuwabara, & Shiraki, 2012).

The oxidative damage to the microbial cell surface can lead to the loss of functionality of the cell membrane and the exposure of the genetic material. Various authors have assessed the microbial decontamination of cold plasma on foodstuffs, such as the outer surface of various fruit and vegetables (Baier et al., 2014; Critzer, Kelly-Wintenberg, South, & Golden, 2007; Misra et al., 2011; Niemira & Sites, 2008) and on apple juice (Montenegro, Ruan, Ma, & Chen, 2002; Surowsky, Fröhling, Gottschalk, Schlüter, & Knorr, 2014). The effect was found to be highly dependent on the operative conditions (type of plasma generator, flow rate, treatment time, gas mixture), type of microorganism and matrix exposed to gas plasma.

Moreover, cold plasma treatments have recently drawn attention as possible treatments for fresh-cut vegetable products with the aim of inactivate endogenous enzymatic activity. In a previous study, Tappi et al. (2014) observed a significant reduction of PPO activity in fresh cut Pink Lady apples (up to 45% compared to the control) and of browning reaction during storage. The observed reduction was probably due to reactions between the enzymes and the radicals produced during treatment. Protein structural modifications upon plasma treatment were observed in different studies, in which modifications in the amino acid side chain and the decrease in the amount of α-helix structures in various enzymes were detected by means of techniques, such as circular dichroism spectroscopy and tryptophan emission fluorescence, and related to the loss of enzymatic activity (Pankaj, Misra, & Cullen, 2013; Surowsky, Fischer, Schlueter, & Knorr, 2013; Takai et al., 2012).

Furthermore, few researches have evaluated the effect of plasma on bioactive compounds and antioxidant activity in lamb's lettuce (Grzegorzewski, Ehlbeck, Schlüter, Kroh, & Rohn, 2011) and fresh-cut kiwifruit (Ramazzina et al., 2015).

However, the differences among the types of plasma and the operating conditions used in these studies make difficult the comparison of the obtained results.

In this contest, the aim of this research was to evaluate the effects of cold plasma, generated by a DBD device, on fresh-cut melon quality and safety. In particular, the effect of different treatment times has been evaluated on qualitative, metabolic and microbiological aspects of fresh-cut melon during controlled storage.

2. Materials and methods

2.1. Raw material, handling and storage

Melons (*Cucumis melo* L. var. *Reticolatus* cv. 'Raptor') grown in the Emilia-Romagna region of Italy were harvested in July 2013. The fruits were stored in plastic bins at 2 ± 0.5 °C and approximately 100% RH in air for 2 weeks. After this period, 20 kg of fruits free from defects were selected, transported to our laboratory, placed in a dark refrigerated chamber at 4 °C and saturated atmosphere for one week. At the beginning of experiments melons had a dry matter content of 15.73 g (±0.29) 100 g fw^{-1}, a soluble solid content of 14.27 (±0.35) % and a titratable acidity of 0.39 mg (±0.03) of malic acid g fw^{-1} measured as an average of 10 fruits.

Before cutting operations, whole melons were washed and scrubbed with a sponge to eliminate dirt from the surface and then immersed for 2 min in a 200 ppm sodium hypochlorite solution, in order to sanitize the peel and avoid cross contamination during processing. Work surface and cutting tools were also sanitized with the same solution prior to use.

Melons were then halved and blossom and stem ends were eliminated. Skin and seeds were also removed. From the central part of each half, 10 mm slices were cut using a sharp knife and each slice was divided in 4 trapezoidal pieces (about 10 g each).

2.2. Gas plasma generator

A dielectric barrier discharge (DBD) generator composed by three parallel pair electrodes made of brass and a 5 mm thick glass covering one electrode of each couple, was used for the treatment as described in previous works (Berardinelli, Vannini, Ragni, & Guerzoni, 2012; Ragni et al., 2010; Ramazzina et al., 2015). The electrodes were confined in a cabinet (about 3×10^{-2} m^3 of air volume) and were DC powered by three independent power supplies at 19 V and about 3 A. The high voltage (about 15 kV peak to peak and a dominant frequency of 12.5 kHz) was generated by switching transistors and transformers.

Air gas was used to generate the discharge (at 22 °C and 60% of RH) and, according to previous studies (Ragni et al., 2010), it led to the formation of OH and N radicals and ions. Samples made of 15 melon pieces were placed at about 70 mm from the electrodes (Fig. 1). The plasma species were directed to the sample surface by three fans mounted over the electrodes (flow rate of about 7×10^{-3} m^3 s^{-1}).

2.3. Gas plasma treatments and sample storage

Preliminary tests were run in order to select treatment time and to avoid visible damages on the melon fresh-cut surface. On the basis of the obtained results, treatment durations of 30 min (15 min for each side) and 60 min (30 min for each side) were selected. Each treatment was repeated in triplicate. For each replication, samples of 30 pieces, obtained from 5 different melons, were randomly divided in two subsamples of 15 pieces each, one used for plasma treatment and one used as control in order to minimize differences due to natural variability.

Control samples were stored at the same temperature and RH conditions for the duration of each treatment. Gas plasma treated

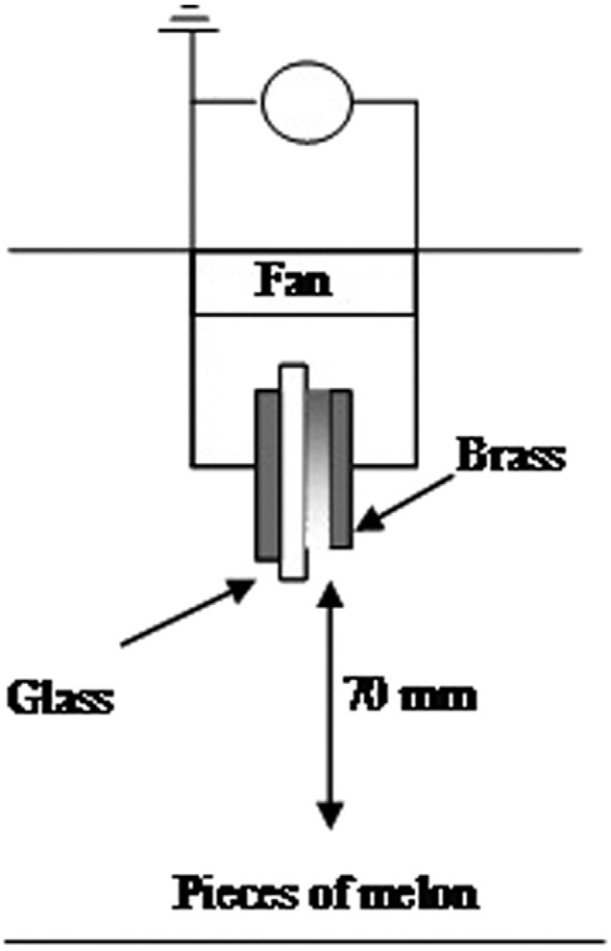

Fig. 1. Schematic representation of the electrode configuration.

samples and control ones were packed in propylene trays, sealed with a micro perforated polypropylene film in order to maintain atmospheric air composition avoiding dehydration, and stored for 4 days at 10 °C and 90% RH. During storage, three packages for each sample were selected after 0, 1, 2 and 4 days for analytical determinations. Microbiological analyses were performed after 0, 1, 2, 3 and 4 days of storage.

2.4. Qualitative determinations

2.4.1. Chemical parameters

Dry matter content was determined gravimetrically by difference in weight before and after drying at 70 °C, until a constant weight was achieved (AOAC International, 2002).

Soluble solid content (SSC) was determined at 20 °C by measuring the refractive index of melon juice with a digital refractometer mod. PR1 (Atago Co. Ltd, Tokyo, Japan), calibrated with distilled water.

Titratable acidity (TA) was determined by titration with NaOH 0.1 N until pH 8.1 was reached (AOAC Official Method 942.15, 2000).

For each sample, SSC and TA were determined in triplicate on the juice obtained by crushing 10 melon pieces, after filtering through Whatman #1 filter paper.

2.4.2. Texture

Penetration tests were performed using a Texture Analyser mod. TA-HDi500 (Stable Micro Systems, Surrey, UK) equipped with a 50 N load cell and a 6 mm diameter stainless steel cylinder. Compression test speed was 0.5 mm s^{-1}, the test ended when a maximum deformation of 90% was reached. Tests were performed on 10 melon pieces for each sample.

Firmness F (N), as the first peak force value representing the limit of the flesh elasticity, and the gradient G between 0 and F were considered.

2.4.3. Colour

A spectrophotocolorimeter (Colorflex, Hunterlab) was used to measure surface colour of melon pieces (D_{65} illuminant and 10° standard observer). For each piece, measurements were performed on each side. The L*, a* and b* parameters of the CIELAB scale were measured, hue angle ($h° = \arctan[{}^{b*}/_{a*}]$) and chroma ($\text{chroma} = \sqrt{a^{*2} + b^{*2}}$) values were also calculated (C.I.E, 1987). Results were expressed as average of 10 measurements for sample.

2.5. Metabolic determinations

2.5.1. Peroxidase (POD) and pectin methylesterase (PME) activities

POD activity was assayed using slight modifications of the spectrophotometric method of Morales-Blancas, Chandia, and Cisneros-Zevallos (2002). 25 g of sample was homogenized with a hand blender with 50 mL of cold (0–2 °C) potassium phosphate buffer 0.1 M (pH 6.5) for 2 min. The homogenized solution was filtered through filter paper and centrifuged for 30 min at 4 °C and 10,000 ×*g*. The supernatant (enzymatic extract) was collected.

POD substrate solution was prepared by mixing 0.1 mL of 99.5% guaiacol, 0.1 mL of 30% hydrogen peroxide, and 99.8 mL of 0.1 M potassium phosphate buffer (pH 6.5). POD activity was tested by adding 150 μL of enzyme extract to 3.35 mL of substrate solution in 10-mm pathlength glass cuvettes. The solution was mixed with a vortex for 3 s and by overturning the cuvette 3 times. POD activity was measured at 25 °C by monitoring the increase in absorbance at 470 nm.

PME activity was assayed using slight modifications of the spectrophotometric method described by Hagerman and Austin (1986). 50 g of sample was homogenised with a hand blender with 50 mL of cold (0–2 °C) NaCl 8.8% (w/v) for 2 min. The homogenized solution was stirred for 15 min, filtered through filter paper, and centrifuged for 30 min at 4 °C and 10,000 ×*g*. The pH of each enzymatic extract was adjusted to pH 7.5 by adding a few drops of 0.1 NaOH.

PME substrate was prepared by mixing 0.5 g of pectin from citrus peel in 100 mL of distilled water. The pH of the substrate was adjusted up to pH 7.5 with NaOH.

PME activity was assayed by adding 2000 μL of substrate, 100 μL of bromothymol blue solution, 740 μL of 3-mM potassium phosphate buffer (pH 7.5), and 160 μL of enzyme extract, directly in 10-mm pathlength glass cuvettes. The solution was mixed with a vortex for 3 s and by overturning the cuvette 3 times.

PME activity was measured at 25 °C by monitoring for 5 min the decrease in absorbance at 620 nm. Reaction rates were calculated from the slope ($\Delta A\ min^{-1}$) of the linear portion of the plot absorbance compared with time. Blank was prepared with water.

Residual enzymatic activity (%) was expressed as ratio of treated sample versus its control, according to Pizzocaro, Torreggiani, and Gilardi (1993), and measured just after gas-plasma treatment on three independent extracts.

2.5.2. Respiration rate

Respiration rate was evaluated using a static method as previously described (Tappi et al., 2014). Four cylinders (4 × 10 mm) were sampled from each melon piece, half were subjected to plasma treatments and half were used as control. Samples were then placed in sealed 20 mL glass ampoules and stored at 10 °C for 24 h. O_2 and CO_2 percentage was measured in the ampoule headspace by a gas analyzer "check point O_2/CO_2" mod. MFA III S/L (Witt-Gasetechnik, Witten, Germany) after 1, 3, 5, 22 and 24 h. Respiration rate was calculated as mg of

consumed O_2 (RRO_2) or produced CO_2 ($RRCO_2$) h^{-1} kg fw^{-1} according to the following equations:

$$RRO_2 = \frac{mm_{O_2} \cdot V_{head} \cdot \frac{(20.8 - \%O_{2,head})}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283}$$

$$RRCO_2 = \frac{mm_{CO_2} \cdot V_{head} \cdot \frac{\%CO_{2,head}}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283}$$

where mm_{O_2} and mm_{CO_2} refer to gas molar masses (g mol^{-1}), V_{head} represents the ampoule headspace volume (dm^3), % $O_{2,head}$ and % $CO_{2,head}$ refer to gases percentages in the ampoule headspace at time t (h); m is the sample mass (kg); and R is the gas constant (8.314472 dm^3 kPa K^{-1} mol^{-1}). For each sample, the average of three replicates was considered.

2.5.3. Metabolic heat by isothermal calorimetry

Isothermal calorimetry allows the evaluation of the metabolic response to stress in fresh cut tissue through the determination of metabolic heat production as reported by Rocculi et al. (2012) and Tappi et al. (2014).

Four cylinders were sampled from each melon piece using a core borer and subjected to the different plasma treatments, and then placed in 20 mL glass ampoule (about 2.5 g). A TAM-Air isothermal calorimeter (TA Instrument, New Castle, USA) previously described by Wadsö and Gómez Galindo (2009) was used to measure the rate of metabolic heat production.

Isothermal measurements were performed at 10 °C for 24 h. For each sample, the average of four replicates was considered. Before and after the measurements, baseline was recorded for 30 min.

2.6. Microbiological analyses

Samples of untreated and plasma-treated melon (10 g) were suspended into sterile 0.1% (w/v) buffered peptone-water solution and homogenized with a Stomacher Lab Blender (Seward, PBI International, Whitstable, Kent, UK) for 2 min at room temperature.

Mesophilic lactobacilli and lactococci were determined on MRS agar (Oxoid Ltd, Basingstoke, Hampshire, UK) containing 0.1% of cycloheximide (Sigma Chemical Co.) and M17 agar (Oxoid Ltd), respectively at 30 °C for 48–72 h under anaerobic conditions. Yeasts were enumerated on Yeast Extract Peptone–Dextrose agar (YPD, Oxoid Ltd), added of 150 ppm chloramphenicol, at 30 °C for 72 h. Viable counts of total aerobic mesophilic and psychrotrophic bacteria were determined on Plate Count Agar (Oxoid Ltd) at 30 °C for 48 h and 4 °C for 10 days, respectively.

Microbial analyses were carried out for melon samples exposed to three independent plasma treatments for each process time, which were analysed in duplicate (i.e. total six analyses for each process time).

2.7. Data analysis

Significant differences in qualitative and metabolic parameters and in microbial loads, at the same sampling time, were assessed using the t-test, and significance of differences was defined at $p \leq 0.05$. Moreover, microbiological data of mesophilic and psychrotrophic bacteria collected over storage were modelled using the Gompertz equation as modified by Zwietering, Jongenburger, Rombouts, and Van't Riet (1990) in order to obtain the microbial growth parameters, i.e. maximum growth rate (μmax), lag phase length (λ) and maximum cell increase attained at the stationary phase (A). The growth parameters derived by the Gompertz equation in relation to plasma treatment times were then used to estimate the product shelf-life, which was calculated as the time necessary to attain a threshold level of 6 Log CFU g^{-1} as a critical cell load for the spoilage-associated microflora.

3. Results and discussion

3.1. Qualitative assessment of fresh-cut melon

As a consequence of minimal processing operations, wounding response promotes an increase in the vegetable product maturation processes due to higher respiration and conversion rate of starch (Beirão-da-Costa, Steiner, Correia, Empis, & Moldão-Martins, 2006).

As reported in Table 1, soluble solid content (SSC), titratable acidity (TA) and dry matter (DM) showed few significant differences among control and plasma treated melon samples. After 2 and 4 days of storage, 30 + 30 plasma treated samples showed higher DM and lower SSC contents than control ones. When significantly different, TA was lower in treated samples (after 0 and 2 days for 15 + 15 treatment and after 2 days for 30 + 30 treatment).

Generally, results seem to point out a higher water loss during storage, which was more evident when the longest plasma treatment was used. Similar results were obtained in previous experiments on fresh-cut kiwifruit (Ramazzina et al., 2015) and various fresh-cut fruit and vegetable (Wang et al., 2012); this behaviour was attributed to the moderate effect of the fan during the treatment.

Various authors (Aguayo, Escalona, & Artés, 2004; Fundo et al., 2014) have reported softening during storage of fresh-cut melon as a consequence of the degradation of the internal structures due to tissue ripening, and the solubilisation of the protopectinic fraction of the cell wall components and to the loss of cell adhesion (Varoquaux, Lecendre, Varoquaux, & Souty, 1990).

In this case, treated samples did not exhibit significantly different textural parameter values (Table 2) compared to control ones, with the exception of samples treated for 15 + 15 min that showed lower values for both firmness and gradient just after the treatment, and samples treated for 30 + 30 min that were characterised by lower values of gradient compared to the control ones after 2 and 4 days of storage.

Tappi et al. (2014) found a slight decrease in the crunchiness of fresh-cut apples subjected to plasma generated by the same DBD generator that was attributed to the destruction of the superficial layer of cells, but no significant differences in textural characteristics of kiwifruit slices were observed (Ramazzina et al., 2015). Other authors (Schnabel, Niquet, Schlüter, Gniffke, & Ehlbeck, 2014) did not detect any difference in apple flesh using a different device for plasma generation.

The colour changes occurring during storage in fresh cut melon can be attributed to the variation of pigment concentration and to induction of a translucent water-soaked tissue (glossy) caused by the loss of cellular compartmentation and water redistribution within the tissues (often tissue softening) since melon is not affected by enzymatic browning (Agar, Massantini, Hess-Pierce, & Kader, 1999). The development of translucency has been found to be the principal visual change of deterioration in fresh-cut melon stored under MAP (Aguayo et al., 2004; Bai, Saftner, Watada, & Lee, 2001; Oms-Oliu, Raybaudi-Massilia Martínez, Soliva-Fortuny, & Martín-Belloso, 2008).

Table 3 shows mean values and standard deviations of colorimetric parameters obtained for plasma treated samples and related controls. During storage, although significant differences were found only after 2 and 4 days between the 15 + 15 min treated and control samples, it can be trendily observed a reduction of all the colour parameters in the plasma treated samples. At the end of storage, treated melon samples appeared darker and more translucent compared to control ones.

The increased translucency may be due to a variation on the structure of the tissue that however did not affect textural parameters.

3.2. Metabolic evaluation

POD is a ubiquitous enzyme in vegetable cells and it can promote several reactions that adversely influence product quality, such as lipid and phenolic oxidations with consequent deterioration of flavour, colour and nutritional quality (Morales-Blancas et al., 2002). It is

Table 1
Changes of soluble solid content (SSC), titratable acidity (TA) and dry matter (DM) in control (C) and plasma treated (T) melon samples during storage.

Treatment	Storage (days)	SSC (%)		TA (mg malic acid 100 g fw^{-1})		DM (%)	
		C	T	C	T	C	T
15 + 15 min	0	1.41 ± 0.01^a	1.41 ± 0.03^a	0.0081 ± 0.0002^a	0.0062 ± 0.0004^b	9.8 ± 0.1^a	8.9 ± 0.3^a
	1	1.44 ± 0.05^a	1.45 ± 0.18^a	0.0058 ± 0.0007^a	0.0058 ± 0.0002^a	9.8 ± 0.4^a	9.1 ± 0.2^a
	2	1.51 ± 0.02^a	1.48 ± 0.04^a	0.0075 ± 0.0007^a	0.0050 ± 0.0003^b	8.8 ± 0.2^a	9.3 ± 0.3^b
	4	1.45 ± 0.07^a	1.40 ± 0.06^a	0.0102 ± 0.0002^a	0.0093 ± 0.0003^a	9.0 ± 0.5^a	8.9 ± 0.3^a
30 + 30 min	0	1.50 ± 0.07^a	1.64 ± 0.08^a	0.0080 ± 0.0003^a	0.0081 ± 0.0003^a	9.1 ± 0.6^a	8.9 ± 0.6^a
	1	1.41 ± 0.06^a	1.47 ± 0.07^a	0.0059 ± 0.0002^a	0.0055 ± 0.0003^a	9.2 ± 0.4^a	9.1 ± 0.3^a
	2	1.54 ± 0.03^a	1.35 ± 0.02^b	0.0099 ± 0.0004^a	0.0065 ± 0.0002^b	8.4 ± 0.2^a	10.2 ± 0.3^b
	4	1.46 ± 0.03^a	1.36 ± 0.02^b	0.0109 ± 0.0001^a	0.0110 ± 0.0002^a	8.5 ± 0.3^a	9.2 ± 0.2^b

Different letters indicate significant differences between control (C) and plasma treated sample (T) for each storage time ($p \leq 0.05$).

characterized by a high stability to thermal (Anthon, Sekine, Watanabe, & Barrett, 2002; Lemmens et al., 2009) and pressure treatments, and by a substantial number of different isoenzymes.

As shown in Fig. 2a, POD activity underwent a significant reduction in plasma treated melon samples proportional to the treatment time, as the residual activity was found 91% (15 + 15) and 82% (30 + 30) compared to the control sample.

PME is a cell wall bound enzyme which is able to de-esterify pectins producing methanol and pectins with a lower degree of esterification (Alandes, Pérez-Munuera, Llorca, Quiles, & Hernando, 2009), that are further degraded by other pectolitic enzymes, causing tissue softening.

PME activity (Fig. 2b) was not affected by the 15 + 15 min treatment but after the 30 + 30 min the residual activity was found to be 94%.

The obtained reduction in the enzymatic activity is lower compared to the results obtained in our previous study on polyphenoloxidase (PPO) in Pink Lady apples (up to 54% after 15 + 15 min of treatment) (Tappi et al., 2014). It can hence be assumed that different enzymes present a different resistance to denaturation by plasma agents, possibly due to their different structure and to the presence of isoenzymes. The type of fruit tissue, the specific microstructure, and porosity (e.g. 13.3 ± 0.6 % for melon and 27.3 ± 1.1 % for apple, as found by Muujica-Paz, Valdez-Fragoso, Loopez-Malo, Palou, and Welti-Chanes (2003)) can also play a role in the different treatment response.

Furthermore, the reduction observed in the enzymatic activity did not seem to have any relationship with colour and textural results.

In Table 4 data on respiration rate of fresh-cut plasma treated and control melon samples measured during 24 h at 10 °C are reported. In terms of percentages, at the end of the experiment, the oxygen level within the ampoules was in the range of 14.8 and 17.3% in the control and in the range of 16.1 and 17.8% in the treated samples. Moreover, for either control or treated samples, CO_2 level never exceeded 5% that, according to Iversen, Wilhelmsen, and Criddle (1989) is the threshold for triggering anaerobic metabolism in vegetable tissues. Hence, it is possible to assume that the ratio between product and headspace amount was appropriate to maintain aerobic metabolism during the 24 h considered. However, the treatment effect has changed the normal respiration pathway of the product in aerobic conditions.

Table 2
Textural parameters of control (C) and plasma treated (T) melon samples during storage.

Treatment	Storage (days)	Firmness (N)		Gradient (N s^{-1})	
		C	T	C	T
15 + 15 min	0	13.70 ± 4.47^a	9.28 ± 1.63^b	3.88 ± 0.82^a	3.16 ± 0.87^b
	1	9.84 ± 2.31^a	8.30 ± 2.33^a	2.17 ± 0.64^a	1.86 ± 0.43^a
	2	9.64 ± 2.01^a	9.91 ± 1.25^a	2.32 ± 0.40^a	2.53 ± 0.49^a
	4	9.31 ± 2.80^a	8.23 ± 2.49^a	2.34 ± 0.72^a	2.08 ± 0.51^a
30 + 30 min	0	8.42 ± 1.84^a	9.68 ± 2.61^a	2.51 ± 0.54^a	2.60 ± 0.53^a
	1	9.31 ± 1.47^a	9.44 ± 3.27^a	2.05 ± 0.32^a	2.00 ± 0.59^a
	2	10.18 ± 2.34^a	9.33 ± 2.94^a	2.63 ± 0.51^a	2.15 ± 0.42^b
	4	9.96 ± 3.10^a	9.62 ± 2.95^a	2.30 ± 0.64^a	1.82 ± 0.50^b

Different letters indicate significant differences between control (C) and plasma treated sample (T) for each storage time ($p \leq 0.05$).

Actually, plasma treatment seemed to promote an increase in CO_2 production if compared to control sample, that was statistically significant after 22 h in the 15 + 15 sample and after 1, 3, 5 and 24 h in the 30 + 30 one. O_2 consumption, when significantly different (after 3 h in the 15 + 15 treated sample and after 5 h in the 30 + 30 treated sample), was lower in the treated samples than in the control ones. Generally, it seems that the plasma treatment has caused a higher CO_2 production and a lower O_2 consumption, as can happen as a consequence of a partial conversion of the tissue respiratory metabolism from aerobic to anaerobic. These results confirmed what we found in a previous study on apple tissue subjected to the same kind of treatment (Tappi et al., 2014), hence contributing to highlight the complexity of the tissue response to plasma treatments.

Specific thermal power profiles of melon tissue cylinders during 24 h of analysis at 10 °C are reported in Fig. 3.

For limitations of the instrument sample holders (22 mL vials), it was not possible to evaluate metabolic heat production on samples of the same size and surface–volume ratio as the one used for qualitative evaluation. Hence, considering that plasma treatment is considered to be mainly a surface treatment, the response of the tissue in larger pieces could be different. Nevertheless, the results can give useful information about the comparison of the effect of different treatment times on the melon tissue metabolism, particularly in terms of metabolic activity of regular cylindrical samples.

The heat production of the treated samples was lower compared to the controls for all the 24 h of analysis, proportionally to the treatment time. Metabolic profiles obtained by isothermal calorimetry have been integrated in order to calculate the total metabolic heat produced by fruit tissues during the first 12 h and during 24 h at 10 °C. Differences among samples were more pronounced after 24 h of analysis than after 12 h, as it can be observed in Fig. 4. The variation in the respiratory pathway observed by the respiration rate results could be the cause of the decrease of the heat production detected. Plasma treatment could also affect cell vitality by decreasing it, as it has been observed for different minimal processing operations such as blanching for fresh carrot (Gómez, Toledo, Wadsö, Gekas, & Sjöholm, 2004) and osmodehydration for kiwifruit (Panarese, Tylewicz, Santagapita, Rocculi, & Dalla Rosa, 2012).

3.3. Microbiological evaluation

In order to evaluate the effects of gas plasma treatments on the microbial traits of melon samples, cell viability immediately after treatments and over refrigerated storage was measured for the spoilage-related microflora, i.e. total mesophilic and psychrotrophic bacteria, lactococci, lactobacilli and yeasts (Table 5 and Fig. 5).

Initial populations of total aerobic mesophilic and psychrotrophic microorganisms were about 3.4 and 2.5 Log CFU g^{-1} respectively, while lactic acid bacteria ranged between 2 and 3 Log CFU g^{-1}. On the other hand yeasts were roughly above the detection limit. Such values are in agreement with results reported by other authors for fresh-cut "Piel de Sapo" (Fernández, Picouet, & Lloret, 2010) and cantaloupe

Table 3
Colorimetric parameters of control (C) and plasma treated (T) melon samples during storage.

Treatment	Storage (days)	L*		a*		b*		Hue angle		Chroma	
		C	T	C	T	C	T	C	T	C	T
15 + 15 min	0	58.7 ± 3.3^a	57.4 ± 2.4^a	18.0 ± 1.8^a	17.0 ± 1.0^a	37.3 ± 2.8^a	36.9 ± 2.0^a	244.3 ± 1.3^a	245.2 ± 0.9^b	41.4 ± 3.2^a	40.6 ± 2.2^a
	1	57.4 ± 5.6^a	57.0 ± 5.0^a	17.4 ± 2.2^a	16.7 ± 2.3^a	36.1 ± 3.4^a	34.2 ± 2.7^a	244.2 ± 1.4^a	244.0 ± 1.8^a	40.1 ± 3.9^a	38.1 ± 3.3^a
	2	58.6 ± 4.1^a	58.2 ± 5.5^a	18.1 ± 2.5^a	16.1 ± 1.5^b	38.5 ± 3.5^a	36.1 ± 3.1^a	244.8 ± 1.3^a	245.9 ± 0.7^b	42.5 ± 4.2^a	39.5 ± 3.4^b
	4	57.9 ± 4.7^a	54.4 ± 3.5^b	20.8 ± 1.7^a	17.5 ± 2.7^b	46.6 ± 3.1^a	34.0 ± 3.7^b	245.9 ± 1.1^a	242.7 ± 0.8^b	51.0 ± 3.4^a	38.2 ± 4.5^b
30 + 30 min	0	59.4 ± 2.6^a	59.5 ± 2.9^a	17.2 ± 1.1^a	18.0 ± 1.8^a	37.2 ± 1.8^a	37.7 ± 2.7^a	245.1 ± 0.9^a	244.4 ± 1.0^a	41.0 ± 2.1^a	41.8 ± 3.1^a
	1	56.6 ± 3.4^a	57.3 ± 3.9^a	17.6 ± 1.8^a	17.0 ± 2.1^a	37.6 ± 3.2^a	35.8 ± 3.3^a	244.9 ± 0.7^a	244.6 ± 1.1^a	41.5 ± 3.7^a	39.6 ± 3.8^a
	2	57.7 ± 4.6^a	55.6 ± 3.1^a	17.5 ± 1.2^a	17.0 ± 2.3^a	38.2 ± 2.4^a	36.6 ± 3.7^a	245.3 ± 0.6^a	245.2 ± 1.1^a	42.0 ± 2.6^a	40.4 ± 4.3^a
	4	55.6 ± 6.6^a	52.8 ± 8.0^a	18.6 ± 2.9^a	17.2 ± 3.3^a	35.8 ± 5.0^a	33.6 ± 5.3^a	242.6 ± 1.0^a	242.9 ± 1.1^a	40.4 ± 5.7^a	37.8 ± 6.2^a

Different letters indicate significant differences between control (C) and plasma treated sample (T) for each storage time ($p \leq 0.05$).

(Fang, Liu, & Huang, 2013) melons, except for yeasts that were found at lower levels than literature data.

Overall, gas plasma treatments resulted in marked immediate reductions in cell viability of the indigenous bacteria by increasing the treatment time. The highest inactivation levels were observed for the mesophilic and lactic acid bacteria whose cell loads were under the detection limit in the 30 + 30 min treated samples which correspond to 3.4 and 2 log reductions, respectively. On the other hand cell load reductions not exceeding 1 Log CFU g^{-1} were recorded for the psychrotrophs.

The microbial inactivation observed after plasma exposure has been attributed to the effect of the various reactive species generated during the discharge. As reported in previous studies (Ragni et al., 2010; Ramazzina et al., 2015), the discharge generated by the DBD device showed the presence of nitrogen (N_2^+, NO•) and oxygen (OH•) reactive species. The inactivation mechanism has been related to plasma membrane damage due to oxidation of membrane lipids, and leakage of the intracellular components. Also, the oxidation of amino acids and nucleic acids can contribute to cell injury or death (Critzer et al., 2007).

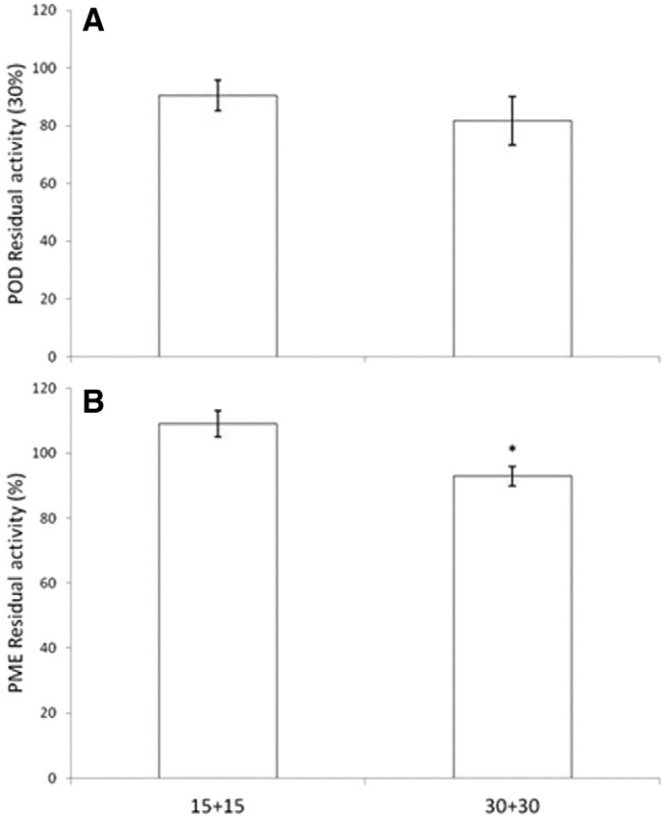

Fig. 2. (a) Peroxidase (POD) and (b) pectin methylesterase (PME) residual activity of melon samples treated with plasma for 15 + 15 and 30 + 30 min. * indicates samples that were significantly different from the control sample at a p-level < 0.05.

The fate of the surviving cells over the refrigerated storage was quite different among the various microbial groups also in relation to plasma processing time. Yeasts showed the worst growth ability as the maximum cell loads attained in the gas plasma treated samples did not exceed 3.5 Log CFU g^{-1} regardless of the treatment time, which was significantly ($p < 0.05$) lower compared to the control fruit (7.5 Log CFU g^{-1}; Fig. 5a). Lactobacilli remained under the detection limit up to 2 days in the plasma treated samples, while they displayed a cell growth over 6 log units in untreated samples after 3 days of storage (Fig. 5b). Nevertheless final counts of 5.4 and 7 Log CFU g^{-1} were attained in 15 + 15 min and 30 + 30 min treated samples, respectively. Similar final cell loads were achieved also by lactococci although they showed slower growth dynamics compared to lactobacilli (Fig. 5c). Total mesophilic and psychrotrophic bacteria presented the highest growth ability reaching levels of 7–7.8, 5.4–5.9 and 7.3–7.6 Log CFU g^{-1} in the control, and in the 15 + 15 min and 30 + 30 min samples, respectively. To better evaluate their recovery dynamics over storage and the effect on the products shelf-life, their cell count data were modelled with the Gompertz equation as modified by Zwietering et al. (1990). Table 5 reports the Gompertz parameters for mesophilic and psycotrophic bacteria in relation to gas plasma treatments as well as the time necessary to reach the value of 6.0 Log CFU g^{-1}, which was chosen as a spoilage threshold according to literature data (Patrignani, Vannini, Kamdem, Lanciotti, & Guerzoni, 2010). As expected, *K* values, corresponding to the initial levels of bacteria surviving the gas plasma processes, decreased by increasing the plasma exposure time. Also λ values were negatively affected by plasma treatments being significantly higher than in the control samples in the case of psychrotrophic bacteria. These data clearly indicate that plasma treatments resulted in an inactivation and/or severe damages which however were repaired by microbial cells during storage. On the contrary, an opposite effect was detected for μmax values. In fact, significantly higher growth rates were found for treated melon samples compared to the control ones, and particularly for those corresponding to the longest process time.

Table 4
Respiration rate expressed as oxygen consumed and carbon dioxide produced during 24 h of storage at 10 °C.

Treatment	Storage time	$RRCO_2$ (mg h^{-1} kg fw^{-1})		RRO_2 (mg h^{-1} kg fw^{-1})	
		C	T	C	T
15 + 15 min	1	59.5 ± 11.19^a	49.1 ± 6.91^a	59.7 ± 0.21^a	48.5 ± 7.44^a
	3	38.2 ± 12.99^a	38.3 ± 1.23^a	34.5 ± 2.22^a	23.6 ± 1.59^b
	5	35.4 ± 3.659^a	39.2 ± 4.58^a	32.0 ± 0.54^a	27.4 ± 4.44^a
	22	25.5 ± 0.85^a	31.9 ± 2.10^b	22.1 ± 0.69^a	18.6 ± 0.24^b
	24	25.3 ± 4.37^a	29.4 ± 0.19^a	21.2 ± 1.82^a	19.2 ± 1.42^a
30 + 30 min	1	41.0 ± 15.51^a	66.8 ± 6.43^b	27.8 ± 4.93^a	35.5 ± 2.03^a
	3	29.5 ± 3.54^a	57.2 ± 2.12^b	25.9 ± 1.75^a	26.4 ± 1.16^a
	5	30.6 ± 2.68^a	44.1 ± 4.72^b	24.7 ± 1.77^a	21.2 ± 0.29^b
	22	19.5 ± 3.26^a	32.5 ± 10.59^a	16.9 ± 2.44^a	18.4 ± 0.29^a
	24	20.8 ± 2.20^a	36.9 ± 5.52^b	17.5 ± 1.61^a	17.1 ± 0.29^a

Different letters indicate significant differences between control (C) and plasma treated sample (T) for each storage time ($p \leq 0.05$).

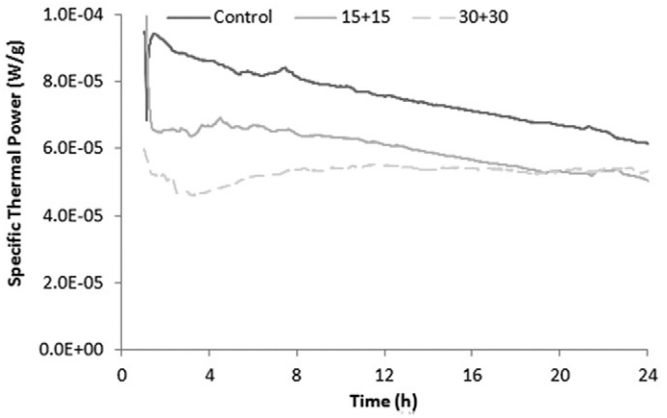

Fig. 3. Specific thermal power profiles of melon tissue cylinders in relation to gas plasma treatments during 24 h of analysis at 10 °C.

Compared to the rinsing with traditional or emerging sanitizers (Silveira, Aguayo, & Artés, 2010), both the plasma treatments provided higher reductions of the initial contaminating microflora. In addition, the processing conditions used in this study negatively affected the fate of lactic acid bacteria and yeasts which are usually reported to be the main spoilage species for fruits including fresh-cut melon (Zhang et al., 2013). Likewise the growth of the mesophilic and psychrotrophic bacteria was markedly limited following the 15 + 15 min treatment thus leading to a significant increase in the melon samples shelf-life. However, unexpectedly the increase in processing time, although it allowed an immediate higher microbial inactivation, did not offer any additional advantage in terms of microbial shelf-life, despite no appreciable differences were observed for the qualitative indices. The current literature on cold plasma treatments on several raw fruit and vegetables reports that the inactivation level is generally time-dependent although non-linear inactivation curves are reported (Baier et al., 2014; Lee, Kim, Chung, & Min, 2015), but the evaluation is often limited to the immediate effect after the exposition and ignores the behaviour of the surviving microbial cells during further storage.

The overall effect observed in this study after the longest plasma treatment on microbial shelf-life was negligible if compared to that of untreated samples and comparable shelf-life values were obtained. Conversely, the 15 + 15 min exposure to gas plasma gave rise to a significant extension of the microbial shelf-life. In fact, the critical spoilage threshold was reached beyond four days in the treated samples, while the untreated melons were spoiled after 2.5–3 days.

This phenomenon could be related to tissue damages (e.g. cell wall weakening) caused by prolonged treatments resulting in the higher

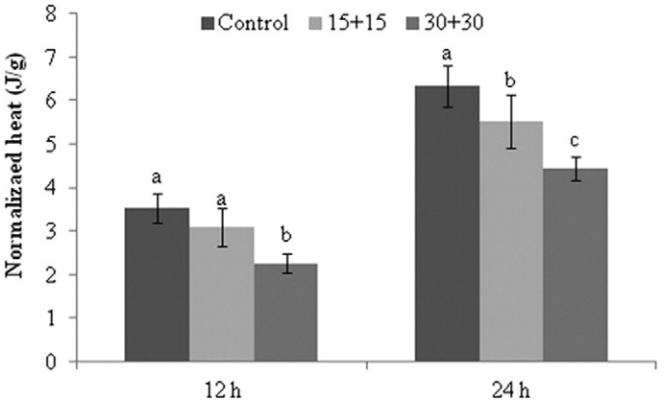

Fig. 4. Normalized heat produced by melon samples during 12 and 24 h of analysis at 10 °C. Different letters indicate samples that were significantly different at p-level < 0.05.

Table 5
Gompertz parameters of mesophilic and psychrophilic bacterium recovery dynamics in melon samples during storage at 10 °C in relation to the gas plasma treatment.

Microbial group	Gas plasma treatment time (min)	K	A	μmax	λ	R	Shelf-life (days)**
Mesophilic bacteria	Control	3.36	4.44	1.24	0.14	0.973	2.3
	15 + 15 min	1.48	3.92	1.55	0.20	0.998	>4§
	30 + 30 min	n.d. *	7.61	2.39	0.03	0.991	2.9
Psycotrophic bacteria	Control	2.48	4.34	1.45	0.07	0.987	2.9
	15 + 15 min	2.08	3.82	2.59	1.83	0.985	>4§
	30 + 30 min	1.48	5.77	3.69	1.25	0.999	2.6

Data are the mean of three different samples. The variability coefficients ranged between 5% and 7%.
K = initial cell level (Log CFU g^{-1}) after gas plasma treatments.
A = maximum cell increase attained at the stationary phase with respect to the initial cell load (Log CFU g^{-1}).
μmax = maximum growth rate (Δ Log [CFU g^{-1}] per day).
λ = lag phase length (days).
R = correlation coefficient.
* n.d. Under the detection limit.
** Time necessary to attain a cell count of 6 Log CFU g^{-1}, calculated using the predicted Gompertz parameters.
§ Since the threshold level of 6 Log CFU g^{-1} was not attained within 4 days, shelf-life could not be predicted by using mathematical models.

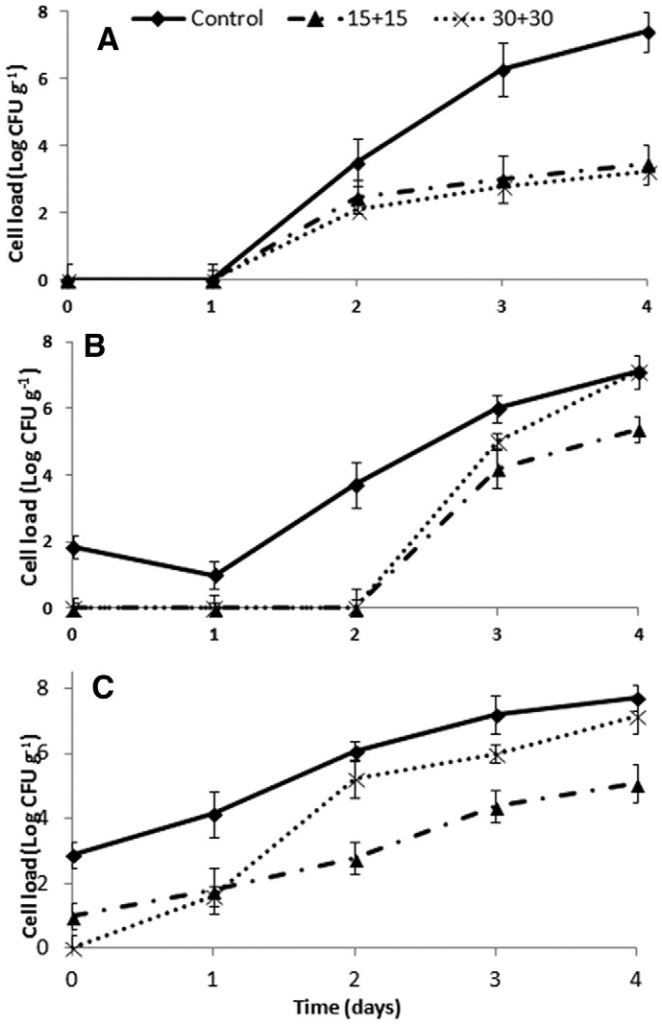

Fig. 5. Cell numbers of yeasts (A), lactobacilli (B) and lactococci (C) during storage at 10 °C of melon samples.

water loss recorded for the 30 + 30 min treated melons which probably made fruit more susceptible to microbial spoilage.

These results highlight the necessity of modulating treatment time not only by its immediate effects but also on the further shelf-life evaluation.

4. Concluding remarks and future perspectives

Among the fresh-cut products, melon is considered as highly perishable and potentially hazardous food, as demonstrated by the number of foodborne disease outbreaks registered in the recent years in developed countries.

The overall results obtained in this study indicate that the tested cold plasma treatment is very promising in order to stabilize fresh-cut melon, allowing efficient decontamination. Moreover, by modulating the treatment time a significant enhancement of microbial shelf-life was achieved due to a delayed growth over storage of the surviving spoilage microflora.

As far as quality characteristics are concerned, the treatment effect was very limited and mainly related to a slight increase of both dry matter content and translucent appearance during storage.

In addition, a slight reduction of enzymatic activity was observed but this effect was dependent on the type of enzyme considered, and did not seem to have any positive reflection on related qualitative parameters. A reduction of metabolic heat was obtained along with an alteration of the respiratory pathway, indicating a stress response of the tissue to the treatment that should be further clarified.

The potential application on in-packed cold plasma technology showed by Pankaj et al. (2014) makes this technique very encouraging for fresh-cut commodity stabilization, aiming to replace traditional chemical sanitizers such as chlorine and hydrogen peroxide.

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Paper VI

Effect of cold plasma treatment on the functional properties of fresh-cut apples

Manuscript

Effect of cold plasma treatment on the functional properties of fresh-cut apples

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Abstract

The atmospheric double barrier discharge (DBD) plasma technology is a promising tool in food industry as an alternative to traditional food preservation methods. However, the effect of the reactive chemical species generated during the treatment on the quali-quantitative content of bioactive compounds in food is still little studied. In addition, to the best of our knowledge there are no studies concerning the effect of treatment-generated chemical species on physiological cell functions.

The aim of this study is to investigate the effect of plasma technology on antioxidants content and antioxidant activity of minimally processed Pink Lady apples. Moreover, we assessed the role exerted by polyphenolic extracts from treated apples on cell viability and cell response to oxidative stress. According to the obtained results, the plasma treatment causes only a slight reduction of antioxidant content and antioxidant capacity. Noteworthy, the plasma treated polyphenolic extracts do not impair the cellular response triggered by redox homeostasis imbalance.

Keywords: fresh-cut apples, cold gas plasma, polyphenols content, in-vivo, ex-vivo, antioxidant activity

1.Introduction

The major issue for the food science is to maintain important food quality attributes, to increase the level of food safety and to enhance the products shelf-life. In the last decade, non-thermal technologies for food stabilization have been developed in response to the worldwide interest for more fresh-like and natural food products, minimizing the typical thermal alterations such as sensorial changes, formation of off-flavours and losses of nutritional components (Pereira, & Vicente, 2010; Surowsky, Schlüter, & Knorr,, 2014).

Among non-thermal treatments, cold gas plasma presents several advantages. Gas plasma is an ionised gas characterized by active particles such as electrons, ions, free radicals and atoms which are both in fundamental and excited states; the ionization occurs by applying energy to a gas or to a gas mixture (Moreau, Orange, & Feuilloley, 2008). Actually, when atmospheric air is used as working gas, reactive oxygen species (ROS) and reactive nitrogen species (NOS) are formed. These oxidative species can cause lipids peroxidation and proteins and DNA oxidation (Montie, Kelly-Wintenberg, & Roth, 2000) and may potentially interact with bioactive compounds altering their content/effect in food products.

Operative and configuration conditions of the atmospheric plasma generators and the assessment of the efficacy of the ionized gas on model systems or real food products, in terms of microorganism decontamination, were extensively reviewed (Moreau Orange, & Feuilloley, 2008; Scholtz, Pazlarova, Souskova, Khun, & Julak, 2015; Surowsky, Schlüter, & Knorr, 2014). In addition, a lot of studies have been published concerning the impact of plasma on the quality of both solid and liquid foods (Surowsky, Schlüter, & Knorr, 2014). These applications were mainly addressed to the stabilization of model system or food products, through the reduction of the activity of oxidative enzymes, such as polyphenol oxidase in fresh-cut apples (Bußler, Schnabel, Ehlbeck, & Schlüter 2013; Tappi et al., 2014), peroxidase in tomatoes (Pankaj, Misra, & Cullen, 2013) and polyphenol oxidase and peroxidase in a polysaccharide gel model food system (Surowsky, Fischer, Schlüter, & Knorr, 2013b).

Nevertheless the effect of gas plasma treatment on the bioactive compounds and nutritional properties of food products has to be deeply studied, particularly for its application to fresh-like commodities.

Among them, minimally processed fruit and vegetables are one of the major growing segments in food retail establishments, playing an important role on the antioxidant intake of an increasing number of consumers (Kanlayanarat et al. Acta Hort. 746, ISHS 2007). For this products, that present an endogenous metabolic activity during the shelf-life period, it is crucial to understand not only the direct effect of plasma treatment on the level and the antioxidant properties of their bioactive components, but also their modification during storage as consequence of the tissue response to the processing stress.

In this context, interesting works regard the influence of cold plasma reactive species on the stability of pure phenolic compounds and on the total phenolic content of lamb's lettuce (Grzegorzewski, Ehlbeck, Schlüter, Kroh, & Rohn, 2011; Grzegorzewski et al., 2009). The authors demonstrated by high-performance liquid chromatography a time- and structure-dependent degradation of phenolic compounds, although the plant matrix seemed to protect them from oxidation caused by plasma-induced reactive species. Double barrier discharge (DBD) cold plasma treatments of minimally processed kiwifruit, in addition to no induction of textural changes and to the improvement of the product visual quality, did not promote significant changes in antioxidants (ascorbic acid and polyphenols) content and antioxidant activity of the product (Ramazzina et al., 2015). Plasma treatment applied to freshly squeezed orange juice had almost no effect on vitamin C content. This study showed how the effect of plasma on ascorbic acid was strongly dependent on the type of food matrix (Surowsky, Schlüter, & Knorr, 2014).

In this regard, the possible preservation of food polyphenols and vitamins after plasma treatment on minimally processed fruit and vegetable is of particular interest due to their potential effect on health properties. Oxidative stress is involved in many diseases, such as atherosclerosis, diabetes, neurodegenerative diseases, aging and cancer (Francini & Sebastiani, 2013; Lall, Syed, Adhami, Khan, & Mukhtar, 2015; Rahman, Hosen, Islam, & Shekhar, 2012). A growing number of studies point out ROS as key compounds of resilience and human pathologies (de Roos & Duthie, 2014).

Dietary polyphenols, in particular flavonoids, may protect against oxidative stress by scavenging ROS, chelating trace elements involved in free radical generation and inducing cellular antioxidant defence by modulation of redox-sensitive gene expression (Kumar & Pandey, 2013; Rahman Hosen, Islam, & Shekhar,2012). However, pro-oxidant activities of polyphenols have also been reported (de Roos & Duthie, 2014).

According to Niemira (2012), the U.S. Food and Drug Administration has not yet allowed the use of cold plasma for food processing because of the lack of knowledge on the primary modes of action and on the effects on sensory and nutritional properties of the products. In particular, researches about the impact on antioxidant properties and bioactive compounds, on the potential chemical residue effects and on the formation of toxicants are therefore needed in order to provide sufficient information to assess the health-related implication of the process.

Previously, we provided evidence that gas plasma treatment on fresh-cut Pink Lady apples causes an inhibition of polyphenol oxidase activity with a consequent improvement of the product visual quality (Tappi et al., 2014). Nevertheless, the consequence of treatment on bioactive compounds content has not yet been investigated. The purpose of this study was to determine DBD cold plasma effects on antioxidants content and antioxidant activity of fresh-cut Pink Lady apples. The phenolic composition of treated and control samples were analysed by HPLC-MS (high-performance liquid chromatography-mass spectrometry) in order to better understand the plasma effect on the single polyphenols level. Additionally, the *in vitro* antioxidant activity was evaluated through a multimodal approach, combining different assays for the analysis of antiradical activity and reducing activity of antioxidants. Moreover, for the longest treatment time investigated (30 min) we assessed the ability of polyphenolic extracts from treated and untreated apples to protect Caco2 cells, which show biochemical characteristic of normal adult enterocytes, against oxidative stress.

2. Materials and methods

2.1 Chemicals

Chemicals of analytical grade were purchased from Sigma-Aldrich (Steinheim, Germany) except for hydrochloric acid and methanol, which were purchased from Romil (Feltham, UK).

Theaphenon E®, a standardized green tea extract preparation ((-)-epigallocatechin-3-gallate (EGCG), 68.58%; (-)-epigallocatechin (EGC), 10.56%; (-)-epicatechin (EC), 4.31%; (-)-epicatechin-3-gallate (ECG), 5.95%; (-)-gallocatechin-3-gallate (GC) and other trace catechin derivatives) was a kind gift from Doctor Hara (Tea Solutions, Hara Office Inc). Fresh 1 mg/mL Polyphenon E® stock solution was prepared in de-ionized sterile water and diluted immediately in complete medium at the final concentration required for each experiment.

2.2 Raw material, handling and storage

Apples (*Malus domestica* cv. 'Pink Lady®') harvested two weeks before, were provided by the local market. Fruits free from defects were transported to our laboratory and stored in a refrigerated chamber at 5±1°C and saturated atmosphere in darkness for one week. Apples were characterized by a dry matter content of 15.73 (± 0.29) g 100 g^{-1} fw, a soluble solid content of 14.27 (± 0.35) °Brix and a titrable acidity of 0.39 (± 0.03) mg malic acid g^{-1} fw.

2.3 DBD gas plasma generator and treatments

Cold plasma was generated by a Dielectric Barrier Discharge (DBD) device that was already described and characterized by Ragni et al. (2010). It consists of an hermetic chamber containing three parallel pair of electrodes (brass) supplied by a DC power supply and powered by high voltage transformers and power switching transistors. A 5 mm thick glass was used as dielectric material. As feed gas, atmospheric gas driven at 1.5 slm was chosen. Frequency of oscillation was 12.7 kHz and the supply power was in the range of 150 W.

The discharge was characterized by emission spectroscopy in previous studies (Ragni et al., 2010) that showed the presence of oxygen and nitrogen radicals and ions as commonly detected when atmospheric air is used to generate plasma.

2.4 Sample preparation and plasma treatments

Apple slices (40×10×10 mm) were manually obtained from apple flesh using a sharp blade. For each treatment time, 15 slices were used. Samples were exposed to cold plasma at a distance of 70 mm from the electrodes for a total of 10 (5+5 on each side), 20 (10+10 on each side) and 30 (15+15 on each side) min.

In the treatment chamber, temperature was 22°C and RH 60%. Control sample were stored at the same temperature and humidity conditions for the duration of the treatment.

Treatment time was stressed to 120 (60 + 60) min of processing only for polyphenols and *in-vitro*-antioxidant activity determinations.

2.5 Physico-chemical parameters

Water content was obtained gravimetrically on about 5 g of finely chopped apples exactly weighted, after drying at 70°C until constant weight (AOAC International 2002). Soluble solid content was measured on the juice obtained from apple slices, after filtering through Whatman #1 filter paper, with a digital refractometer (Atago Co. Ltd, Tokyo, Japan). Titratable acidity was determined according to AOAC (International Method 942.15, 2000) Maturity index was expressed as the ratio between SSC and TA (Sweeney, Chapman, & Hepner, 1970) and apple juice pH was measured with a Crison pH-meter.

For each sample, soluble solids, titratable acidity and pH were carried in quintuple for each sample on the juice obtained by nine apple slices, taken from the three replicated treatments.

Surface colour was measured in control and treated samples using a Chroma Meter CR-400 reflectance colorimeter (Minolta Italia, Milano, Italy) with a D65 illuminant and the 10° standard observer. In order to verify the effect on enzymatic browning, colour was monitored just after treatment and every hour up to the following 4 h on six slices for sample.

The remained 30 apple slices (in total 45; 15 x treatment x three repetitions) for each treatment condition have been immediately freeze dried and used for the phenolic and antioxidant analysis.

2.6 Polyphenolic content by HPLC

Polyphenolic extract preparation

Freeze-dried apple powder (250 mg) was weighed into an eppendorf tube. A total of 1.5 mL of 60% of aqueous methanol with 1 % (v/v) of formic acid was added, and the suspension was vortexed vigorously for 2 min. Tubes were left 60 min in a sonic bath. The extract was centrifuged for 20 min (20.878 *g*), and supernatant was collected at 4°C and transferred to a vial before the injection into the HPLC system.

High-performance liquid chromatography and mass spectrometry analysis

Before analysis 20 µl of each internal standard were added to the samples (genistein: 580 µg/ml; genistin: 380 µg/ml).

HPLC separations were carried out by means of a SUNSHELL C18 (2.1 i.d. × 100 mm) column, 2.6 µm particle size with mobile phase, pumped at a flow-rate of 0.3 mL/min, consisting of a mixture of acidified acetonitrile (0.1% formic acid) (solvent A) and 0.1% aqueous formic acid (solvent B). Following 0−2 min, 2% B; 2−13 min, 2% to 30% B; 13−20 min, 30% to 80% B; 20−22 min, 80% to 2% B; 22−30 min, 2% isocratic; this step was followed by the washing and reconditioning of the column.

The identity of the phenolic compounds was confirmed using a triple quadrupole mass spectrometer (Thermo Scientific, TSQ Vantage) with a heated electrospray ionization (H-ESI II) operating in the negative ionization mode. The capillary temperature was 270 °C; the sheath gas and auxiliary gas were 40 and 5 arbitrary units, respectively; and the source voltage was 3kV, Vaporizer Temperature 200°C argon was used for MS MS experiments with a Collision Pressure of 1.0.
For the identification a full scan analyses was performed scanning from m/z 100 to 950, while a product ion scan experiment was applied for ions not fully identified in the previous method. Identification was performed by comparing the mass spectra with literature data, and whenever possible, the identification was confirmed by using pure standards of the components.
Quantification was achieved in Selected Ion Monitoring mode according to the concentrations of a corresponding internal standard, respectively, genistin for glucoside and genistein for aglicone.

2.7 *In vitro*-Antioxidant activity and total phenolics index

Antioxidant activity and total phenolics index of apple samples was assessed by different *in vitro* microplate assays, using the methods previously reported (Ramazzina et al., 2015). The antioxidant activity was performed by ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), DPPH (2,2-diphenyl-1-picrilhydrazyl) and FRAP (ferric reducing antioxidant power) methods. The total polyphenols content was quantified on both amphiphilic and hydrophilic extract by the Folin-Ciocalteu phenol reagent.

2.8 *Ex vivo*-antioxidant activity

Preparation of extracts

For ex-vivo analysis, 3 g of freeze-dried apple samples were added with methanol 60% (v/v) and vortexed for 2 min. mixtures were vigorously shaken for 10 min and then centrifuged for 10 min at room temperature (10000g). the supernatant was collected and the pellet was subjected to a second extraction. The total supernatant was dried in a rotary evaporator .

Cell line and culture

Caco2 cells were a kind gift from Professor Bussolati (University of Parma, Parma, Italy). Cells were routinely grown in 1:1 mixture of Ham's F12:DMEM medium. Culture media was supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37°C under a 5% CO_2 atmosphere. Cell harvesting was performed by Trypsin/EDTA (Sigma-Aldrich, Steinheim, Germany) treatment.

For the reactive oxygen species determination and luciferase assay the Caco2 cells were grown in 1:1 mixture of Ham's F12:DMEM medium without red phenol (Sigma-Aldrich, Steinheim, Germany).

WST-1 assay

Inhibition of cell proliferation by polyphenols extracts was measured by WST-1 assay (Roche, Lewes, United Kingdom). The assay is based on the reduction of tetrazolium salt WST-1 to soluble formazan by electron transport across the plasma membrane of actively dividing cells. Formazan formation was detected at 450 nm spectrophotometrically. Caco2 cells were plated in triplicate in 96-well microplates at a density of $4x10^4$ cells/well and allowed to adhere overnight. Cells were treated with increasing concentrations of Theaphenon E® or polyphenolic apple extracts. After 5 h of incubation the WST-1 assay was performed.

Reactive oxygen species determination

The production of intracellular ROS was detected using the 2,7-dichlorofluorescein diacetate (DCFH-DA) assay. Briefly, Caco2 cells were seeded in black 96-well plates ($4x10^4$ cells/well) and allowed to attach overnight. After 5 hours of treatment with increasing concentrations of Theaphenon E® or polyphenolic apple extracts, cells were washed twice with PBS and loaded with 20 mM DCFH-DA in PBS for 15 minutes at 37°C. After incubation, cells were washed with PBS and ROS generation was measured by the fluorescence intensity of dichlorofluorescein (DCF, exc. 475 nm, em. 535 nm) using an Enspire Multimode Plate Reader (Perkin Elmer, Waltham, Massachusetts). Inside the cells, DCFH-DA is cleaved by nonspecific esterases forming non-fluorescent DCFH, which is oxidized to the fluorescent compound DCF by ROS. In the same wells the total protein content was quantified using the Bio-Rad DC Protein assay (Bio-Rad, Berkeley, California).

Plasmid construction and luciferase assay

To generate the recombinant plasmid pGL4-NQO1, genomic DNA was extracted from human liver using the QIAamp DNA mini Kit (Qiagen, Venlo, the Netherlands) according to manufacturer's protocol. The upstream promoter region of the Nqo1 gene (635 pb) was PCR amplified using the following primers: fw 5'-ACCTGCCTTGAGGAGCAGGGGTGGTGCAG-3', rv 5'-GGCTCTGGTGCAGTCCGGGGCGCTGATTGG-3' (Dhakshinamoorthy & Porter, 2004). The PCR product was subcloned in the pCR®2-TOPO vector (Invitrogen, Carlsbad, California). The

KpnI/XhoI restriction fragment obtained from digestion of TOPO-NQO1 was then ligated into the pGL4.10 vector (Promega, Madison, Wisconsin) and the resulting plasmid was sequenced.

Caco2 cells were seeded in a 96-well white microplate at a density of $2x10^4$ cells/well and transfected using Viafect (Promega, Madison, Wisconsin), using 0,2 μg of pGL4-NQO1 and pGL4.10 empty vectors. Transfection efficiency was monitored by pEGFP-N1 transfection (Clontech Laboratories, Mountain View, California).

The luciferase activity was measured after 5 hours incubation with increasing concentrations of Theaphenon E® or polyphenolic apple extracts using the Britelite™ plus reactive (PerkinEmler, Waltham, Massachusetts) and the EnSpire® Multimode Plate Readers (PerkinEmler, Waltham, Massachusetts). The luciferase activities were normalized to the total protein content after checking for equal transfection efficiency in each well. The results are representative of three independent experiments run in quadruplicate.

Determination of cellular reduced glutathione (GSH) content

Caco2 cells were seeded in a 96-well white microplate at a density of $2x10^4$ cells/well; the cellular GSH and GSSG were quantified under basal condition or after 5 hours incubation with increasing concentrations of Theaphenon E® or polyphenolic apple extracts using GSH/GSSG-Glo Assay (Promega, Madison, WI), according to manufacturer's protocol. GSH and GSSG levels were normalized to protein concentrations and the GSH/GSSG ratio was calculated.

RNA extraction, reverse transcription and quantitative real-time PCR

Caco2 cells were seeded in a 35 mm dishes at a density of $6x10^5$ and allowed to attach overnight. After 5 hours of treatment with two different concentrations of treated or untreated polyphenolic apple extracts, total RNA was extracted with the Trizol Reagent (Fisher Molecular Biology, Rome, Italy) and cleaned-up with the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. For reverse transcription reaction, 100 ng of total RNA from each experimental condition was combined with 1 μL of random primers (0.2 μg/μL) and heated up to 65°C for 5 min. Following a brief chill on ice, the Reverse Transcription mix (Thermo Scientific, Boston, MA) was incubated at 25°C for 5 min, 45°C for 60 min and 70°C for 10 min. The first strand synthesis reaction was diluted 1:2 than 2 μL of each cDNA preparation were used for quantitative real-time PCR with the set of primers described below. The thermal cycling comprised an initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s. Analysis of results was performed by DNA Engine Opticon 4 (MJ Research, Walthman, MA) using the 2X SYBR Premix Ex Taq (Takara Bio

Inc, Japan). Relative quantification was calculated by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeper gene for data normalization. The results are expressed as mean ± SD of two independent determinations each performed in duplicate.

Primers sequences used for GST members were the following (Scharmach, Hessel, Niemann, & Lampen, 2009):

GSTT2-fw 5'-CTTTCCTGGGTGCTGAGCTA-3' GSTT2-rv 5'-GGTGTTGGGAGGGTTTTCTT-3'

GSTP1-fw 5'-GGAGACCTCACCCTGTACCA-3' GSTP1-rv 5'-CTGCTGGTCCTTCCCATAGA-3'

GSTA4-fw 5'-TCCGTGAGATGGGTTTTAGC-3' GSTA4-rv 5'-GGTGGTTACCATCCTGCAAC-3'

Primers sequences used for UGT members were the following (Ohno & Nakajin, 2009):

UGT1A1-fw 5'-AATAAAAAAGGACTCTGCTATGCT-3' UGT1A1-rv 5'-ACATCAAAGCTGCTTTCTGC-3'

UGT1A4-fw 5'-GAACAATGTATCTTTGGCCC-3' UGT1A4-rv 5'-ACCACATCAAAGGAAGTAGCA-3'

UGT2B7-fw 5'-GGAGAATTTCATCATGCAACAGA-3' UGT2B7-rv 5'-CAGAACTTTCTAGTTATGTCACCAAATATTG-3'

2.9 Statistical analysis

Data are expressed as mean values ± SD for the indicated number of independent determinations. One way (ANOVA) for the *in vitro* analysis was carried out to test the significance of the effects of treatment time. Significant differences ($p<0.05$) between mean values were tested by the LSD test. ANOVA for the *ex vivo* analysis was carried out to test the significance of the effects of treatments versus control. Significant differences ($p<0.001$) between mean values were tested by Holm-Sidak method. Statistical analyses were carried out using the software STATISTICA for Windows (StatsoftTM, Tulsa, Oklahoma).

3. Results and discussion

3.1 Chemical parameters

Chemical parameters of apple samples are reported in Table 1. Water content and maturity index (SSC/TA) were not affected by the treatment and did not show any significant difference compared to control sample at all treatment times.

Table 1. Physico-chemical parameters of Pink Lady apples as affected by plasma treatment time.

	time (min)			
	0	10	20	30
Water content (%)	83.73± 0,29[a]	83.53 ± 0,14[a]	84.84 ± 0,43[a]	83.63 ± 0,40[a]
MI (SSC/TA)	34.88 ± 0.5[a]	33.08 ± 0.64[a]	37.68 ± 1.62[a]	34.54 ± 0.40[a]
pH	3.73 ± 0.03[a]	3.64 ± 0.05[b]	3.56 ± 0.04[b]	3.65 ± 0.05[b]
L*	77.89 ± 1.05[a]	76.93 ± 1.52[a]	76.87 ± 0.88[a]	79.48 ± 1.51[b]
a*	0.59 ± 0.84[a]	0.08 ± 0.70[a]	-0.56 ± 1.00[a]	-2.49 ± 0.89[b]
b*	26.75 ± 2.71[a]	27.03 ± 2.02[a]	27.16 ±1.49[a]	24.54 ± 2.20[b]

Values followed by different letters within the same row are significantly different at a $p<0.05$ level.

A slight acidification was observed upon plasma exposure independently by treatment time. While some other quality parameters, such as colour and texture have been studied in a wide range of food products, the impact of plasma on product pH has not been investigated often. No differences were observed by Gurol, Ekinci, Aslan, & Korachi (2012) in milk or other model liquid systems treated with argon plasma. However, Satoh, MacGregor, Anderson, Woolsey, & Fouracre (2007) found a strong decrease of PBS buffer pH from 7.3 to 3 to 4 and an increase of conductivity as a consequence of pulsed plasma exposure. A slight acidification was observed also in pork loins treated with a DBD device with helium and oxygen as working gas (Kim, Yong, Park, Choe, & Jo., 2013). The authors suggested that those changes were due to the dissolution of acidogenic moleculs such as nitrogen oxides, generally generated in plasma discharges, in the food system.

Colour was measured four hours after treatment in order to evaluate a possible effect on fresh cut apples browning. No differences were observed up to 20 min of treatment; however samples exposed for 30 min were characterized by higher L* values and lower a* and b* values compared to untreated sample.

These results confirm those obtained in a previous study of plasma effects on browning inhibition (Tappi et al., 2014), where at the same experimental condition, a reduction in browning was observed after 30 min treatment by image analysis. In the same study, an inhibition of polyphenoloxidase (PPO) activity proportional to treatment time up to 57% for 30 min was reported.

Reduction of enzymatic activity upon plasma exposure has been observed in various studies (Pankaj, Misra, & Cullen, 2013 Surowsky, Fischer, Schlueter, & Knorr, 2013) and it has been

attributed to the oxidation of reactive side-chain of the amino acids by plasma radicals, in particular OH, O_2^-; HOO and NO, that promote a change in the secondary protein structure and the modification of some amino acids side chains of the enzyme (Deng, Shi, Chen, & Kong,. 2007; Takai, Kitano, Kuwabara, & Shiraki, 2012). In particular, Surowsky, Fischer, Schlueter, & Knorr, (2013) found a variation in the relative amounts of alfa- helix structures and β-sheet content upon plasma exposure, that was strongly correlated to the loss of enzymatic activity. Generally the inhibitory effect is dependent on the type of enzyme considered and on the matrix effect (Tappi et al., 2015).

3.2 Phenolic content

The phenolic content of apples was measured by HPLC-MS/MS analysis and the content of each detected phenolic is reported in Table 2. The phenolic content of Pink Lady apples was of 2.14 mmol $kg^{-1}_{f.w.}$ (144 mg $kg^{-1}_{f.w.}$) which is higher than literature data (90 mg $kg^{-1}_{f.w.}$) reported in a previous study (Veberic et al., 2005). However these values are lower than those of the most widely studied variety, Golden Delicious, whose values range between 233 and 417 mg $kg^{-1}_{f.w.}$ depending on agricultural practices and harvesting years (Lee, Kim, Kim, Lee, & Lee, 2003; Tsao, Yang, Young, & Zhu, 2003; Chinnici, Gaiani, Natali, Riponi, & Galassi, 2004; Ceymann, Arrigoni, Schärer, Nising, & Hurrell , 2012).

In order to verify if the DBD plasma treatment, carried out in the experimental conditions of this study, could have determined a significant decrease of polyphenols, the product was over treated for 120 (60 + 60) min of processing and a significant decrease (20% ca.) of total phenolic was observed.

At the investigated treatment time, suitable for fresh-cut apple stabilization (Tappi et al., 2015), the treatment significantly affected the total phenolic content of apples with a significant increase (21% ca.) after 10 (5 + 5) min of processing, slightly reduced in the 20 (10+10) min treated sample, to reach values after 30 (15 + 15) min non significantly different from those of raw apples (Table 2).

Specifically, the initial phenolic increase after 10 min of processing was mainly due to a significant increase of procyanidin B trimer, flavonols, hydoxycinnamic acids and phloretin xylosyl glucoside.

The increase of procyanidin B trimer is the most outstanding in relative terms and corresponds, in molar terms, to a reduction of catechin, epicatechin and dimers B1 and B2, suggesting the occurrence of polymerization reaction. When calculated in percentage on total procyanidins, the content of trimeric procyanidins increased while those of dimeric and monomeric procyanidins decreased. According to Nicoli, Calligaris, & Manzocco (1999), the formation of procyanidins upon catechins polymerization occurs in apples due to both enzymatic and chemical oxidation reactions;

the latter could easily take place during plasma treatment, thanks to the production of radical species and the availability of oxygen in the atmosphere.

Table 2. Content of phenolics (μmol $kg^{-1}_{f.w.}$) of Pink Lady apples as affected by plasma treatment time.

		time (min)				
	Compound	0	10	20	30	120
Flavan-3-ols	Catechin	46.6^{ab}	40.5^{bc}	48.8^{a}	36.9^{c}	26.8^{d}
	Epicatechin	365^{a}	353^{ab}	398^{a}	303^{b}	229^{c}
	Procyanidin dimer B1	40.1^{a}	37.8^{a}	39.0^{a}	31.2^{b}	17.8^{c}
	Procyanidin dimer B2	138^{a}	135^{ab}	141^{a}	113^{b}	70.9^{c}
	Procyanidin dimer B4	9.61^{a}	9.86^{a}	9.40^{ab}	7.63^{b}	4.63^{c}
	Procyanidin B trimer	8.62^{a}	8.14^{a}	7.81^{a}	6.57^{b}	3.53^{c}
	Procyanidin B trimer 2	15.3^{a}	14.8^{a}	14.4^{a}	11.4^{b}	7.01^{c}
	Procyanidin B trimer 3	49.6^{a}	49.0^{a}	46.3^{a}	37.0^{b}	20.3^{c}
	Procyanidin B trimer 4	7.00^{a}	7.30^{a}	6.50^{ab}	5.40^{b}	2.68^{c}
	Procyanidin B trimer 5	0.39^{b}	15.05^{a}	13.52^{b}	0.14^{b}	0.05^{b}
Hydroxycinnamic acids	Caffeic acid	0.96^{a}	0.55^{b}	1.13^{a}	0.98^{a}	1.02^{a}
	Caffeoylquinic acid isomer 1	292^{b}	384^{a}	291^{b}	263^{bc}	238^{c}
	Caffeoylquinic acid isomer 2	613^{bc}	832^{a}	685^{b}	620^{bc}	591^{c}
	4-Coumaroyl quinic acid	91.9^{a}	99.2^{a}	97.7^{a}	77.9^{b}	80.8^{b}
	Coumaroyl quinic acid	247^{b}	302^{a}	268^{ab}	257^{a}	283^{ab}
Dihydrochalcones	Phloretin-2'-O-(2''-O-xylosyl)glucoside	145^{b}	196^{a}	187^{a}	197^{a}	139^{b}
	Phloridzin	35.7^{c}	49.9^{bc}	54.5^{b}	81.2^{a}	25.8^{c}
Flavonols	Myricetin rhamnoside	2.89^{bc}	4.95^{a}	3.64^{b}	2.94^{bc}	2.30^{c}
	Quercetin	2.77^{bc}	4.22^{a}	3.49^{ab}	3.55^{a}	2.33^{c}
	Quercetin-O-glucoside	2.90^{b}	6.22^{a}	3.70^{b}	2.97^{b}	2.28^{b}
	Quercetin-O-rhamnoside	27.0^{b}	37.2^{a}	32.8^{ab}	33.2^{a}	24.2^{b}
	Rutin	0.13^{a}	0.06^{ab}	0.14^{a}	0.05^{b}	0.00^{c}
Total phenolics	hydrophilic + amphiphilic	2142^{b}	2588^{a}	2352^{ab}	2092^{b}	1774^{c}

Values followed by different letters within the same row are significantly different at a $p<0.05$ level.

High molecular weight procyanidins (from tetramers to decamers) where not found in this study, but the extraction of polyphenols from freeze dried samples for the HPLC-MS/MS analysis was carried out in methanol:water, while these compounds are generally extracted using acetone:water mixtures (Foo & Lu, 1999; Lazarus, Adamson, Hammerstone, & Schmitz, 1999).

The increase of trimer concentration could also have other explanation since the extraction of procyanidins in apple could be favoured by the activity of some carbohydrate-hydrolyzing enzymes, such as pectinase, cellulase, hemicellulase, and glucanase, which help the release of polyphenols complexed with cell walls (Landbo & Meyer, 2001; Sørensen, Pedersen, Anders, & Meyer, 2005, Pinelo, Zornoza, & Meyer, 2008), which are mainly catechin and procyanidins. Polysaccharides-polyphenols complexes exist as a consequence of the non-covalent binding of polyphenols, which are mainly located in vacuoles, to extracellular cell walls upon decompartmentation (Jiménez-Escrig, 2014).

Since 10 min of cold plasma processing in the same experimental conditions of this study, were proven to slightly affect (12% ca. reduction) the enzymatic activity of apples (Tappi et al., 2014), it is not clear if other naturally occurring apple enzymes (e.g. pectinases) are likely to exert their hydrolytic activity in this lapse of time. In this study, no catechin and dimeric procyanidin increase was observed after 10 min of treatment; this fact does not suggest the occurrence of any enzyme-mediated release of procyanidins, because the content of B2 dimeric procyanidin has to increase as a consequence of enzymatic hydrolysis (Pinelo, Zornoza, & Meyer, 2008; Zheng Hwang, & Chung 2009).

However, since cell walls polysaccharides form a higher number of complexes with high molecular weight procyanidins than low molecular weight ones (Poncet-Legrand et al., 2010), the increase of trimeric procyanidins (17%), which could be caused by a polymerization reaction upon oxidation, could also have been influenced by the enzymatic-mediated release from cell walls-polyphenols complexes during 10 min of processing.

Chlorogenic acid and phloridzin showed a 30 and 40% increase of their initial concentration after 10 min of cold plasma treatment. The increase of hydroxycinnamic acids and chalcones in plant tissues is associated to wounding response, that induces an increase of the content of phenolic compounds, likely due to their biosynthesis through the phenyl-propanoid pathway, as a plant defence mechanism (Uritani & Asahi, 1990; Heredia & Cisneros-Zevallos, 2009). Selected antifungal polyphenols such as chlorogenic acid, isochlorogenic acid and chalcones, among which phloridzin, could be synthesized after an elicited increased activity of the key enzymes (phenylalanine ammonia lyase and chalcone synthase) of the phenyl-propanoid biosynthetic pathway (Uritani & Asahi, 1980; Lattanzio Lattanzio, & Cardinali, 2006). In this study the increase

in chlorogenic acids seems solely dependent on biosynthesis consequent to the wounding response to minimal processing (peeling and cutting), and not to the effect of hydrolases activity. In fact, contrarily of what we observed (Table 2), a dramatic increase of caffeic acid (as a consequence of chlorogenic acid hydrolysis) and no increase of chlorogenic acid (hydroxycinnamic acids are, generally, retained not able to bind with cell walls) was previously found, as a consequence of carbohydrate hydrolases-assisted polyphenol extraction in apples (Pinelo, Zornoza, & Meyer,, 2008; Zheng Hwang, & Chung ,2009). On the other hand, Pinelo and co-workers (2008) attributed the increase of phloridzin and rutin that we found until 30 min of treatment (Table 2) to enzymatic assisted hydrolysis.

Moreover, also the quercetin and quercetin glucoside increase observed as a consequence of 10 min of plasma treatment could be a consequence of the hydrolysis of the cell wall (Zheng et al., 2009). In general a 40% to 110% increase of polyphenols conjugated to the monomeric constituents of the cell walls, among which rhamnosides and xylosides in particular, was observed (Table 2), but these types of polyphenols are not considered as constituents of the cell walls (Pinelo et al., 2006).

With some exception, at treatment time longer than 10 min, all the polyphenols showed a progressive reduction.

When calculated in percentage on total procyanidins, trimer reduction was higher than that of monomers and dimers. In addition to oxidation and polymerization, as previously discussed, catechin and procyanidins could also undergo binding to the cell walls, and oxidized procyanidins and high molecular weight procyanidins could bind better than the reduced and monomeric compounds respectively (Poncet-Legrand et al., 2010).

The total phenolic index (TPI) (Table 3) of apple products has been estimated by measuring their ability to reduce the Folin-Ciocalteu reagent, an extensively used method for the estimation of total phenolics, after solid phase extraction on C-18 cartridges, considered suitable technique for the separation of phenolic compounds (Antolovich et al., 2000; Nackzk and Shaidi, 2004).

In this study, a total TPI of about 12.6 μmol $g^{-1}_{d.w.}$ (2.2 mg GAE $g^{-1}_{d.w.}$) was measured in the amphiphilic fraction, after SPE separation; also in this case, this value is lower than that of Golden Delicious apples but comparable with literature results (Tsao Yang, Young, & Zhu, 2003; Sacchetti Cocci, Pinnavaia, Mastrocola, & Dalla Rosa, 2008; Ceymann Arrigoni, Schärer, Nising, & Hurrell., 2013).

The DBD plasma treatment significantly affected the TPI of apples with a significant increase (+8.5% ca.) after 10 min of processing, followed by a progressive decrease with the increasing of processing time to reach significantly lower values (about -9%) than those of raw apples after 30 min and of about -35% in the sample over treated for 120 min (Table 3).

The spectrophotometric determination of total polyphenols underestimated the initial polyphenols increase after 10 min of processing, but overestimated the final polyphenol decrease after 120 min of processing, when compared to HPLC-MS/MS analysis. This happened probably because the TPI assay is based on the capacity of phenolic compounds to reduce the Folin-Ciocalteu reagent under basic conditions, being the mechanism of the TPI assay based on an oxidation/reduction reaction. This result roughly indicates that the polyphenols which increase during the first 10 min of processing show a generally low reducing power, whilst those which undergo oxidation with increasing processing time show an high reducing power.

3.3 Antioxidant activity

In the present study, the antioxidant activity of apple samples was investigated with a variety of methods aimed to measure their RSA (ABTS and DPPH assay) and reducing power (FRAP and TPI). Results are presented in table 3. As mentioned, the total phenolic index is a method measuring the reducing power of the phenolic extract; for this reason it can be used to investigate the reducing power of a polyphenol mixture, being considered an antioxidant method (Prior et al., 2005).

Table 3. Total phenolic content (TPI) and antioxidant activity (μmol $kg^{-1}_{f.w.}$) of Pink Lady apples as affected by plasma treatment time.

		time (min)				
	Extract	0	10	20	30	120
TPI[a]	amphiphilic	12633[b]	13702[a]	12460[b]	11383[c]	8207[d]
TPI[a]	hydrophilic	855[b]	917[ab]	981[ab]	1050[a]	623[c]
TPI[a]	hydrophilic + amphiphilic	13484[b]	14622[a]	13357[b]	12242[c]	8790[d]
ABTS[b]	amphiphilic	14265[b]	15076[a]	14734[ab]	13526[b]	10098[c]
ABTS[b]	hydrophilic	855[a]	644[b]	840[ab]	686[b]	803[ab]
ABTS[b]	hydrophilic + amphiphilic	15120[ab]	15720[a]	15574[a]	14212[b]	10900[c]
DPPH[b]	hydrophilic + amphiphilic	22543[a]	23008[a]	21311[a]	21868[a]	16608[b]
FRAP[c]	hydrophilic + amphiphilic	26900[b]	30123[a]	27781[b]	25913[b]	17024[c]

Values followed by different letters within the same row are significantly different at a $p<0.05$ level.
[a](μmol GAE $kg^{-1}_{f.w.}$), [b](μmol TE $kg^{-1}_{f.w.}$), [c](μmol Fe^{2+} $kg^{-1}_{f.w.}$)

The ABTS and TPI assays were conducted on both the amphiphilic and hydrophilic extracts; the former showed a radical scavenging activity much higher than the latter, which accounts for about the 6% of total TEAC (sum of TEAC values of the amphiphilic and hydrophilic extracts). Similarly, the reducing power of hydrophilic extract, as measured by the TPI, accounted for about the 6% of

total TPI. This result is due to the fact that apple is very poor of water soluble polyphenols and ascorbic acid, which could filtrate through the cartridge set upon washing with the acidulated water extract prior to the elution of amphiphilic compounds.

The DBD plasma treatment significantly affected the TEAC of amphiphilic compounds of apples with a significant increase (6% ca.) after 10 min of processing, followed by a progressive decrease with the increasing of processing time, to reach values lower than those of raw apples (-30%) when over treated (120 min) (Table 2). The total TEAC showed an initial increase of 5% ca. and a final decrease of 28%, since no initial increase of the TEAC of hydrophilic compounds of the raw apple and a lower final decrease of the latter index were observed during plasma processing.

The DPPH• assay did not evidence any difference in antioxidant activity after 10 min of processing whilst it evidenced an antioxidant activity decrease after 120 min of processing (Table 2), showing a lower sensibility of this method than the TEAC assays.

The DBD plasma treatment significantly affected the TPI of amphiphilic compounds of apples as well a total TPI, which showed both a significant increase (8.5% ca.) after 10 min of processing, followed by a progressive decrease with the increasing of processing time, to reach values lower than those of raw apples (-30%) when over treated (Table 2).

Differently from the ABTS assay, the TPI assay evidenced an initial increase of the reducing power of hydrophilic compounds of the raw apple and a final decrease of this index during plasma processing. These two assays differs for the mechanisms of action which are radical scavenging for ABTS and single electron transfer for TPI, and both the polyphenols formed and consumed during plasma treatment showed lower radical scavenging activity than reducing power. The FRAP assay showed values similar to those of the TPI assay, but was more sensitive since it evidenced an initial reducing power increase of 11% after 10 min and a 37% reduction after 120 min of processing.

The initial increase of antioxidant activity after 10 min of treatment could be ascribed to the increase of selected polyphenols as a response to wounding. Several studies have been performed regarding the effect of wounding response on antioxidant activity (Kang & Saltveit, 2002; Reyes & Cisneros-Zevallos, 2003; Reyes Villareal, & Cisneros-Zevallos, 2007; Heredia & Cisneros-Zevallos, 2009) and the results observed in this study are similar to those reported by Heredia & Cisneros-Zevallos (2009). On the other hand, the initial increase of antioxidant activity after 10 min of treatment could be also due to catechins oxidation which causes the formation of procyanidins (e.g. trimers) having and higher antioxidant activity than the former compounds (Nicoli Calligaris, & Manzocco, 1999; Di Mattia et al., 2013). An initial increase of antioxidant activity followed by its reduction was also observed during the oxidation of processed apple products by Sacchetti

Cocci, Pinnavaia, Mastrocola, & Dalla Rosa (2008), who observed that this increase depends on both the activity of oxidases and the concentration of polyphenols.

3.4 Effect of treated and untreated apple polyphenolic extracts on Caco2 cells viability

In order to investigate the effect of plasma technology on cell viability we performed the WST-1 colorimetric assay. Caco2 cells were seeded in a 96-well microplate and incubated for 5 h in the presence of different concentrations of treated or untreated apple polyphenolic extracts. As shown in Fig. 1A, no reduction on cell proliferation was observed for both plasma treated and untreated samples, even at the highest concentration tested. As shown in Fig. 1B, the polyphenolic extracts obtained from plasma treated samples do not induce significant changes in cell proliferation in comparison with extracts obtained from untreated apple slides.

The Caco2 cells viability was also evaluated in the presence of different concentrations of Theaphenon E®. The concentration range examined in our study comprises the concentration achievable in human plasma (Chow, Hakim, Vining, Crowell, Ranger-Moore, Chew, et al., 2005). The results indicated that the concentration range (28 mg/mL and 75 mg/mL) chosen for the further experiments did not significantly contribute to alter cell proliferation in human cultured cells.

The concentrations tested are also comparable with the polyphenol content of the apple extracts prepared by us. As shown in Fig. 1C, the extract did not cause cytotoxicity. In vitro studies have demonstrated a cell-specific and dose-dependent cytotoxic response to EGCG treatment (Kim, Quon, & Kim, 2014). In particular, Salucci and co-workers have reported that in Caco2 cells EGCG is not toxic at the concentrations ranging between 100-250 μM, whereas a 60% cytotoxicity occurrs at 500 μM (Salucci, Stivala, Maiani, Bugianesi, & Vannini, 2002).

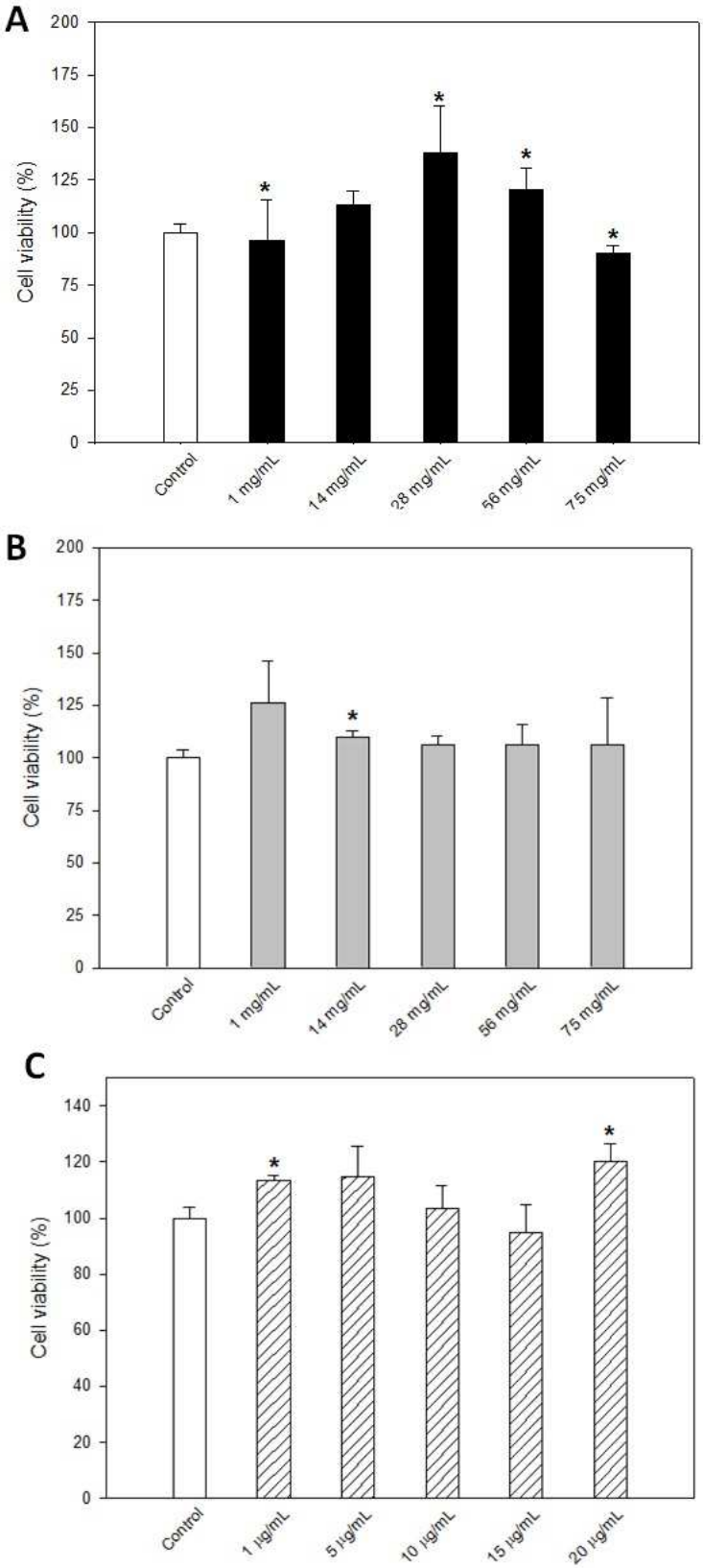

Fig 1. Effects of polyphenolic extracts from untreated (A) and treated for 30 min (B) apple and Theaphenon E® (C) on Caco2 cells viability. Caco2 cells were treated with different concentrations of polyphenols for 5 h. Cell viability was determined by WST-1 assay. Data are presented as means ± SD from four replicate wells of three different experiments as percentage of control sample. Statistical significance versus control was calculated by two-sided Student's t-test. *, $p<0.05$.

3.5 Effect of treated and untreated apple polyphenolic extracts on ROS production

Intracellular ROS levels affect cell viability and high ROS concentrations can cause cellular damage. Using the DCFH-DA assay, we evaluated the modulation of intracellular ROS in Caco2 cells after 5 h incubation with treated and untreated apple polyphenolic extracts. As shown in Fig. 2, the production of oxidizing species is dependent on extracts concentration. The treated apple extract caused a statistically significant decrease in ROS production as compared to untreated apple extract. The plasma treated sample pointed out a decrease in ROS level of about 1.8-fold with respect to the untreated sample at the concentration of 75 mg/mL. However, it is important to underline that these concentrations exerted no effects on cell viability.

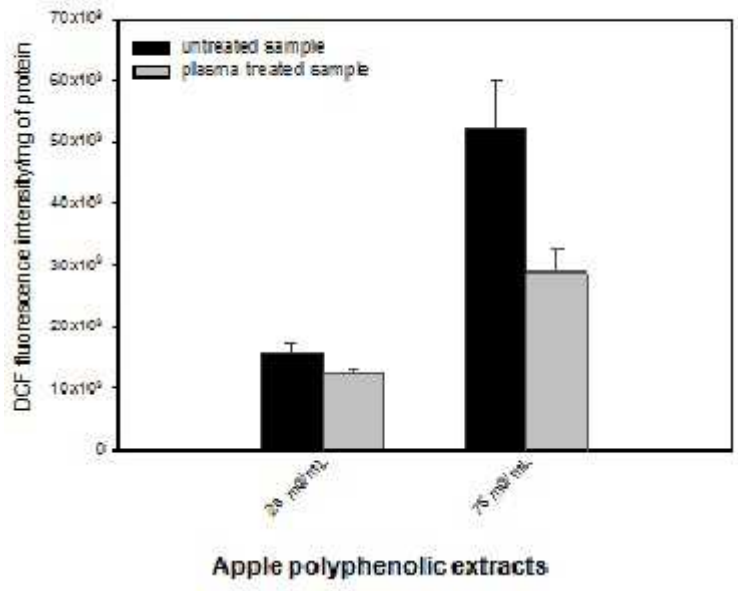

Fig. 2 Effects of polyphenolic extracts from treated (30 min) and untreated apple on Caco2 cells ROS production. Caco2 cells were treated with different concentrations of polyphenols for 5 h. ROS production was determined by DCFH-DA assay. Data are presented as means ± SD from eight replicate wells of three different experiments. Statistical significance versus untreated samples was calculated by two-sided Student's t-test. **, $p<0.001$.

Noteworthy, the pro-oxidative and antioxidative properties of plant-derived polyphenols are well documented (Babich, Schuck, Weisburg, & Zuckerbraun, 2011; Elbling, Herbacek, Weiss, Gerner, Heffeter, Jantschitsch, et al., 2011; Rizzi, Naponelli, Silva, Modernelli, Ramazzina, Bonacini, et al., 2014). The imbalance of the redox homeostasis, which occurs after the administration of EGCG (the major constituent in green tea) in different cell lines, showed a dose-dependent trend (Babich, Schuck, Weisburg, & Zuckerbraun, 2011; Elbling, et al., 2011; Rizzi, et al., 2014). Accordingly, incubation of Caco2 cells for 5 h with concentrations of Theaphenon E® higher than 5 μg/mL produced a significant increase in ROS production without reducing cell viability (data not shown). The same biological effect has been reported on Caco2 cells after incubation with high concentrations of apple extracts (Bellion, Digles, Will, Dietrich, Baum, Eisenbrand, et al., 2010).

3.6 Effect of treated and untreated apple polyphenolic extracts on phase II defence enzymes

To further determine whether plasma treatment could generate reactive species able to modulate the response of phase II defense enzymes, we performed both gene-reporter assay and qPCR.

Phase II enzymes perform conjugation reactions with the aim of transforming toxic endogenous compounds and xenobiotics in hydrophilic compounds that can be more easily excreted. They play also an important role in the metabolic inactivation of pharmacologically active substances. The Nrf2/EpRE pathway is one of the main cell signaling system involved in the safeguard against oxidative stress. It regulates the expression of key protective enzymes such as glutathione peroxidase (GPX), glutathione S-transferase (GST), NADPH quinine oxidoreductase 1 (NQO-1) and UDP-glucuronosyltransferase (UGT) (de Roos & Duthie, 2014; Zhang, An, Gao, Leak, Chen, & Zhang, 2013).

A DNA fragment containing the EpRE sequence was subcloned upstream a firefly luciferase into a suitable promoterless reporter plasmid (pGL4 vector). After transient transfection with the expression plasmid, different concentrations of untreated and plasma treated polyphenolic extracts were added to the cell culture and incubated for 5 h. As shown in Fig. 3A, there is no statistically significant difference in the induction of luciferase activity between untreated and plasma treated extract. Although the plasma technology generates ROS and NOS (Montie, Kelly-Wintenberg, & Roth, 2000), their concentrations does not seem to modify the Nrf2/ARE pathway response with respect to untreated apple extract. However, the recombinant plasmid used for the gene reporter assay contains just one copy of the EpRE motif and previous data showed a good correlation between the number of EpRE repeats in the reporter plasmid and the level of luciferase activity (Wang, Hayes, & Wolf, 2006). Noteworthy, incubation of Caco2 cells for 5 h with different concentrations of Theaphenon E® determines a statistically significant increase in luciferase activity (Fig. 3B). The different response obtained with the gene reporter assay is probably due to the presence of contaminants in the apple extracts able to quench the luciferase signal.

In order to confirm the harmless effect of plasma technology, we quantified by qPCR the mRNA levels of genes belonging to GST and UGT family enzymes, which are targets of the Nrf2/EpRE pathway. GST family members are able to catalyze the conjugation of the sulfhydryl moiety of glutathione (GSH) with a broad range of endogenous and exogenous electrophilic substrates (Tew & Townsend, 2012). UGT family members are endoplasmic reticulum-bounded enzymes that catalyze glucuronidation of endogenous and exogenous substrates, like bilirubin, bile acids, steroids and xenobiotics (Shuji Ohno and Shizuo Nakajin 2009).

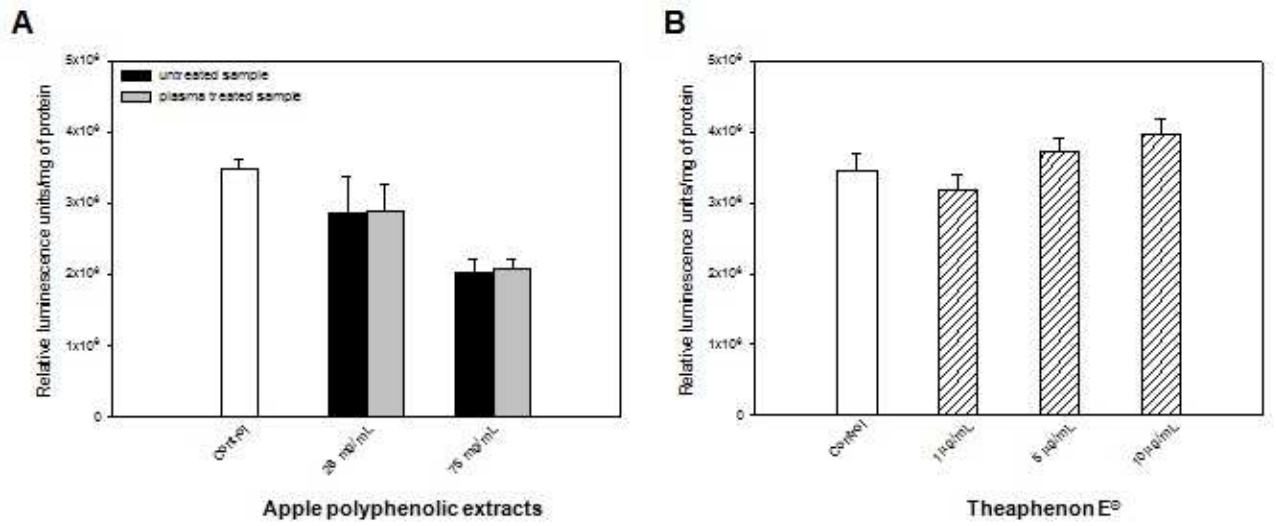

Fig. 3. Effects polyphenolic extracts from treated (30 min) and untreated apple (A) and Theaphenon E® (B) on Caco2 cells ARE luciferase activity. Caco2 cells were treated with different concentrations of polyphenols for 5 h. The luciferase activity was normalized for the total protein content. Data are presented as means ± SD from four replicate wells of three different experiments. Statistical significance versus untreated sample was calculated by two-sided Student's t-test. *, $p<0.05$.

Previous data have shown that in different colon tumor cell lines apple polyphenols (Veeriah, Miene, Habermann, Hofmann, Klenow, Sauer, et al., 2008) and digitoflavone (Yang, Cai, Yang, Sun, Hu, Yan, et al., 2014) are able to induce gene expression of detoxification enzymes. As shown by comparison of plasma treated and untreated apple extracts (Fig. 4A and B), the polyphenolic extracts are able to induce a different response in Nrf2/ARE in a dose dependent manner. At the lowest concentration tested (28 mg/mL) of plasma treated apple extract, the mRNA levels of both GST and UGT family members are less induced with respect to untreated apple extract. The low concentration of ROS revealed by DCFH-DA assay is not enough to induce a specific cell response to oxidative stress. In contrast, when used at a concentration up to 75 mg/mL, the treated apple extract induces a phase II enzyme response, in accordance with the increase of ROS level. Concerning the GST family members, while the GSTT2 mRNA level is comparable in Caco 2 cells exposed to both treated and untreated samples, the GSTP1 mRNA level increases and the GSTA4 mRNA level remains less induced (Fig. 4A) in cells incubated with plasma treated apple extract. Within the UGT family, our results evidenced an increase of the UGT1A4 mRNA level after incubation with plasma treated apple extract, whereas the UGT1A1 and UGT2B7 mRNAs levels are comparable in both treatment groups (Fig. 4B). These data point out that after administration of apple polyphenols extracts derived from the plasma treatment, Caco 2 cells are able to maintain the physiological response to moderate oxidative stress by increasing the transcription of phase II detoxifying enzymes.

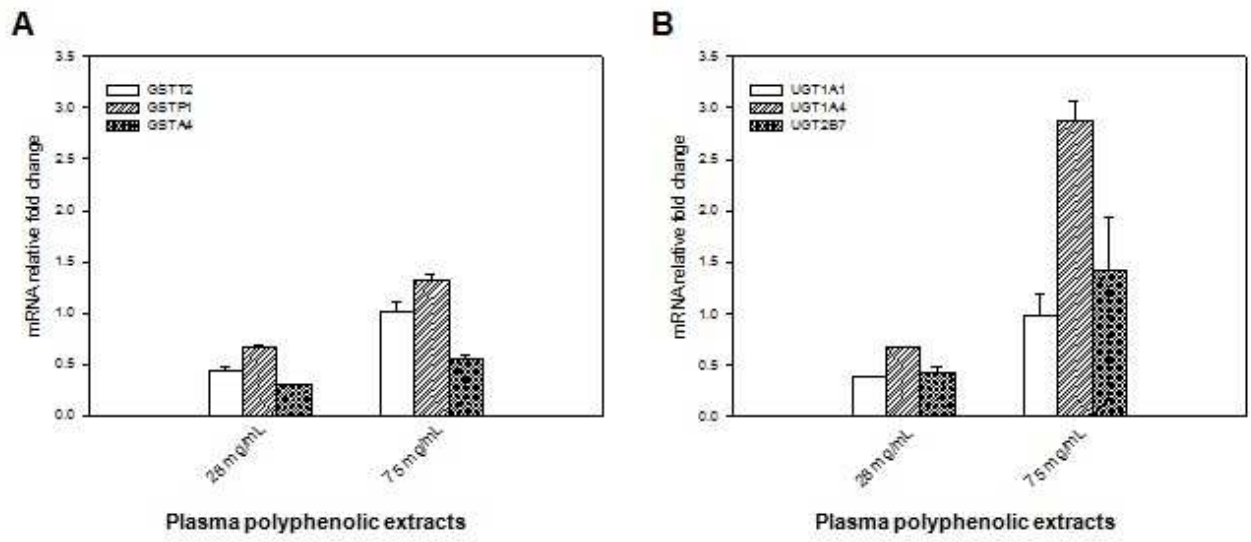

Figure 4. Relative expression of mRNA levels of Nrf2-activated phase II enzymes in Caco2 cells loaded for 5 h with different concentrations of polyphenols extracted from plasma treated (30 min) apples. The relative expression of gene transcripts was calculated by the $2^{-\Delta\Delta CT}$ method using untreated apples extract as reference sample. Data are presented as means ± SD from three different experiments. Statistical significance versus untreated sample was calculated by two-sided Student's t-test. *, $p<0.05$.

4. Conclusions

The effect of plasma treatment on physico-chemical parameters was mainly observed on a slight acidification of the tissue, and in a reduction of browning after the longer exposure time (30 min), probably due to enzymatic activity inhibition showed in previous study (Tappi et al., 2014).

As a consequence of catechin polymerisation products and of the increase of hydroxycynnamic acids and chalcones, phenolic profile of fresh-cut apples was significantly affected by 10 min treatment, both in quantitative (about 20% of increase) and qualitative terms.

After 30 min of treatment, when plasma effect on enzymatic browning inhibition was significant, flavan-3-oils content was lower compared to not treated apples, while dihydrochalcones and flavonols evidenced a slight increase.

As expected, considering its strict connection between polyphenols amount, antioxidant activity evaluated by different *in-vitro* methods followed a similar trend, increasing after 10 min of processing. The DPPH• showed a lower sensibility compared with TEAC assays, while FRAP showed similar values to TPI assay.

Moreover, this research represents the first approach in assessing the effect of chemical species generated during the gas plasma treatment on human cell line. We demonstrated that the polyphenolic extract obtained from plasma treated apple does not induce significant changes in cell proliferation in comparison with untreated apple. Furthermore, Caco 2 cells exposed to moderate oxidative stress induced by the polyphenols extracts administration are able to protect themselves through the expression of phase II detoxifying enzymes.

In conclusion, the DBD plasma treatment is a very promising tool to preserve the qualitative properties and the phytochemical profile of fresh-cut Pink Lady apples. Further, apple exposure to gas plasma does not seem to generate chemical species harmful to human cells. Other studies in cellular models are needed to confirm this preliminary data.

Further studies of the product microstructure and metabolomics features are needed. Firstly to understand the chemical or enzymatic nature of polyphenols modification phenomena, secondly to clarify the induced metabolic response promoted by the treatment to the fresh apple tissue. This has to be focused on the product packed in real packaging (passive modified atmosphere packaging) and storage conditions (during 10-15 days of refrigerated storage).

Acknowledgments

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Paper VII

Optimization of vacuum impregnation with calcium lactate of minimally processed melon and shelf-life study in real storage conditions

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Optimization of vacuum impregnation with calcium lactate of minimally processed melon and shelf-life study in real storage conditions

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Abstract

Vacuum impregnation (VI) is a process that allows the impregnation of fruit and vegetable porous tissues with a fast and more homogeneous penetration of active compounds compared to the classical diffusion processes. The objective of this study was to investigate the effect on VI treatment with calcium lactate on qualitative parameters of minimally processed melon during storage. For this aim, the present work was divided in two parts. Initially, the optimization of process parameters was carried out in order to choose the optimal VI conditions for improving texture characteristics of minimally processed melon that were then used to impregnate melons for a shelf-life study in real storage conditions. On the basis of a 2^3 factorial design, the effect of Calcium lactate (CaLac) concentration between 0 and 5 % and of minimum pressure (P) between 200 and 600 mbar were evaluated on colour and texture. Processing parameters corresponding to 5% CaLac concentration and 600 mbar of minimum pressure were chosen for the storage study, during which the modifications of main qualitative parameters were evaluated. Despite of the high variability of the raw material, results showed that VI allowed a better maintenance of texture during storage. Nevertheless, other quality traits were negatively affected by the application of vacuum. Impregnated products showed a darker and more translucent appearance on the account of the alteration of the structural properties. Moreover microbial shelf-life was reduced to four days compared to the seven obtained for control and dipped samples.

Practical application: Minimally processed fruit represent a growing sector for the fresh produce industry, on the account of their health properties combined with a high convenience value, but they are characterized by a shorter shelf-life compared to the intact product. Therefore, preservation processes able to increase the product shelf-life are of great interest to fruit processing industries. Vacuum impregnation with calcium has been studied to improve product texture properties of various fruit and vegetable. Nevertheless researches were often limited to the immediate effect of the treatment not taking in consideration the evolution of the product quality during storage in real conditions.

Keywords: vacuum impregnation, minimally processed melon, calcium, shelf-life.

1. Introduction

Vacuum impregnation (VI) is a process that allows the impregnation of fruit and vegetable porous tissues, exploiting a mass transfer known as hydrodynamic mechanism (HDM) (Fito *et al.*, 1996). A

two-step pressure change is implicated in this mechanism. At first, vacuum pressure is applied to a solid-liquid system (vacuum step), causing the expansion of the gas inside the product pores and its partial outflow until mechanical equilibrium is achieved. In the second step (atmospheric step), atmospheric pressure is restored and the compression of the residual gas in the pores leads to the penetration of the external liquid into the pores (Tylewicz *et al.*, 2012). Compared to the classical diffusion processes, carried out by simple dipping or prolonged immersion of the product in the solution at atmospheric pressure, VI presents the advantage of a fast and more homogeneous penetration of the active compounds directly into the internal structure of the product (Saurel, 2004).

Various substances can be introduced inside the food matrix such as nutritional, nutraceutical and/or functional compounds, antimicrobial and antioxidant substances, organic acids, structuring substances, etc. (Betoret *et al.*, 2003). Depending on the type of component chosen for the impregnation, the final product will be characterized by modified/improved sensorial or nutritional quality or an extended shelf-life.

One of the most critical issue for quality maintenance of minimally processed melon during refrigerated storage is its susceptibility to tissue softening. The application of calcium dips has been widely investigated in pears (James *et al.*, 2002), kiwifruit (Agar *et al.*, 1999), nectarines and peaches (Gorny *et al.*, 1999) and melons (Luna-Guzmán *et al.*, 1999; Silveira *et al.*, 2011). The structuring effect of calcium is due to its ability to form cross-links or bridges between free carboxylic group of the pectin chains, resulting in strengthening of the cell wall and at the same time, preserving the structural and functional integrity of membranes.

The use of VI with calcium salts has been considered for fortification of various fruit and vegetables (Fito *et al.*, 2001; Gras *et al.*, 2003; Tapia *et al.*, 1999), in order to obtain food with higher calcium content suitable for consumers with specific health needs.

Few works have been carried out on the impregnation with calcium to improve texture of minimally processed products. Occhino *et al.* (2011) investigated the effect of calcium and other structuring compounds in zucchini slices, while Degraeve *et al.* (2003) combined calcium and pectinesterase for strawberry impregnation finding a remarkable synergistic effect in increasing firmness.

Nevertheless, generally published studies are limited to the immediate effect of the treatment and do not take in consideration the evolution of the product quality during storage in real conditions.

The objective of this study was to investigate the effect on VI treatment with calcium lactate on qualitative parameters of minimally processed melon during storage. For this aim, the present work was divided in two parts. Initially the optimization of process parameters was carried out in order to choose the optimal VI conditions for improving texture characteristics of fresh melon. In the second

part of the research, the optimal processing conditions were used to impregnate melons, and evaluate modifications of main qualitative parameters during storage, compared to fresh cut samples and to samples dipped in the same calcium solution at atmospheric pressure.

2. Materials and Methods

Sample preparation

Two different batches of about 50 melons (*Cucumis melo* var Reticulatus) were harvested at commercial maturity during August 2013 and 2014 in Emilia-Romagna region (Italy); care was taken to select fruits with similar ripening degree, on the basis of their soluble solid content and titratable acidity. The first batch was used for the optimization study, the second for the storage study. The fruits were brought to our laboratory where they were held at 4°C for 48 h before processing.

Prior to processing, selected whole fruits were washed with water, scrubbed with a sponge to eliminate dirt on the external surface and then dipped for 1 min in a 200 ppm chlorine solution.

Fresh samples were characterized by soluble solid content, colour, textural parameters and porosity by means of methodology reported below.

After water removal with tissue paper, each fruit was halved longitudinally, stem ends were eliminated and the central part was cut in 2 cm thick slices. From each slice cylindrical samples (ø=2 cm, h=2 cm) were obtained from melon flesh using a manual sharp cork borer.

VI system and experimental plan

The impregnation process was performed using automatic vacuum controller system (AVCS, S.I.A., Bologna, Italy), a programmable device designed to control the pressure acting on the impregnating solution during the impregnation process. The AVCS is connected to the impregnation chamber by a Teflon tube and to a vacuum pump.

The experiment was divided into two parts as follows:

a. Optimization of VI process parameters for improving qualitative characteristics of fresh-cut melon

Among calcium salts, lactate (CaLac) was selected on the account of literature, as it is able to improve/preserve the texture attributes of fresh-cut melon during storage, having lower impact on the organoleptic properties compared to other salts (chloride, carbonate and propionate) (Aguayo *et al.*, 2008; Lamikanra & Watson, 2004; Luna-Guzmán *et al.*, 1999).

The impregnation (at minimum pressure) and the relaxation (after atmospheric pressure restoration) times were 5 min each. The time was selected as the minimum time able to obtain a level of

impregnation corresponding to the product real porosity, as too long exposure to high vacuum level can cause tissue deformation (Mújica-Paz *et al.* 2003).

A 2^3 full experimental plan was applied in order to evaluate the singular, quadratic and interactive effects of the selected variables on some qualitative parameters. Each factor had 3 levels ranging from 0 to 5% for CaLac and from 200 to 600 mbar for minimum pressure, as shown in table 1. The highest salt concentration has been selected after preliminary tests, aimed at evaluating the maximum level not detected by sensorial analysis. The impregnation medium was isotonic regarding native soluble solids content of melon to avoid water transfer phenomenon.

The minimum absolute pressure reached during the treatment was obtained applying a stepwise protocol chosen on the basis of preliminary experiments that involved the application of vacuum pressure in different steps of 200 mbar each. During intermediate steps, samples were held at the relevant pressure value for 30 seconds. Each treatment was repeated in three independent replicates, each with 50 g of products and a ratio product:solution of 1:4.

Table 1. Coded and real values of the independent variables in the experimental plan

Coded value	-1	0	1
CaLac concentration (%)	0	2.5	5
Minimum absolute pressure (mbar)	200	400	600

Impregnated melon samples were tested for porosity, weight gain (WG), colour and textural parameters. On the basis of the obtained results on quality parameters, the combination of the process parameters was chosen for the shelf-life tests.

b. Study of the modification of qualitative characteristics of vacuum impregnated fresh cut melon during storage

Vacuum impregnated melons were obtained according to the protocol chosen during the first part of the experiment (600 mbar and 5% CaLac). Fresh cut melon was used as control (C). In order to evaluate the effect of the vacuum treatment, VI samples were also compared to fresh-cut melon dipped at atmospheric pressure in the same CaLac solution (D) for the same duration. About 45 g for package of each type of melon sample (VI, D and C) were packed in trays sealed with a micro-perforated film of polyethylenetereftalate (PET) 75 μm thick, and subjected to storage for 10 days at 10°C. This temperature was chosen in order to simulate the temperature abuse commonly occurring at retail stores and to accentuate any beneficial or negative effects of the postharvest treatments (Saftner *et al.*, 2003). In each package, headspace was about 900 mL.

During storage, at 0, 2, 4, 7 and 10 days the samples were analysed for gas composition in the headspace, soluble solid content, titrable acidity, dry matter, colour, texture and the microbial cell loads. At each sampling time, three packages were evaluated for each sample (C, D and VI).

Analytical determinations

Chemical parameters

- Sample porosity was determined using pycnometric method measuring real and apparent density according to Gras *et al.*, (2003). Total or real porosity (εr) constitutes a measure of the empty spaces in the fruit tissue, and represents the maximum space that could be impregnated with an isotonic solution. It was determined on five different melon cylinders.
- The impregnation parameter weight gain (WG) was calculated according to (Tylewicz *et al.*, 2012) with the following equation:

$WG = 100 \times (m - m_0)/m_0$

where m is the mass of the impregnated sample (g) and m_0 is the initial mass (g).

- Soluble solids content (SSC) was determined at 20 °C by measuring the refractive index with a digital refractometer mod. PR1 (Atago Co. Ltd, Tokyo, Japan) calibrated with distilled water.
- Titratable acidity (TA) was determined by titration with NaOH 0.1 N until pH 8.1 was reached (AOAC Official Method 942.15, 2000). Results were expressed as ml of malic acid/100 g.

For each sample, SSC and TA were determined in triplicate on the juice obtained from 10 melon slices, after filtering through Whatman #1 filter paper

- Dry matter content was determined gravimetrically by difference in weight before and after drying at 70 °C, until a constant weight was achieved (AOAC International, 2002) on three different melon cylinders.

Gas composition

O_2 and CO_2 percentage was measured in the packages headspace by a gas analyzer "check point O_2/CO_2" mod. MFA III S/L (Witt-Gasetechnik, Witten, Germany). At each sampling time, it was measured in three packages for each sample.

Colour

Surface colour was measured with a spectrophotocolorimeter (HUNTERLAB ColorFlexTM, A60-1010-615, Reston, Virginia) using the D65 illuminant and the 10° standard observer. For each treatment the average of 15 measurements was calculated. The L*, a* and b* parameters of the

CIELAB system were measured, a* and b* parameters were further used to calculate Hue angle ($h_0 = \left(arctan\left[\frac{b^*}{a^*}\right]/2\pi\right) x\ 360$

Texture

Mechanical parameters were measured with a penetration test using a Texture Analyser TA-HDi500 (Stable Micro Systems, Surrey, UK) equipped with a 50 N load cell and a 6 mm diameter stainless steel cylinder until a maximum deformation of 90% of sample thickness. For each treatment the average of 15 measurement was calculated.

The acquired curves (Force, N, versus time, s) were analysed and the following mechanical parameters were extracted: firmness - the first peak force (N) value and the linear distance (LD) as an index of crispness. LD was determined, using the "linear distance" function (1) within Texture Expert Exceed software (version 2.61, Stable Micro Systems) on a plot Force (F) in Newton versus distance (D) in millimetres (Gregson & Lee, 2003).

$$L_d = \sum_{x=1}^{x=n} \sqrt{\left[F(x+1) - F(x)\right]^2 + \left[D(x+1) - DS(x)\right]^2} \quad (1)$$

This function automatically calculates the line length by summing the lengths computed between consecutive data points using the Pythagoras equation. The software calculate Ld by summing the length of the straight segments connecting each point acquired (500 points/s) between selected times or distances. Although a length is calculated, distance units cannot be used because one axis has force units. Generally, an increase of Ld corresponds to an increase of crispness.

Microbiological analysis

Melon samples (10 g) were suspended into sterile 0.1% (w/v) peptone-water solution and homogenized with a Stomacher Lab Blender (Seward, PBI International, Whitstable, Kent, UK) for 2 min at room temperature.

Mesophilic lactobacilli and lactococci were determined on MRS agar (Oxoid Ltd, Basingstoke, Hampshire, UK) containing 0.1% of cycloheximide (Sigma Cemical Co.) and M17 agar (Oxoid Ltd), respectively, at 30 °C for 48-72 h under anaerobic conditions. Yeasts were enumerated on Yeast Extract Peptone-Dextrose agar (YPD, Oxoid Ltd), added of 150 ppm chloramphenicol, at 30 °C for 72 h. Viable counts of total aerobic mesophilic and psychrotrophic bacteria were determined on Plate Count Agar (Oxoid Ltd) at 30 °C for 48 h and 4°C for 10 days, respectively.

Microbiological counts were carried out in triplicate for all the samples at each storage time.

Statistical analysis

For the optimization of parameters, a 2^3 full experimental plan was applied. The experimental design allows to establish a second order polynomial by :

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$$

The statistical analysis was performed using the software Statistica 8.0 (Statsoft Inc., Tulsa, UK) to obtain the coefficients of the polynomial, F-values, standard errors (SE) and the explained variability percentage (R^2), in order to evaluate the global fitting of the model to the obtained experimental values.

During storage, significant differences among samples in qualitative parameters and in microbial loads, at the same sampling time, were assessed using the analysis of variance (ANOVA), and significance of differences was defined according to the LSD post-hoc test at $p \leq 0.05$.

3. Results and discussion

Results of the chemico-physical parameters of the raw materials of the two different batches are reported in Table 1. Even if in terms of SSC and TA the two batches were very similar; regarding colour, fruits of the first batch presented a lighter and less orange colour.

Table 2. Colorimetric and textural parameters of the 2 batches of fresh melon samples.

	1st batch		2nd batch	
	$\bar{x}$	SD	$\bar{x}$	SD
SSC	12.5	0.2	12.3	0.1
TA	0.11	0.01	0.10	0.01
L*	68.41	3.75	61.22	4.01
h°	73.22	0.97	69.86	2.02
Firmness	7.28	2.5	5.8	0.9
LD	17.27	2.1	30.5	3.5
porosity	9.78	1.75	5.74	1.15

These melons were characterized by higher firmness, and their averaged porosity (9.78 %) was almost the double of the one of the second batch fruits (5.74 %). In any cases, these values are in agreement with previous literature studies, that reported porosity of melon in the range of 4-13%

(Fito *et al.*, 2001; Mújica-Paz *et al.*, 2003), also considering the variability of this parameter as a function of cultivar, agricultural practices and harvesting year.

a. Optimization of VI process parameters for improving qualitative characteristics of fresh-cut melon

Table 3 reports weight gain (WG), textural and colorimetric parameters of samples impregnated according to the experimental plan. The second order polynomials for the response parameters, determined by data analysis, are shown in table 4. These models allowed evaluating the effects of linear, quadratic, and interactive terms of the independent variables (pressure level - P and CaLac concentration - C) on the chosen dependent variables. Apart from LD, all other models showed high R^2 values (from 0.795 to 0.999).

To better understand the interactive effects of the independent variables, surface plots based on these models were drawn. As examples, the representation of WG and Firmness as a function of P and C are shown in Figures 1 and 2.

Table 3. Experimental plan factor values; mean values and standard deviations of weight gain (WG), textural and colorimetric parameters of each fresh cut melon samples.

Sample	Pressure	CaLac concentration	WG (%)		Firmness (N)		Linear Distance (LD)		L*		h°	
	mbar	%	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
1	200	0	10.66	1.29	10.06	3.85	22.94	5.33	45.89	3.04	74.18	0.60
2	200	2.5	5.48	1.14	11.18	2.96	25.46	4.14	49.86	2.03	74.47	0.87
3	200	5	3.82	0.52	14.27	5.18	31.86	8.95	50.21	2.20	74.86	1.02
4	400	0	6.89	0.59	11.13	4.91	21.85	7.05	54.58	4.96	73.64	0.60
5	400	2.5	3.53	0.35	15.03	9.32	25.91	11.19	57.95	3.41	73.63	0.43
6	400	5	1.84	0.53	11.64	6.91	28.18	10.51	57.98	1.84	73.62	0.71
7	600	0	5.42	1.37	13.09	6.40	27.06	10.57	60.48	2.16	73.56	0.75
8	600	2.5	1.55	0.56	18.16	9.57	30.42	10.41	62.99	1.87	72.75	0.65
9	600	5	1.08	0.61	19.32	7.16	33.19	8.43	61.87	2.66	73.02	0.54

WG was in the range of 1.08-10.66 %. According to the VI theory (Fito *et al*., 1996), the extent of pores filling, hence weight gain, depends on the fruit's real porosity and mechanical properties, as well as the vacuum intensity, and application time. Moreover, pressure changes can promote deformations of the tissue because of the viscoelastic properties of the solid matrix. Thus, volume fraction of the product impregnated by the external liquid would be affected not only by the initial

effective porosity, but also by the volume changes at the end of the vacuum and atmospheric steps (Anino *et al.*, 2006).

Table 4. Best-fit equations relative to the effects of pressure level [P], CaLac concentration [C] on weight gain (WG), firmness, linear distance (LD), L* and h° values

Parameter	Model	R^2	F-value	SE
WG	14.8130-0.02469[P]-2.72536[C]+0.22878[C2]+0.00125[P][C]	0.984	98.499	0.3872
Firmness	6.615799+0.012566[P]+1.093556[C]	0.912	?	0.9931
LD	16.6111+0.01889[P]+1.8485[C]	0.661	8.7680	3.4918
L*	33.65+0.06839[P]+2.6046[C]-0.00004[P2]-0.28246[C2]-0.00146[P][C]	0.999	1585.0	0.1899
h°	75.141111-0.00348[P]	0.795	32.067	0.3014

Only terms with $P < 0.05$ were included.

In our study, real porosity of melon samples was found 9.78 ± 1.75, as reported in table 2, in agreement with previous literature studies that reported porosity of melon in the range of 4-13% (Fito *et al.*, 2001; Mújica-Paz *et al.*, 2003). Considering real porosity an indication of the maximum impregnation level, impregnation with only sucrose isotonic solution at 200 mbar (sample 1) allowed a good impregnation level in this sample.

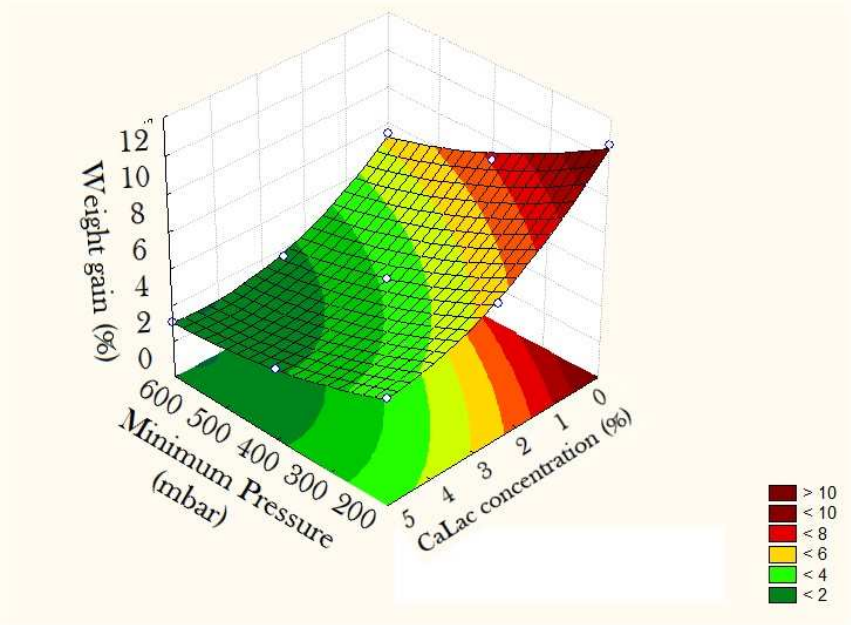

Fig. 1. Three-dimensional contour plots showing the effects of the interactions [P] × [C] on weight gain (WG, %)

However, as it can be observed, the weight variation is influenced negatively by both minimum pressure and CaLac concentration. In particular, it increases by increasing vacuum pressure and it decrease by increasing the impregnating medium concentration. The effect of the vacuum level

applied confirms results obtained by Tylewicz *et al.* (2012) and Mújica-Paz *et al.* (2003) on apples and melons respectively.

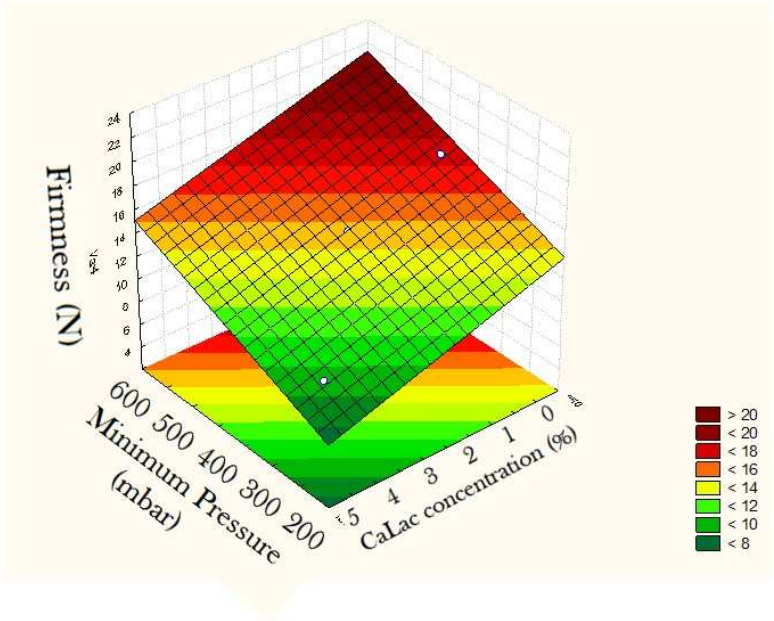

Fig. 2. Three-dimensional contour plots showing the effects of the interactions [P] × [C] on Firmness (N).

By the Beta coefficients, that indicate the relative weight of the independent variables on the dependent one, it can be appreciated that the CaLac concentration is the most influential (1.95 for C and 0.34 for P). This result may be due to the viscosity of the medium that increases by increasing its concentration. Gras *et al.* (2003) did not find that impregnation levels in eggplant, carrot and oyster were affected by the presence of calcium in solution, but the concentration of calcium used was limited to 20 g/L, while in our experiment reached 50 g/L.

Viscosity has been reported to make penetration of solutes into fruit structures more difficult by different authors (Guillemin *et al.*, 2008; Mújica-Paz *et al.*, 2003; Saurel, 2004). Guillemin *et al.* (2008) found that solute distribution was heterogeneous and confined to the surface when a 2% sodium alginate solution was used. Compared to less viscous solutions a longer holding time after vacuum release was necessary to obtain a higher impregnation level and a homogeneous penetration of the solutes.

However, the solution tested by the authors were highly viscous, ranging from 9 to 116 mPa s. With highly viscous solutions, liquid intake may be restricted because of the equilibrium between the relaxation force of the matrix and the friction force results in sample deformation instead of impregnation (Barat *et al.*, 2001). In our experiments, the solution viscosity varied from 1.02 to 1.21 mPa s. Nevertheless, at these low values, the increase of the calcium lactate concentration promoted a slight increase of solution viscosity, restricting the impregnation of the tissue.

All impregnated samples showed higher firmness and LD values compared to the fresh tissue. Martínez-Monzó *et al.* (1998) observed, by CryoSEM, that the impregnation of apple tissue with isotonic sucrose solution did not cause alteration to turgor, size, shape and intracellular volume of cells, but the intercellular spaces were flooded by the external solution. Nevertheless, it can be expected that the gas-liquid exchange may induce variations in the mechanical and structural properties of products.

Igual *et al.* (2008) studied the effect of VI on mechanical characteristics of cut persimmons obtained by two different cultivars and found that while it had a softening effect on one cultivar, the second one was hardened by the treatment. The deformation and relaxation mechanisms caused by the application of the vacuum can affect the solid matrix in a different way, depending on the viscoelasticity of the product (Fito *et al.*, 1996).

As it can be observed in table 3, the effect of VI on firmness and LD was less pronounced when the vacuum applied was higher, probably because the increase of vacuum lead to some irreversible deformation of the solid matrix. On the other hand, calcium had a visible effect on texture, proportional to its concentration, as demonstrated by the coefficient relative to both parameters. Hence, although it decreased the impregnation level, a high concentration of calcium in the solution was able to increase hardness and crispness of the tissue.

Previous studies on the effect of VI with calcium salts reported contrasting results. (Anino *et al*., 2006) found that the impregnation process decreased the resistance to compression in apple tissue, when isotonic solution was used, and the presence of calcium salt made tissue damage even more pronounced, probably for the occurrence of rupture of membranes and plasmolysis observed by microscopic analysis. On the contrary, Gras *et al.* (2003) found that after VI with isotonic solution containing calcium, samples of eggplant and carrot were characterized by a higher stiffness and fragility and hypothesized the formation of bonds in middle lamellae and cell walls. The effect was not observed in oyster tissue, probably because, not containing pectin, the structure was minimally affected by the presence of calcium. (Occhino *et al.*, 2011) studied VI treatment with different combination of calcium with maltodextrins in order to improve texture of zucchini. While the reduction of shear and energy force were decreased by the application of vacuum, the presence of $CaCl_2$ in the solution limited the hardness loss when in combination with the other solutes, even having an hardening effect when alone.

Studying the distribution of calcium, Anino *et al.* (2006) found that impregnation occurs mainly in the large intercellular spaces of the tissue, and only to a lesser measure inside the cells of eggplant. However, calcium concentration in the impregnating solution seems to have an effect on the way

calcium is transferred and located in the tissue, and probably on the effect on the final textural characteristics of the product.

All samples after the treatment showed significant differences in the colour parameters compared with the control (C). In particular, samples underwent to a general darkening (decrease of L* value), probably due to the alteration of the structural properties of the tissue as a consequence of vacuum application and/or of the gas-liquid exchange that can cause a change in the refraction index (Muntada *et al.*, 1998; Tapia *et al.*, 1999). This decrease was dependent only on the level of vacuum pressure applied, both in its individual and quadratic effect. Increasing the vacuum lead to a higher impregnation with a higher gas-liquid exchange promoting the change in the colour coordinates. Also the increase of calcium concentration had a positive effect on sample lightness, probably related to the lower extent of impregnation obtained.

Hue angle values were affected only by pressure but variations were minimal. As reported by Fito & Chiralt (2000), although variations in a* and b* coordinates can be observed after VI, generally the hue is only slightly modified. The authors also reported that the variations induced by VI treatments did not have a detrimental effect on consumer perception.

On the basis of the overall results obtained in this first part of the study, the treatment conditions of sample 9 (600 mbar and 5% CaLac concentration) were chosen for the further storage study.

b. Study of the modification of qualitative characteristics of vacuum impregnated fresh cut melon during storage

In the second part of the study the effect of the best selected vacuum treatment (VI) conditions, compared to a conventional dipping at atmospheric pressure in the same CaLac solution (D) for the same duration and to the control ones (C) on some quality aspects of fresh-cut melon samples during storage were analysed.

Due to the respiratory metabolism, fresh vegetable products consume O_2 and produce CO_2, promoting changes in the gas composition within the package. At the same time, packaging materials were usually semi-permeable and allow the exchange with the external atmosphere. Headspace composition depends on the sum of these two phenomena. As it can be observed by table 5, at the end of the 10 days of storage oxygen concentration was in the range of 19.5-19.8% while CO_2 was in the range of 2.3-3.1%, avoiding the anaerobic conditions until the end of the storage period. Generally significant differences observed among samples were minimal and did not indicate a clear trend.

Table 5. O_2 and CO_2 concentration in the headspace of control (C), dipped (D) and vacuum impregnated (VI) minimally processed melons during storage at 10°C

	Days of storage							
	2		4		7		10	
Sample	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
				O_2 (%)				
C	20.1[a]	0.2	19.7[b]	0.1	18.2[ab]	0.7	19.7[a]	0.6
D	20.2[a]	0.1	20.4[a]	0.1	17.8[b]	0.8	19.2[b]	0.7
VI	20.1[a]	0.1	19.8[b]	0.1	18.5[a]	0.6	19.5[ab]	0.7
				CO_2 (%)				
C	2.4[a]	0.3	3.0[a]	0.0	6.2[a]	1.0	3.1[a]	0.6
D	2.4[a]	0.1	2.3[b]	0.1	5.8[a]	0.6	2.8[a]	0.3
VI	2.5[a]	0.1	2.9[a]	0.0	4.7[a]	0.5	2.7[a]	0.6

Different letters indicate statistically significant values among samples at the same storage time ($p<0.5$).

Table 6 reports some physico-chemical parameters of melon samples during storage. Generally, samples underwent at the end of storage to an increase of dry matter, a decrease of titratable acidity and little variation of soluble solid content.

Water loss in minimally processed product is enhanced by the disruption of the tissues due to peeling and cutting operation (Rolle & Chism, 1987). In the present study, no differences were found initially and dry matter of samples increased up to about 3-5 % in all samples.

Table 6. Physico-chemical parameters of control (C), dipped (D) and vacuum impregnated (VI) minimally processed melon samples during storage at 10°C

	Days of storage									
	0		2		4		7		10	
Sample	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
					Dry matter (g/100 g)					
C	11.66[a]	± 2.54	9.18[b]	± 0.84	13.98[a]	± 1.47	9.71[b]	± 0.30	15.40[a]	± 0.92
D	12.33[a]	± 2.48	11.59[ab]	± 2.11	12.54[a]	± 0.65	15.43[a]	± 2.95	13.67[b]	± 0.06
VI	11.99[a]	± 0.14	14.19[a]	± 0.97	13.54[a]	± 2.37	17.59[a]	± 2.83	14.12[ab]	± 0.95
					Titratable acidity (ml malic acid/100 g)					
C	0.11[a]	± 0.01	0.028[a]	± 0.001	0.014[b]	± 0.001	0.049[c]	± 0.007	0.082[a]	± 0.004
D	0.09[a]	± 0.07	0.028[a]	± 0.014	0.019[ab]	± 0.008	0.084[b]	± 0.014	0.070[a]	± 0.014
VI	0.12[a]	± 0.01	0.037[a]	± 0.008	0.028[a]	± 0.002	0.131[a]	± 0.016	0.051[b]	± 0.008
					Soluble Solid content (%)					
C	12.3[a]	± 0.1	11.3[a]	± 0.1	11.8[a]	± 0.1	10.1[b]	± 0.2	11.1[a]	± 0.1
D	12.2[a]	± 0.4	12.2[a]	± 0.1	11.7[a]	± 0.1	11.5[a]	± 0.1	10.9[a]	± 0.1
VI	12.5[a]	± 0.1	11.7[a]	± 0.1	11.5[a]	± 0.1	10.8[ab]	± 0.2	11.6[a]	± 0.1

Different letters indicate statistically significant values among samples at the same storage time ($p<0.5$).

Titratable acidity and soluble solid content are parameters related to the ripening stage of the fruit and the rate of their variation is also increased by minimal processing operations, although

microbial spoilage may play a part in their evolution during storage. The changes observed during storage and among samples in the present study, are only slight and do not seem to present a pattern attributable to the treatments.

Colour coordinates of lightness and hue angle of samples during storage are shown in Figure 3.

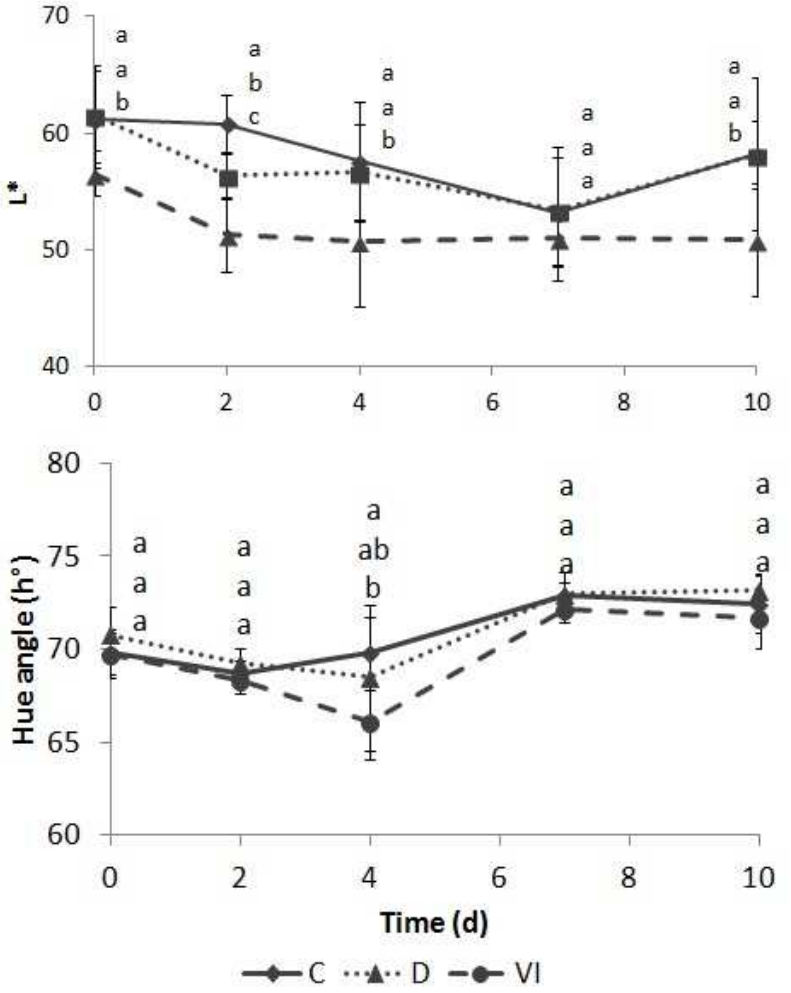

Fig 3. Colour coordinates (L* and h°) of melon samples during storage. C: control, D: dipped; VI: vacuum impregnated sample. Different letters indicate statistically significant values among samples at the same storage time ($p<0.5$).

Immediately after treatment, L* values of samples subjected to VI were lower compared to C and D samples while h° was unchanged. These results are in agreement with the ones found in the first part of this study that reported a product darkening as a result of vacuum application. During storage, lightness decreased in all samples. The development of translucent appearance is found to be one of the main changes in minimally processed melon during storage, connected to both visual quality and texture loss. Since melon is not generally affected by enzymatic activity, the variation of L* values can be ascribed to the variation of pigments concentration and to the induction of a

translucent water-soaked tissue (glossy) caused by the loss of cellular compartmentation and water redistribution within the tissues (Aguayo *et al.*, 2004).

During storage, while almost no significant differences were found between C and D samples, sample VI was characterized by constantly lower values. No differences were observed in hue angle values, confirming that this chromatic characteristic was not strongly influenced by vacuum impregnation.

Figure 4 reports the evolution of textural parameters of melon samples during storage. Just after treatment, firmness values were 5.8 ± 0.9 N for sample C, 6.5 ± 0.5 N for D and 7.5 ± 0.4 N for VI, while linear distance values were 30.53 ± 3.5 for sample C, 37.5 ± 1.5 for D and 36.4 ± 2.5 for VI. Increase of hardness in cut melon after dipping in calcium solution has been widely documented (Lamikanra & Watson, 2004; Lamikanra & Watson, 2007; Luna-Guzmán *et al.*, 1999). The increase of both parameters observed after VI is higher compared to D samples probably for a higher impregnation favoured by the application of vacuum, but was noticeably lower compared to the one obtained in the first part of the study. The discrepancy is probably due to the lower porosity of the second batch of melons that allowed for a lower impregnation of samples (1.08% vs. 0.58% WG at the selected process parameters). Nevertheless the obtained values were significantly higher than the ones obtained both for control and dipped samples.

Softening of minimally processed melon during storage can be considered a consequence of the degradation of the internal structures due to tissue ripening, and the solubilisation of the protopectinic fraction of the cell wall components and to the loss of cell adhesion (Aguayo *et al.*, 2004; Fundo *et al.*, 2014). A substantial firmness loss was mainly observed in the first four days in all samples, while values remained quite constant for the rest of the storage, although VI and D samples were always significantly higher than control samples.

Linear distance underwent a slow decrease in control samples indicating a decrease in sample crunchiness. A similar trend was observed in dipped samples although values were constantly higher. VI samples, on the other hand, showed an increase of linear distance during storage.

Firming and resistance to softening have been observed to be a consequence of various calcium treatments. The effect was attributed to the stabilization of membrane systems and to the formation of Ca-pectates, which increase rigidity of the middle lamella and cell wall to increased resistance to polygalacturonase attack, on the pectic substances of the middle lamella and cell wall and to improved turgor pressure (Luna-Guzmán *et al.*, 1999).

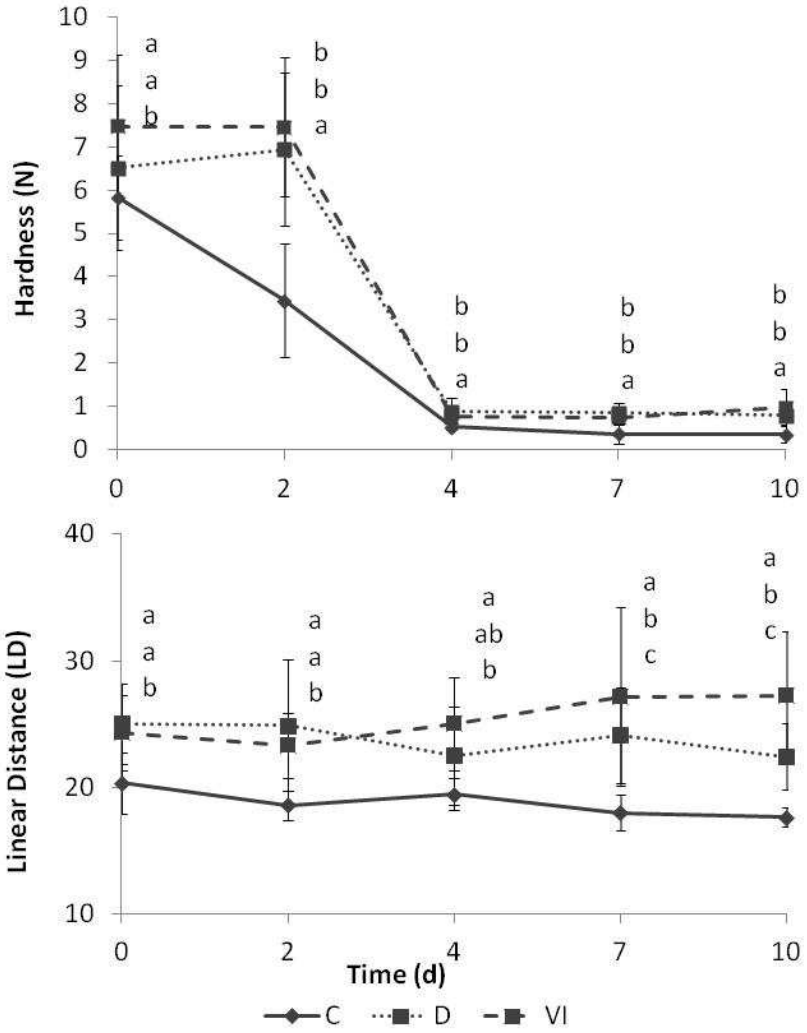

Fig. 4 textural parameters (hardness and linear distance) of melon samples during storage. C: control, D: dipped; VI: vacuum impregnated sample Different letters indicate statistically significant values among samples at the same storage time ($p<0.5$).

Results obtained during storage showed that the application of vacuum combined with calcium allowed to obtain a firming effect on minimally processing melon and in particular, a higher crunchiness during storage compared to control and dipped samples.

In order to evaluate the effects of the treatments on the melon naturally occurring microflora, the cell viability of the principal spoilage agents (total mesophilic and psychrotrophic bacteria, lactic acid bacteria and yeasts), was measured over refrigerated storage (Table 7) .

Total aerobic mesophilic and psychrotrophic microorganisms were about 2 and 2.2 Log CFU g^{-1}, respectively, while lactic acid bacteria ranged between 0.8 and 1.9 Log CFU g^{-1} and yeasts were found at 1 Log CFU g^{-1}. Similar results were found by other authors for fresh-cut "Piel de Sapo" (Fernández *et al.*, 2010) and cantaloupe (Fang *et al*., 2013; Tappi *et al.*, 2016) melons.

Table 7. Microbial loads of of control (C), dipped (D) and vacuum impregnated (VI) minimally processed melons during storage at 10°C.

	Days of storage									
	0		2		4		7		10	
Sample	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
	Mesophilic Bacteria (Log CFU g^{-1})									
C	1.83 [a]	± 0.25	3.32 [a]	± 0.56	4.52 [b]	± 0.34	8.13[a]	± 0.64	8.71[a]	± 0.54
D	2.14 [a]	± 0.37	3.20 [a]	± 0.41	4.43 [b]	± 0.21	7.51[a]	± 0.58	7.26[a]	± 0.71
VI	1.95 [a]	± 0.6	3.31 [a]	± 0.36	6.68 [a]	± 0.74	8.02[a]	± 0.61	7.70[a]	± 0.69
	Psycotrophic Bacteria (Log CFU g^{-1})									
C	2.10[a]	± 0.14	2.37[a]	± 0.42	4.20[b]	± 0.20	7.95[a]	± 0.58	8.19[a]	± 0.45
D	2.19[a]	± 0.12	3.39[a]	± 0.35	4.18[b]	± 0.25	7.88[a]	± 0.61	7.87[a]	± 0.47
VI	2.45[a]	± 0.28	3.10[a]	± 0.44	5.98[a]	± 0.30	7.37[a]	± 0.51	8.41[a]	± 0.57
	Yeasts (Log CFU g^{-1})									
C	1.00[a]	± 0.20	1.00[a]	± 0.20	2.40[a]	± 0.63	4.89[b]	± 0.60	4.64[b]	± 0.48
D	1.00[a]	± 0.20	1.00[a]	± 0.20	1.00[a]	± 0.34	4.92[b]	± 0.71	4.32[b]	± 0.61
VI	1.00[a]	± 0.20	1.00[a]	± 0.20	2.30[a]	± 0.36	6.79[a]	± 0.54	6.79[a]	± 0.59
	Lactic Acid Bacteria (Log CFU g^{-1})									
C	0.85[a]	± 0.21	1.85[b]	± 0.20	3.70[b]	± 0.56	7.99[a]	± 0.60	8.06[a]	± 0.45
D	1.39[a]	± 0.55	2.69[ab]	± 0.20	3.99[b]	± 0.62	7.68[a]	± 0.71	6.54[b]	± 0.47
VI	1.86[a]	± 0.19	3.16[a]	± 0.20	6.77[a]	± 0.45	7.61[a]	± 0.54	7.48[ab]	± 0.57

Different letters indicate statistically significant values among samples at the same storage time ($p<0.5$).

No difference was observed among DIP and VI treatments compared to the control sample for any of the microbial groups immediately after the treatment and after 2 days of storage. During the rest of the storage, it can be observed that the growth of all microbial groups was favoured in the VI sample. At the fourth day, VI sample showed higher values compared to other samples for mesophilic, psycotrophic and lactic acid bacteria, that were respectively 6.68, 5.98 and 6.77 Log CFU g^{-1}, while higher yeasts loads were observed after seven days. Finally, at the end of the storage, sample VI showed higher values for yeasts loads of 6.79 compared to 4.64 and 4.32 Log CFU g^{-1} of C and D samples respectively, while no difference was observed for the other microbial groups.

According to literature (Patrignani *et al*., 2010), the value of 6.0 Log CFU g^{-1} can be considered as a spoilage threshold for minimally processed fruit and vegetables. In C and D samples, this value was reached after 7 days for mesophilic, psycotrophic and lactic acid bacteria and was not exceeded for yeasts until the end of the storage. On the other hand, VI samples reached the spoilage threshold after four days for mesophilic, psycotrophic and lactic acid bacteria and after seven days for yeasts.

Therefore, it seems that microbial shelf-life of minimally processed melons was slightly reduced in samples subjected to VI treatments. To our knowledge, the influence of the VI treatment on microbial growth has not been studied directly. We can hypothesize that the deformation-relaxation

phenomena that can cause irreversible alteration to the visco-elastic properties of the fruit tissues upon the application of a vacuum pressure, may enhance nutrients availability for microbial growth. Nevertheless, the presence of calcium should promote the formation of bond in the middle-lamellae and cell walls, leading to a stiffer and more fragile cellular network (Gras *et al.* 2003). In the present experiment, according to the results obtained in the optimization plan, the lowest level of vacuum was applied combined with the highest CaLac concentration. Although, this combination seemed to have had a structuring effect on melon, it seems to negatively affect microbial shelf-life. Considering this finding, further studies are needed in order to clarify these results, particularly considering that minimally processed melon is considered a highly perishable and potentially hazardous product on the account of its high pH (5.2-6.7) and water activity (0.97-0.99) values.

4. Conclusions

The results obtained in this study allowed to evaluate the influence of calcium concentration and vacuum pressure on colour and textural parameters of minimally processed melons. The high variability of the raw material appeared to be highly influencing the impregnation process, in terms of impregnation levels and of impact on the textural properties.

Nevertheless VI with calcium confirmed to improve textural maintenance during shelf-life, compared to both untreated and dipped samples. However, other quality traits were negatively affected by the application of vacuum. Impregnated products were characterised by a darker and more translucent appearance on the account of the alteration of the structural properties. Moreover microbial shelf-life was reduced to four days compared to the seven obtained for control and dipped samples.

Further studies are in due course in our lab in order to prolong the shelf life of minimally processed melon subjected to vacuum impregnation, by the combination of natural antimicrobial with calcium lactate in the impregnating solution.

Acknowledgments

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Paper VIII

Preliminary study on the quality and stability of minimally processed apples impregnated with green tea polyphenols during storage

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Preliminary study on the quality and stability of minimally processed apples impregnated with green tea polyphenols during storage

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Abstract

Vacuum impregnation (VI) is a technology that allows the replacement of the gas into the porous food structure with an aqueous solution, allowing the direct formulation of porous foods promoting compositional changes in short treatment times; thus it can be exploited for functional products development.

Green tea (GT) is a high source of flavanoids, known to have strong antioxidant properties which has been widely studied both in *in-vitro* and *in-vivo* trials. In the present study, a GT extract (GTE) was used to enrich minimally processed apples by VI in order to obtain a nutritionally fortified product.

Apples were impregnated at 200 mbar for 5 min, with isotonic sucrose solutions with the addition of 1% GTE and/or 1% of ascorbic acid (AA). The impact of GTE enrichment on quality characteristics of the product and the stability of the antioxidant compounds and their *in-vitro* activity during refrigerated storage were assessed.

Results showed that a good impregnation of minimally processed apples was achieved and resulted in a strong increase of the antioxidant compound content and activity. While other qualitative parameters were only slightly affected, colour of samples was influenced just after the VI treatment with an increase of the yellow/orange colour components but also during storage with a higher degree of browning development. However, the addition of 1% of AA allowed to better preserve colour and antioxidant properties during storage limiting oxidative phenomena. Enrichment of apples with GT catechins and AA seemed to be promising in order to obtain a nutritionally fortified fruit product, even if the results obtained in this study are only a first step in this direction.

Key words: minimally processed apples, vacuum impregnation, green tea catechins, quality, antioxidant activity

1. Introduction

Vacuum impregnation (VI) is a technology that allows the replacement of the gas into the porous food structure with an aqueous solution. This occurs through the application of vacuum pressure exploiting a mass transfer, known as hydrodynamic mechanism (HDM) as described and modelled by Fito, Andrés, Chiralt, & Pardo (1996).

VI has been recognised as an effective non-thermal technology that allows the direct formulation of porous foods promoting compositional changes in short treatment times; thus it can be exploited for functional products development. The main studies carried out on the enrichment of fruit and

vegetable products by vacuum impregnation with various bioactive compounds have been reviewed by Alzamora et al. (2005) and include mainly fortifications with probiotics or minerals. More recently, fruit juices with a high content in bioactive compounds have been used for fruit enrichment (Betoret et al., 2012; Castagnini, Betoret, Betoret, & Fito, 2015; Diamante, Hironaka, Yamaguchi, & Nademude, 2014).

Green tea (GT) is a high source of flavanoids, in particular catechins that include mainly epicatechin (EC), epigallocatechin (EGC), epicatechin-gallate (ECG) and epigalloctechin gallate (EGCG). These compounds are known to have strong antioxidant properties which has been widely studied both in *in-vitro* and *in-vivo* trials. A large number of studies have demonstrated the link between green tea catechins consumption and the prevention of different kinds of cancer such as skin, lung, liver, pancreatic, gastrointestinal, breast, and prostate cancers (Khan & Mukhtar, 2007; Wheeler & Wheeler, 2004) and the prevention of cardiovascular diseases (CVD), microbial diseases, diabetes, and obesity (Zaveri, 2006). Moreover, other properties of GT catechins such as antihypertensive and hypolipidemic were observed (Henry & Stephens-Larson, 1984).

However, the amount of GT that needs to be consumed daily in order to obtain the mentioned health benefits is rather large and difficult to reach (Vuong, Stathopoulos, Nguyen, Golding, & Roach, 2011). In this direction, the fortification of food products with GT could help to reach the right amounts of catechins able to exert beneficial effects on human health.

Nevertheless, researches about its potential use for food fortification are still quite limited. Some studied investigated the addition of GTE as a natural antioxidant compound in order to improve the shelf-life of products susceptible to fat oxidation such as sausages (Bozkurt, 2006; Martínez, Cilla, Beltrán, & Roncalés, 2006; Siripatrawan & Noipha, 2012) and surimi (Pérez-Mateos, Lanier, & Boyd, 2006).

On the other hand, nutritional fortification with GT extracts has been studied in bakery products such as bread (Bajerska, Mildner-Szkudlarz, Jeszka, & Szwengiel, 2010; Wang & Zhou, 2004; Wang, Zhou, & Isabelle, 2007; Wang, Zhou, Yu, & Chow, 2006), biscuits (Sharma & Zhou, 2011), noodles (Li et al., 2012), in an apple product at intermediate moisture (Lavelli, Corey, Kerr, & Vantaggi, 2011; Lavelli, Vantaggi, Corey, & Kerr, 2010) and in probiotic yogurt (Muniandya, Shorib, & Babaa, 2016) monitoring the evolution of the catechins and of the antioxidant activity during processing and storage. Tea catechins are in fact very reactive compounds and can undergo degradations, enzymatic or chemical, leading to a variation of the antioxidant activity. Thus, it is fundamental to assess the stability of the compounds used for fortification within the matrix in which they are included during product shelf-life. Moreover, the addition of GT to a product's formulation may lead to variations of quality parameters such as colour and texture and impact on

the product sensorial profile. While Li et al. (2012) found that overall acceptability of fresh noodles was not affected by the addition of three different quantity of GTE in the dough, Bajerska et al. (2010) identified a good compromise to combine technological properties and sensory characteristics of GT enriched rye breads.

Minimally processed fruit are one of the major growing segments in food retail establishments, playing an important role on the antioxidant intake of an increasing number of consumers. The production of minimally processed apple enriched with GT catechins could allow to combine the health properties of both components, in order to obtain an innovative foodstuff. Nevertheless various aspects have to be taken in consideration. For this products, that present an endogenous metabolic activity during the shelf-life period, it is crucial to understand not only the direct effect of VI enrichment on the level and the antioxidant properties of their bioactive components, but also their modification during storage, as consequence of the tissue response to the processing stress and to the passive atmosphere modification of the package headspace.

In the present study, a GT extract was used to enrich minimally processed apples by vacuum impregnation in order to obtain a nutritionally fortified product. The impact of GTE enrichment on quality characteristics of the product and the stability of the antioxidant compounds and their *in-vitro* activity during refrigerated storage were assessed.

2. Materials and methods

2.1 Raw materials

Apples (*Malus domestica* Borkh) of the Cripps Pink variety harvested one week before were purchased at the local market and stored at 5±1°C for two weeks, during which the experimental research was carried out. Apples were characterized by a soluble solids content of 13.7±0.3 g/100g, dry matter of 12.5±0.5 g/100g and porosity of 25.34± 1.36%. Cylindrical samples (20-mm diameter, 20-mm length) were cut with a manual cork borer and a manual cutter designed for the purpose.

2.2 Solutions for impregnation

Impregnating solutions were prepared at isotonic concentration compared to apples with sucrose, ascorbic acid and green tea extract (GTE) (Polyphenon 60-Sigma Aldrich) in different combinations as reported in **Table 1**. The amount of GTE was chosen in order to obtain after impregnation a concentration of catechins in the final product equal to the quantity found in a cup of tea (50-110 mg), according to (Lavelli et al., 2011), considering a 50 g apple portion. The addition of 1% (w/v)

of ascorbic acid (AA) to the sucrose solution was chosen on the basis of previous literary reports in which it was observed a preservation of phenolic compounds, thank to this antioxidant power when applied by dipping (Cocci, Rocculi, Romani, & Dalla Rosa, 2006) or by VI (Blanda et al., 2008) in apples. Solutions were characterized for pH, viscosity and colour parameters.

Table 1. Composition of the solutions used for VI of apples

Coded name	Composition
S	Sucrose
SG	Sucrose + 1% GTE
SA	Sucrose + 1% AA
SAG	Sucrose + 1% AA + 1% GTE

2.3 Vacuum impregnation treatment

An automatic vacuum controller system (AVCS, S.I.A., Bologna, Italy) connected to a closed chamber and a vacuum pump was used for the impregnation process. Impregnation was carried out at the pressure of 200 mbar for 5 min, before restoring atmospheric pressure. A relaxation time of 5 min was applied. Pressure value and processing times were chosen after preliminary tests as the minimum values that allowed to obtain a level of impregnation corresponding to the product real porosity, as too long exposure to high vacuum levels can cause tissue deformation (Mújica-Paz, Valdez-Fragoso, López-Malo, Palou, & Welti-Chanes, 2003).

Samples were immersed in the solutions in a ratio of 1:4 (w/v). At the end of the relaxation time, samples were removed from the solution, blotted with absorbing paper, and weighed. At least three independent impregnation cycles were carried out for each sample. Obtained cylinders were than randomly divided into packages for storage.

2.4 Packaging and storage

VI samples and a fresh untreated one (F) were packed in polypropylene (PP) trays and hermetically sealed with a high permeability PP micro-perforated film. The content of each package (about 50 g) was weighed before sealing. Packages were stored in thermostatic chambers at 10°C. The 10°C temperature was chosen in order to simulate the abuse of temperature that commonly occurs at retail stores and to accentuate any beneficial or negative effects of the applied postharvest treatments (Saftner, Bai, Abbott, & Lee, 2003).

For each samples, three packages were removed after two, five and seven days in order to carry out the analytical determinations.

2.5 Analytical determinations

2.5.1 Physico-chemical parameters

pH of the solution was measured with a pH-meter (Crison, Barcelona, Spain), while viscosity was determined through a vibrational viscometer (mod. Viscolite VL7, Hydramotion Ltd, York, England)

After impregnation the product weight gain (WG) was calculated using the following equation according to (Tylewicz et al., 2012):

$WG = 100 \times (m - m_0)/m_0$

where m is the mass of the impregnated sample and m_0 is the initial mass.

At every selected storage time, the contents of three packages were weighed and weight loss was evaluated compared to the initial value (%).

Soluble solids content was determined at 20°C with a digital refractometer (PR1, Atago, Japan) by measuring the refractive index of the apple samples' juice, obtained by squeezing 10 cylinders after filtration through Whatman #1 filter paper.

2.5.2 Gas composition in the packages headspace

The composition in O_2 and CO_2 (%) of the packages headspace was determined by a gas analyzer "check point O_2/CO_2" mod. MFA III S/L (Witt-Gasetechnik, Witten, Germany). At each sampling time, it was measured in three packages for each sample.

2.5.3 Texture

Mechanical properties were measured with a penetration test using a Texture Analyser TA-HDi500 (Stable Micro Systems, Surrey, UK) equipped with a 50 N load cell and a 6 mm diameter stainless steel cylinder until a maximum deformation of 90%. For each treatment and sampling time, the average of 10 measurements was calculated.

The acquired curves (Force (N) *vs*. time (s)) were analysed and the following mechanical parameters were extracted: hardness, the first peak force (N) value and the linear distance (LD), as an index of crispness (Tappi et al., 2014).

2.5.4 Colour

Surface colour was measured with a spectrophotocolorimeter (HUNTERLAB ColorFlexTM, A60-1010-615, Reston, Virginia) using the D65 illuminant and the 10° standard observer. For each solution measurements were replicated in triplicate, while for apple samples, for each sample and

storage time, the average of 10 measurements was calculated. The L*, a* and b* parameters of the CIELAB system were measured, a* and b* values were further used to calculate the hue as follows:

Hue angle ($h_0 = \left(arctan\left[\frac{b^*}{a^*}\right]/2\pi\right) x\ 360$

2.5.5 Total phenolic content

Total phenolic content (TPC) was measured according to the Folin-Ciocolteau method proposed by (Singleton & Rossi, 1965).

Phenols were extracted from previously freeze dried samples. Briefly, 0.1 g of freeze-dried samples were added to 2 ml of methanol 60% (v/v), vortexed for 2 min and shaken at room temperature for 10 min. The mixture was centrifuged for 10 min at 14000*g*. The supernatant, opportunely diluted, has been used for the analysis.

100 µl of each extract were added to 500 µl of Folin-Ciocoltau reagent. Afterwards, the samples were kept 5 min in the dark, 2 ml of $CaCO_3$ 15% (w/v) and 7.4 ml of distilled water were added. After an incubation of 120 min at room temperature in the darkness, the absorbance was measured at 700 nm with a spectrophotometer (UV-1601, Shimadzu). Methanol 60% and gallic acid were respectively used as the blank sample and as standard. The standard curve was linear in the range 0 – 800 mg/L gallic acid. Results were expressed as mg of gallic acid equivalents (GAE)/100 g of fresh weight.

2.5.6 Antioxidant activity (AOA) determination

AOA was evaluated according to the method proposed by Brand-Williams, Cuvelier, & Berset (1995) with some modifications as follows. The measurement consisted of a solution absorbance decrease as a result of scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by the antioxidants present in apple samples. The concentration of extract, required to reduce a half of free radicals defined as efficient concentration (EC50), was estimated as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%.

For this determination, five extract concentrations in the range of 0.02– 2.00 mg of dry matter/ml for samples impregnated with GT and of 1.00 – 10.00 mg of dry matter/ml for other samples were prepared. Subsequently, 200 µl of each concentration were added to 3 mL of $6{\cdot}10^{(-5)}$ M of DPPH solution and inserted into each test tube and the content was briefly stirred. Samples were kept in darkness for 30 min then the absorbance was read at 515 nm against 60% methanol used as a blank sample.

2.6 Statistical analysis

The significance of the variations observed among samples and during storage was tested according to one-way variance analysis (ANOVA) and the post-hoc Fisher's LSD test ($p<0.05$) using the software STATISTICA 8.0 (Statsoft Inc., Tulsa, UK).

3. Results

3.1 Qualitative parameters

Physico-chemical parameters of the solutions used for impregnation are reported in **Table 2** together with the WG obtained in the sample. pH was noticeably affected by the addition of GTE but above all, as expected, by the addition of ascorbic acid. Viscosity of the solutions was in the range of 1.456 and 1.473 cP; it was reduced by the addition of ascorbic acid while it was slightly increased by the addition of GTE. Nevertheless the weight gain obtained in samples after VI was not influenced by the solution composition, being in the range of 24 - 26 %. Considering that the apple porosity was about 25%, it can be affirmed that all solutions lead to good impregnation of the apple cylinders.

Table 2. Physico-chemical parameters of the solutions of sucrose (S), sucrose and GTE (SG), sucrose and AA (SA) and sucrose, AA and GTE (SAG) used for apple VI treatment.

Solution	pH	Viscosity (cP)	WG	L*	h°
S	$6.5^{a} \pm 0.2$	$1.470^{a} \pm 0.004$	$24^{a} \pm 1$	$81.4^{b} \pm 0.3$	$152.7^{a} \pm 0.8$
SG	$5.8^{b} \pm 0.1$	$1.473^{a} \pm 0.002$	$25^{a} \pm 2$	$68.5^{d} \pm 0.1$	$82.7^{d} \pm 0.1$
SA	$2.7^{c} \pm 0.1$	$1.456^{b} \pm 0.005$	$25^{a} \pm 2$	$82.85^{a} \pm 0.03$	$129.6^{b} \pm 0.1$
SAG	$2.7^{c} \pm 0.1$	$1.462^{ab} \pm 0.009$	$25^{a} \pm 2$	$73.56^{c} \pm 0.09$	$84.6^{c} \pm 0.3$

Different letters show significant differences among samples at the same storage time, while different uppercase letters show significant differences during the storage within the same sample (P-level < 0.05).

Compared to the simple sucrose solution, the addition of ascorbic acid promoted an increase of that parameter but a neat variation toward yellow tonality as indicated by the decrease of hue angle. The addition of GTE promoted a decrease of about 15 unit of L* and of about 60-70 units of hue angle of the solutions, that appeared characterized by the typical yellow/orange colour of green tea catechins.

Table 3. O_2 and CO_2 (%) content within the packages head space and soluble solid (°Bx) and weight loss (%) of of fresh apple (F) and vacuum impregnated samples with sucrose (S), sucrose and GTE (SG), sucrose and AA (SA) and sucrose, AA and GTE (SAG) during storage time.

	Storage time (days)				Storage time (days)			
Sample	**2**	**5**	**7**		**0**	**2**	**5**	**7**
	O_2 (%)			**Sample**	**Soluble solid content (°Brix)**			
F	$20.1^{aA} \pm 0.7$	$17.7^{abB} \pm 0.3$	$14.5^{cC} \pm 0.7$	**F**	$13.7^{bA} \pm 0.1$	$13.5^{cB} \pm 0.1$	$13.7^{cA} \pm 0.2$	$13.5^{cB} \pm 0.1$
S	$19.2^{bcA} \pm 0.1$	$17.0^{abAB} \pm 2$	$13.6^{abB} \pm 0.1$	**S**	$13.3^{dC} \pm 0.1$	$13.6^{cA} \pm 0.1$	$13.4^{eB} \pm 0.1$	$13.1^{dD} \pm 0.1$
SG	$19.9^{abA} \pm 0.5$	$17.7^{abAB} \pm 0.3$	$17.6^{aB} \pm 0.1$	**SG**	$14.0^{aB} \pm 0.1$	$14.1^{bAB} \pm 0.1$	$14.1^{bA} \pm 0.1$	$14.1^{bA} \pm 0.1$
SA	$19.0^{bcA} \pm 1$	$17.3^{abB} \pm 0.3$	$16.0^{bcB} \pm 3$	**SA**	$13.4^{cC} \pm 0.2$	$13.3^{deD} \pm 0.1$	$13.6^{dB} \pm 0.1$	$14.1^{bA} \pm 0.2$
SAG	$19.4^{bcA} \pm 0.0$	$17.9^{abB} \pm 0.2$	$16.5^{bcC} \pm 0.6$	**SAG**	$13.8^{bC} \pm 0.1$	$14.7^{aA} \pm 0.1$	$14.5^{aB} \pm 0.1$	$14.5^{aC} \pm 0.1$
	CO_2 (%)				**Weight loss (%)**			
F	$1.0^{bC} \pm 1$	$4.2^{aB} \pm 0.3$	$6.0^{abA} \pm 0.6$	**F**	-	$0.5^{aA} \pm 0.4$	$0.9^{aA} \pm 0.3$	$1.1^{aA} \pm 0.5$
S	$2.0^{aA} \pm 0.1$	$4.0^{aA} \pm 1$	$7.3^{aA} \pm 0.0$	**S**	-	$0.7^{aA} \pm 0.5$	$0.57^{aA} \pm 0.07$	$0.7^{aA} \pm 0.2$
SG	$1.1^{aB} \pm 0.7$	$3.1^{bcA} \pm 0.2$	$3.1^{cA} \pm 0.1$	**SG**	-	$0.7^{aAB} \pm 0.3$	$0.43^{aB} \pm 0.04$	$0.9^{aA} \pm 0.1$
SA	$1.6^{abC} \pm 1$	$3.7^{abB} \pm 0.4$	$5.0^{bA} \pm 2$	**SA**	-	$0.25^{aA} \pm 0.02$	$0.5^{aA} \pm 0.2$	$0.6^{aA} \pm 0.5$
SAG	$2.0^{aC} \pm 0.4$	$3.2^{bcB} \pm 0.1$	$4.1^{bcA} \pm 0.3$	**SAG**	-	$0.6^{aA} \pm 0.3$	$1.0^{aA} \pm 0.6$	$0.47^{aA} \pm 0.02$

Different lowercase letters show significant differences among samples at the same storage time, while different uppercase letters show significant differences during the storage within the same sample (P-level < 0.05).

During storage, gas composition within the packages head space has been evaluated and the content (%) of O_2 and CO_2 are reported in **Table 3**. At the beginning of the storage, it appeared that fresh sample had a lower respiration rate compared to the impregnated samples, but during storage this trend was inverted and after seven days, it showed a lower content of O_2 and the highest of CO_2.

As expected, due to the respiration metabolism of the living apples tissues, O_2 content decreased during storage while CO_2 increased, reaching at the end of the seven days considered, values respectively between 13.6 and 17.6% and between 3.1 and 7.9%.

Samples impregnated with GTE showed consistently a lower oxygen decrease and CO_2 accumulation within the packages headspace compared to the correspondent sample impregnated without the extract.

Weight loss of apple samples (**Table 3**) was limited (up to 1.09% compared to the initial weight) during storage and showed only few differences among samples; similarly differences found in soluble solid content were slight (spanned between 13.2 and 14.5%), not indicating a trend but more probably due to the natural variability of the raw material.

Textural parameters of apples during storage are reported in **Table 4**. Hardness of fresh sample decreased during storage from 11.6 ± 0.5 to 6.9 ± 0.6, while crispness, indicated by the Linear Distance parameter slightly increased. Generally, all impregnated samples followed a similar trend, and at the end of the storage they showed few significant differences only for LD.

Table 4. Textural parameters of of fresh apple (F) and vacuum impregnated samples with sucrose (S), sucrose and GTE (SG), sucrose and AA (SA) and sucrose, AA and GTE (SAG)during storage

	Storage time (days)			
	0	**2**	**5**	7
Sample	**Hardness (N)**			
F	$11.6^{abA} \pm 0.5$	$9.0^{bB} \pm 2$	$7.0^{cC} \pm 1$	$6.9^{aD} \pm 0.6$
S	$11.6^{aA} \pm 0.4$	$11.0^{aAB} \pm 2$	$10.0^{abAB} \pm 2$	$10.0^{aB} \pm 2$
SG	$10.8^{aA} \pm 0.3$	$10.0^{abAB} \pm 2$	$9.0^{bB} \pm 2$	$9.0^{aB} \pm 2$
SA	$10.0^{bAB} \pm 2$	$10.0^{aA} \pm 2$	$11.0^{aA} \pm 2$	$9.0^{aB} \pm 2$
SAG	$9.0^{bA} \pm 1$	$11.0^{abA} \pm 2$	$10.0^{abA} \pm 3$	$9.0^{aA} \pm 2$
	Crispness - Linear distance			
F	$36^{aAB} \pm 2$	$34^{aAB} \pm 9$	$32^{aB} \pm 14$	$42^{aA} \pm 6$
S	$33^{abAB} \pm 2$	$32^{aC} \pm 4$	$37^{aAB} \pm 7$	$39^{abA} \pm 7$
SG	$32^{bC} \pm 1$	$33^{aBC} \pm 5$	$37^{aAB} \pm 4$	$40^{abA} \pm 6$
SA	$31^{bA} \pm 3$	$33^{bA} \pm 4$	$30^{aA} \pm 3$	$33^{bA} \pm 2$
SAG	$36^{aA} \pm 3$	$32^{aA} \pm 3$	$37^{aA} \pm 5$	$36^{bA} \pm 7$

Different lowercase letters show significant differences among samples at the same storage time, while different uppercase letters show significant differences during the storage within the same sample (P-level < 0.05).

Lightness (L*) and hue angle (h°) measured in apple samples just after VI treatment are reported in **Figure 1**. After impregnation all samples underwent a decrease of L* values of about 20 units compared to the fresh one, with very little differences among samples. Hue values of S, T and SA samples were the most similar to the untreated sample even if significant differences were observed. Conversely, the addition of GTE to the impregnating solution promoted a decrease of about 10° of hue angle in these samples.

Figure 2 shows the variations of L* and h° of samples during seven days of storage. Impregnated samples did not show differences until the 5th day of storage, while at the last day samples S and SG showed a significant decrease of L* values compared to other samples. At the end of the storage, SA and SAG were characterized by the highest L* values, showing a very limited change during the considered period.

Hue angle in fresh sample underwent a decrease in the first two days and then was quite similar for the rest of storage. S and T samples were characterized by similar colour parameters trends, while SA did not change during the 7 days.

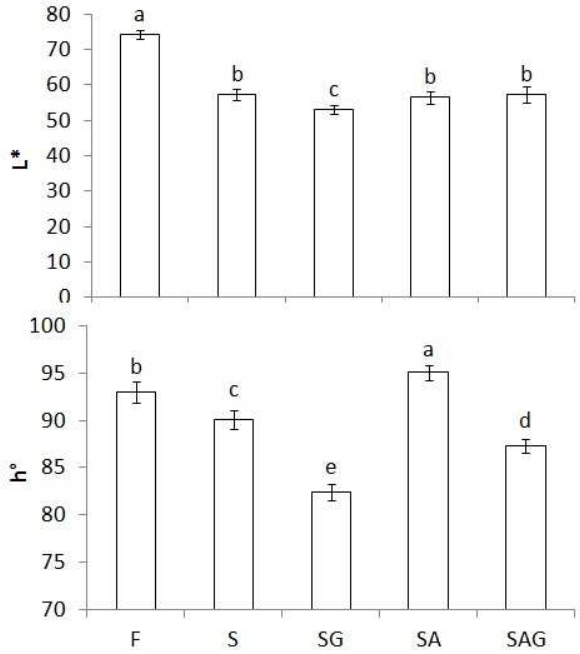

Figure 1. Colorimteric parameters, L* and h°, of of fresh apple (F) and vacuum impregnated samples with sucrose (S), sucrose and GTE (SG), sucrose and AA (SA) and sucrose, AA and GTE (SAG)as affected by VI treatment. Different letters show significant differences among samples (P-level < 0.05).

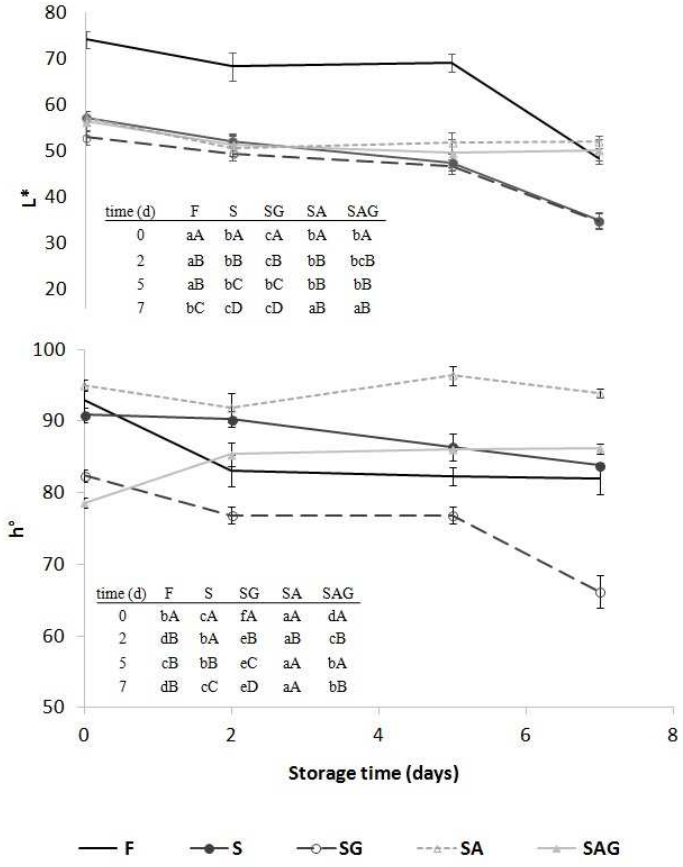

Figure 2. Variations of colorimetric parameters, L* and h°, of samples during 7 days of storage. Different lowercase letters show significant differences among samples at the same storage time, while different uppercase letters show significant differences during the storage within the same sample (P-level < 0.05).

Between samples impregnated with GT, SG showed a sharp decrease of h° during storage until values between 66 and 70°, conversely, in SAG sample h° was characterized by a slight increase during storage and at the end was higher than in the fresh sample.

The colour variations observed in samples impregnated with solutions containing AA were lower, after treatment and during storage, in terms of both L* and h°. Visual examples of F, SG and SAG apple samples at the end of the storage are shown in **Fig. 3**.

Figure 3. Visual examples of F, SG and SAG apple samples at the end of the storage.

3.2 Total phenolic content (TPC) and antioxidant activity (AOA)

As it can be observed in **Table 5**, the impregnation process, both with sucrose and trehalose, caused a reduction of the TPC, probably for a dilution effect. Samples impregnated with GTE showed an increase of TPC with values of 360 and 370 mg of GAE/100 g in respectively sample SG and SAG sample. In SA sample, the TPC increase is reasonably due to the presence of ascorbic acid, that is able to react with the Folin-Ciocolteau reagent, determining an overestimation of the phenolic content.

In the fresh sample, TPC decreased of the 11% at the end of the storage. Conversely, in S sample, although the initial value was lower, it remained quite constant until the 7^{th} day of storage. In SG sample, the initial value underwent a decrease of 20, on the contrary, SAG samples maintained at the end of the storage, TPC content similar to the initial one.

AOA, expressed as EC50 values, of apple samples during storage are reported in table 5. Samples impregnated with GTE showed values ten to twenty fold lower to the correspondent sample impregnated without GTE. EC50 values were constantly lower in SA than in S sample, showing a higher antioxidant activity due to the presence of ascorbic acid.

During storage, it was not possible to observe a clear trend of EC50 values. Moreover, no significant differences were observed among samples impregnated with GTE in any of the storage days considered.

Table 5. Total phenolic content (TPC) and Antioxidant activity (EC50) of fresh apple (F) and vacuum impregnated samples with sucrose (S), sucrose and GTE (SG), sucrose and AA (SA) and sucrose, AA and GTE (SAG) during storage.

	Storage time (days)			
Samples	**0**	**2**	**5**	**7**
	Total Phenolic Content (mg GAE/100 g fw)			
F	$74^{dA} \pm 11$	$56^{dB} \pm 3$	$62^{dB} \pm 2$	$61^{dB} \pm 1$
S	$39^{eB} \pm 3$	$41^{dA} \pm 4$	$40^{eB} \pm 3$	$40^{eB} \pm 3$
SG	$360^{bC} \pm 10$	$353^{bB} \pm 13$	$351^{bA} \pm 4$	$286^{bD} \pm 9$
SA	$117.5^{cB} \pm 0.5$	$123.3^{cA} \pm 0.4$	$105.7^{cD} \pm 0.6$	$115^{cC} \pm 1$
SAG	$373^{aB} \pm 3$	$400^{aA} \pm 4$	$375^{aB} \pm 3$	$379^{aB} \pm 3$
	Antioxidant activity (EC50)			
F	$17.1^{aA} \pm 2$	$15.5^{aA} \pm 0.5$	$16.2^{aA} \pm 0.3$	$16.52^{aA} \pm 0.06$
S	$17.2^{aA} \pm 0.7$	$16^{aA} \pm 2$	$19^{aA} \pm 2$	$15^{abA} \pm 1$
SG	$0.32^{cB} \pm 0.01$	$0.54^{cA} \pm 0.01$	$0.58^{cA} \pm 0.03$	$0.60^{cA} \pm 0.03$
SA	$11.9^{bA} \pm 0.2$	$7.67^{bC} \pm 0.01$	$9.08^{bB} \pm 0.00$	$8.9^{bB} \pm 0.3$
SAG	$0.45^{cAB} \pm 0.09$	$0.41^{cAB} \pm 0.01$	$0.36^{cB} \pm 0.00$	$0.57^{cA} \pm 0.07$

Different lowercase letters show significant differences among samples at the same storage time, while different uppercase letters show significant differences during the storage within the same sample (P-level < 0.05).

4. Discussion

The development and consumption of functional foods, that not only satisfy basic nutrition needs but also allow to obtain health benefits, are increasing (Alzamora et al., 2005). VI has been indicated as an effective non-thermal technology for new products design by exploiting fruit and vegetable porous tissues as new matrices into which functional compounds can be included (Betoret et al., 2003), providing novel functional product categories and new commercial opportunities.

Various literature studies report impregnation of fruit or vegetables with both isotonic or hypertonic solutions or juices containing one or more bioactive substances with the aim of increasing the daily intake to meet nutritional recommendation or health benefits (Betoret et al., 2003; Castagnini et al., 2015; Gras, Vidal, Betoret, Chiralt, & Fito, 2003; Xie & Zhao, 2003). In the present work, we evaluated the fortification of minimally processed apples with green tea catechins and the impact on the stability of some physico-chemical parameters and on antioxidant properties during storage; in our intention, this was a first step for the development of a new functional product.

Physico-chemical parameters of apple samples such as soluble solid content and weight loss during storage did not seem to be affected by VI treatment itself, nor by the different composition of the

solutions. Differences were observed in the evolution of the gas composition in the packages headspaces. After VI samples seemed to have a reduced respiration metabolism during storage compared to the fresh untreated sample. The initial increase of respiration rate may be related to stress due to processing. On the other hand the subsequent decrease may indicate a reduction of the respiratory metabolism. In previous studies (Castelló, Fito, & Chiralt, 2006; Igual, Castelló, Ortolá, & Andrés, 2008) an increase of the respiratory quotient was observed after impregnation of persimmons and strawberries indicating the onset of a endogenous fermentative metabolism, probably due to the inhibition of the oxygen diffusion in the intercellular spaces occupied by the impregnating solution. On the other hand, Sanzana, Gras, & Vidal-Brotóns (2011) observed an increase of respiration rate, both in terms of O_2 consumed and CO_2 produced, in various vegetables after VI treatment, while the respiratory quotient still indicated aerobic metabolism. The authors suggested that the increase in the respiration rate was attributed to a mechanical stress promoted by the application of vacuum to the tissue. The evaluation of respiration rate is important because it is negatively correlated to product shelf-life (Kader, 1987), while the development of anaerobic metabolism is negative for product quality since it may lead to off-odours and off-flavours. In the present study a medium barrier PP film was used, therefore the permeability to both gases played a role in the evolution of the headspace composition, hence it is not possible on the basis of the obtained results to make an accurate evaluation of the respiration rate and of the possible conversion to anaerobic respiration. Nevertheless, it is possible to observe that while VI process slowed down the variation of gas composition inside the packages, the addition of GTE to the VI solutions, consistently decreased the CO_2 accumulation and the O_2 consumption within the headspaces.

These results may indicate an inhibition of the respiratory metabolism of the tissue. Sanzana et al. (2011) also observed a reduction of respiration rate in sample vacuum-impregnated with *Aloe vera* solutions compared to samples impregnated only with sucrose, hypothesising an ability of the bioactive compounds to compensate the stress due to the VI treatment. To our knowledge, previous reports on the impact of the enrichment with polyphenols on the metabolism of fresh fruit tissues are not present in the literature, thus it is difficult to make hypothesis while further studied should be conducted in order to clarify the mechanisms involved.

Textural parameters variations during storage are probably depended on the degradation of cellular structure due to senescence phenomena that in minimally processed fruit are accelerated as a consequence of the mechanical damage caused by processing operations (Toivonen & Brummell, 2008). Nevertheless, the differences found among samples were rather small and did not indicate a clear trend but may have been caused more probably by the high variability of data, typical for this

kind of parameters evaluated on solid heterogeneous product. Hence, it can be assumed that nor the VI process nor the addition of GTE or AA had a significant impact on these qualitative parameters.

Nevertheless, the main variations observed in the samples after treatment and during storage were relative to product colour and antioxidant content, that appeared to be both related to the oxidation of polyphenols.

Colour and visual quality are among the main factors that influences consumers acceptability of minimally fresh fruit products. In these commodities, colour is manly related to the content of some pigments, that during ripening, processing and storage can be subjected to degradation and/or to neo-genesis (Alzamora, Lopez-Malo, & Tapla, 2000) but it is also related to the structural properties of the tissue that can be altered by processing. In the present study, we found that the VI process generally promoted a darkening of the tissue. These results are in agreement with Muntada, Gerschenson, Alazamoea, & Castro (1998) and Tapia, Schulz, Gómez, López-Malo, & Welti-Chanes (2003) that affirmed that the application of vacuum pressure to a tissue promotes alterations of its structural properties and the gas-liquid exchange contributes to a change of the refractive index. On the other hand, the addition of GT extract lead to a variation of hue angle due to the colour of the solutions that were characterised by a yellow/orange hue. The addition of ascorbic acid reduced the variation of colour due to the added tea catechins.

The shelf-life of minimally processed apples is generally limited by enzymatic browning and, as expected, in the fresh product, superficial browning, represented by a decrease of L* value and an increase of hue angle, was observed during storage, mainly due to oxidative phenomena of native phenolic compounds. The enzyme mainly responsible of this phenomenon are polyphenoloxidases (PPO), even if also peroxidase (POD) can play a role (Toivonen & Brummell, 2008). The presence of GT phenolic compounds, of which the main representative is epigallocatechin-gallate (Sang, Lee, Hou, Ho, & Yang, 2005) and of AA, influenced the kinetics of colour variation during storage.

The addition of GTE promoted, in sample SG, an increase of tissue browning that lead to a sharp decrease of luminosity and hue angle at the end of the storage.

Li, Taylor, Ferruzzi, & Mauer (2013) found a colour variation of GT solutions during storage that corresponded to a decrease of L* values and an increase of a* and b* values with a perceived colour, tending to yellow/orange shade, that was attributed to the higher molecular weight of the oxidation products of catechins (Sang et al., 2005).

Lavelli et al. (2011) used GT to enrich an apple product at intermediate moisture and studied the stability of the antioxidant compounds and activity during storage. The authors found a general darkening of the product and an increase of the red parameter due to the addition of the extract, similar to what we found in the present study. Furthermore, the authors observed that during

storage, the enriched product showed different kinetics of colour variation compared to the unfortified one, but, since the products underwent a blanching step during processing and no residual PPO activity was found, these variations were ascribed solely to non enzymatic browning.

Although we did not measure directly PPO activity, since apple samples were not subjected to any thermal treatment, we can assume that enzymatic activity was present and played a role in the colour development of samples during storage, although chemical oxidation may have also occurred. GT catechins are in fact very reactive species, characterised by a high instability being easily oxidised when in aqueous solution. They can undergo both enzymatic and chemical oxidative reactions, following different pathways that generate different degradation compounds.

Enzymatic degradation of tea catechins leads to the formations of various compounds, generally belonging to teaflavins and thearubigins. These groups of compounds can be generated by PPO activity on green tea shoots to produce black tea, and are characterized by a reddish colour (Li et al., 2013; Robertson & Bendall, 1983). On the other side, chemical degradation of EGCG in water solutions may occur during storage forming brown-coloured products and it is mainly caused by two reaction patterns: the epimerisation that occurs at the C2 position generating GCG and the auto-oxidation that involves the B ring that generates EGCG dimers. Which patterns prevails depends on surrounding conditions such as oxygen levels, pH, metal and antioxidants presence (Sang et al., 2005).

The GTE used in this experiment consist of 60% of EGCG that is the most abundant polyphenol compound present in green tea. In the present study we evaluated the TPC of the samples, through the Folin-Ciocolteau method. In order to clarify which oxidative pattern took place and which degradation compounds were present at the end of the storage, a further study on the phenolic characterisation would be necessary.

With regard to TPC, it decreased in fresh sample during the first 2 days of storage, probably because of the enzymatic oxidation carried out by PPO that also determined a browning effect. In impregnated samples instead, although the initial value was lower, it remained quite constant until the end of storage. It could be hypothesised that the impregnation process, limiting the presence of oxygen inside the tissues, also limited the oxidation of phenols, as previously observed by (Xie & Zhao, 2003) on vacuum impregnated apples. In SG enriched product, after an initial increase, TPC content was lower at the end of the storage, probably because of the oxidation of catechins. These results confirmed the ones obtained by colorimetric measurements, as in these samples, the highest degree of browning was observed (**Fig. 2** and **Fig. 3**)

It is known that ascorbic acid is able to inhibit browning reactions, mainly because of its ability to scavenge oxygen and to be oxidised reducing quinones to phenols, before they can participate in

further reactions that lead to coloured pigments. Blanda et al. (2008) observed a reduction in phenolic degradation in apples impregnated with a solution containing 1% of AA during frozen storage. Moreover, Chen, Zhu, Wong, Zhang, & Chung (1998) evaluated the stabilizing effect of AA on GT catechins under various conditions. The results showed that, although these compounds are known to be more stable at acidic pH, the protective effect of AA was not due to the acidification as the addition of citric acid, although it decreases the pH of the solution, did not have any stabilizing effect. Considering that the GT catechins are easily turned into their corresponding semiquinone free radicals, the author hypothesized that AA acted as a reductant restoring their original form, but also that it reduced the oxygen concentration in the solution helping to hinder oxidative reactions. The positive effect on stability was found on all four epicatechin derivatives, but in particular on EGC and EGCG. Hence, we can assume that the ascorbic acid added to the impregnating solutions preserved the phenolic compounds against oxidation, both the native ones, as it can be observed in SA samples, and the added ones, as it can be observed in SAG sample.

Although TPC was found positively correlated to EC50 values (R^2=0.901), the decrease in antioxidant compounds was not always reflected in a decrease of AOA. This discrepancy was also observed by Lavelli et al. (2011) on an apple product fortified with GT catechins. The decrease of AOA during storage was slower and only moderate if compared with the degradation of antioxidant compounds. The authors suggested that the degradation of catechins leads to products with antioxidant properties similar to the original ones. Chemical oxidation was found to increase the chain-breaking activity measured by DPPH method also by Manzocco, Anese, & Nicoli (1998), thanks to the progressive polymerisation of phenolic compounds and the formation of brown macromolecular products. On the other hand, enzymatic oxidation of catechins leads to the opposite effects, diminishing the radical scavenging properties. As already mentioned, the degradation products of enzymatic oxidation of GT catechins are mainly teaflavins and thearubigins, groups of compounds whose AOA is scarcely known (Graham, 1992). Hence, the characterisation of the phenolic fraction in the different samples during storage would help in clarifying also the relationship between antioxidant content and activity.

On the other hand, according to Ramazzina et al. (2015), that compared different *in-vitro* methods for the evaluation of AOA in minimally processed kiwifruit, DPPH method was the less sensitive one. Hence, the results found in the present studies should be integrated with the evaluation of other *in-vitro* or *ex-vivo* antioxidant methods.

5. Conclusions

Aqueous solution containing GTE allowed a good impregnation of minimally processed apples that resulted in a strong increase of the antioxidant compound content and activity. While other qualitative parameters were only slightly affected, colour of samples was influenced just after the VI treatment but also during storage. The addition of catechins, leads to an increase of the yellow/orange colour components of impregnated samples and to a higher degree of browning development. However, the addition of 1% of ascorbic acid allowed to better preserve colour and antioxidant properties during storage limiting oxidative phenomena.

Enrichment of apples with GT catechins and ascorbic acid seems to be promising in order to obtain a nutritionally fortified fruit product, even if the results obtained in this study are only a first step in this direction. The qualitative characterization of the phenolic compounds during storage and their relationship with the antioxidant activity, together with further *in-vitro* and *in-vivo* experiments for the evaluation of the antioxidant properties will need to be carried out. Moreover, aspects related to the tissue metabolism and respiration of the fresh apple tissue need further investigations.

In terms of sensorial acceptability, the impact of apple vacuum impregnation with green tea has to be carefully examined, considering its astringency and bitter taste, taking in consideration the possibility to change in quantitative/qualitative terms the formulation of the impregnating solution.

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Paper IX

Calcium and ascorbic acid affect cellular structure and water mobility in apple tissue during osmotic dehydration in sucrose solutions

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Calcium and ascorbic acid affect cellular structure and water mobility in apple tissue during osmotic dehydration in sucrose solutions

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ABSTRACT

The effects of the addition of calcium lactate and ascorbic acid to sucrose osmotic solutions on cell viability and microstructure of apple tissue were studied. In addition, water distribution and mobility modification of the different cellular compartments were observed. Fluorescence microscopy, light microscopy and time domain nuclear magnetic resonance (TD-NMR) were respectively used to evaluate cell viability and microstructural changes during osmotic dehydration. Tissues treated in a sucrose–calcium lactate–ascorbic acid solution did not show viability. Calcium lactate had some effects on cell walls and membranes. Sucrose solution visibly preserved the protoplast viability and slightly influenced the water distribution within the apple tissue, as highlighted by TD-NMR, which showed higher proton intensity in the vacuoles and lower intensity in cytoplasm-free spaces compared to other treatments. The presence of ascorbic acid enhanced calcium impregnation, which was associated with permeability changes of the cellular wall and membranes.

1. Introduction

The concentration of plant foods by immersing solid food pieces in a hypertonic solution consisting of salt, sugar, glycerol, or other humectants is known as osmotic dehydration (OD) (Sereno, Moreira, & Martinez, 2001). This technique reduces the a_w of the product without a phase change because the flow of water from the product into the concentrated solution is compensated by the solutes migration from the solution into the product (Nieto, Vicente, Hodara, Castro, & Alzamora, 2013). This process permits the formulation of products with intermediate moisture content through dewatering and impregnation of desired solutes (Barrera, Betoret, & Fito, 2004). Because of its versatility, OD has a wide range of applications in the development of minimally processed plant foods or as pretreatment for other preservation methods such as freezing or drying (Alzamora, Cerrutti, Guerrero, & López-Malo, 1995; Garcia Loredo, Guerrero, Gomez, & Alzamora, 2013).

The addition of calcium in osmotic solutions has been widely used in plant foods as fortifier and to enhance firmness (Anino, Salvatori, & Alzamora, 2006; Barrera, Betoret, Corell, & Fito, 2009; Mavroudis, Gidley, & Sjöholm, 2012; Silva, Fernandes, & Mauro, 2014a). Fortification using combinations of substances such as calcium and iron (Barrera et al., 2004) or Ca and vitamin C (Silva, Fernandes, & Mauro, 2014b) has also been investigated.

OD causes physical modifications of cell membranes and cell walls, which affects the rheological properties and state of water (Nieto et al., 2013; Vicente, Nieto, Hodara, Castro, & Alzamora, 2012). Knowledge about the microstructure and mass transport in OD of plant tissues is fundamental for controlling production of foods fortified with vitamins and mineral salts. Mass transfer in cellular tissue is influenced by the osmotic pressure and structure properties such as permeability of the plasma membrane and vacuole membrane, cell wall porosity, or even intercellular porosity. The osmotic pressure, in turn, depends on the solute concentration and the salt and acid dissociation because each substance presents specific transport properties through plasma and vacuole membranes or cell wall pores. When the cellular structure is changed, the tissue selectivity is also modified, so that water mobility and distribution are affected.

Osmotic dehydration of plant foods is largely controlled by the cellular membranes, which have different permeabilities to different substances. Biological membranes are composed of phospholipid

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bilayers with intrinsic proteins. Studies have shown that water can cross plant membranes through proteinaceous channels formed by members of the aquaporin superfamily, also called water channels (Weig, Deswarte, & Chrispeels, 1997). Aquaporins are hydrophobic proteins that enhance the biological membrane's permeability to water. They belong to a group of membrane proteins, the major intrinsic proteins (MIP) family of channels, with a molar mass in the range of 26 and 30 kDa (Tyerman, Niemietz, & Bramley, 2002; Weig et al., 1997). These channels increase the permeability of biological membranes to water compared to the lipid bilayers; they are detected by the low activation energy needed to transport water across water channels (Tyerman et al., 2002).

Calcium ions that occupy spaces outside the plasma membrane (apoplast) have a structural role in the cell wall because they interact with pectic acid polymers to form cross-bridges that reinforce the cell adhesion, thereby reducing cell separation, which is one of the major causes of plant tissue softening (Roy et al., 1994). Moreover, calcium can affect water channel activity; however, the significance of the inhibition of plant aquaporins by calcium is complex and has still not been clarified (Maurel, 2007). Conversely, calcium can also cross membranes through cation channels. A vacuolar non-selective Ca^{2+} channel (Peiter et al., 2005) has been identified as a plasma membrane non-selective cation channel (Tapken et al., 2013) in plant cells.

Ascorbic acid (AA) influences the cell physiology; however, little is known about its role in plant tissue. Exposure of *Arabidopsis thaliana* seedlings to ascorbic acid demonstrated that exogenous AA caused grow inhibition and damage in the cellular structure by increasing the ROS (reactive oxygen species) content (Qian et al., 2014). In addition, a very low pH (2–3) can increase the cell wall porosity (Zemke-White, Clements, & Harris, 2000), which increases diffusion of great molecules in the free spaces of the cellular tissue.

The complexity of osmotic dehydration of plant tissues rises when using a multicomponent solution because all the solutes and their respective concentrations affect the membrane permeability and cell wall. Consequently, monitoring the water distribution can be useful to clarify the behavior of the cellular microstructures as osmotic dehydration proceeds. Time domain nuclear magnetic resonance (TD-NMR) is an analytical method that allows the determination of the water content and its mobility in different cell compartments by proton relaxation times of water in foods (Hills & Duce, 1990). It is a non-invasive method suitable for large tissue samples that relates water content and water properties in different proton pools within the tissue with different transverse relaxation times (T_2) of water (Hills & Remigereau, 1997; Panarese et al., 2012; Tylewicz et al., 2011). In fruit samples, the higher the mobility of a proton bearing molecule, the higher the spin–spin (T_2) relaxation time is expected to be. The intensities of proton pools with different transverse relaxation times are a relative measure of the amount of water corresponding to a specific T_2. This technique has been used in OD of plants to evaluate water mobility and distribution within the cellular tissue (Cornillon, 2000; Panarese et al., 2012; Tylewicz et al., 2011). Microscopic techniques can also be important tools to clarify cell viability by using vital dyes. Protoplasts stained with fluorescein diacetate (FDA) allow the estimation of two types of plasma membrane injuries: lysis and the loss of semipermeability (Halperin & Koster, 2006; Koster, Reisdorph, & Ramsay, 2003). Vacuole membrane alterations can be evaluated by the capacity of intact tonoplasts to retain neutral red and provide contrast to vacuoles (Carpita, Sabularse, Montezinos, & Delmer, 1979; Thebud & Santarius, 1982).

A multianalytical approach that combines several techniques such as micro and ultrastructural microscopy, calorimetry and NMR have been successfully employed in investigations of plant foods subjected to mild processing conditions (Panarese et al., 2012; Rocculi et al., 2012; Tylewicz et al., 2011).

The main objective of this work was to investigate the effects of the addition of calcium lactate (CaLac) and ascorbic acid (AA) to sucrose (Suc) osmotic solutions on mass transfer, cell viability and structure of apple tissue, as well as the consequential water distribution and mobility modification among the different cellular compartments.

2. Materials and methods

2.1. Raw materials

Apples (*Malus domestica* Borkh) of the Cripps Pink variety, popularly known by the brand name Pink Lady (Castro, Barrett, Jobling & Mitcham, 2008), were provided by the local market and stored at 5 ± 1 °C for no longer than 2 weeks, during which osmotic dehydration experiments were performed. The average weight of the apples was 233.5 ± 17.7 g, and the soluble solids content was 13.4 ± 0.3 g · 100 g^{-1}. Apples were cut in cylinders (8-mm diameter) with a manual cork borer and cut to a length of 40 mm using a manual cutter designed for this purpose. Commercial sucrose (refined sugar, Eridania, Italy), L-ascorbic acid (Shandong Luwei Pharmaceutical Co., China) and calcium lactate (calcium-L-lactate 5-hydrate powder, PURACAL® PP Food, Corbion PURAC, Netherlands) were used in the experiments.

2.2. Osmotic dehydration

Apple cylinders were weighed (approximately 0.1 kg) in a mesh basket and immersed in the osmotic solution. Each basket corresponded to a single OD time: 0.5, 1, 2 and 4 h. The OD system consisted of a cylindrical glass vessel containing 4.5 kg of aqueous solution. The cylindrical baskets, coupled with an impeller of a mechanical stirrer, were continuously rotated. Two baskets were prepared for each process time. The syrup-to-fruit ratio was approximately 15:1 (w/w).

The OD was performed with four different aqueous solutions: 40% sucrose (Suc), 40% sucrose + 4% calcium lactate (Suc–CaLac), 40% sucrose + 2% ascorbic acid (Suc–AA) and 40% sucrose + 4% calcium lactate + 2% ascorbic acid (Suc–CaLac–AA). After the pre-established contact period, the samples were removed from the solution, rinsed with distilled water, blotted with absorbing paper, and weighed.

Immediately after the process, analyses of the total solids and soluble solids contents were performed for fresh and osmotically treated samples in triplicate. The proton transverse relaxation time (T_2) was also immediately measured for six replicates. Samples for calcium and ascorbic acid analyses were freeze-dried.

2.3. Analytical methods

The moisture content for 2 g of fresh and treated samples was determined gravimetrically, in triplicate, by drying at 70 °C until a constant weight was achieved. The soluble solids content was determined at 20 °C by measuring the refractive index with a digital refractometer (PR1, Atago, Japan). Water activity was measured in a water activity meter (AquaLab Series mod. CX-2, Decagon, USA).

2.3.1. Ascorbic acid

For ascorbic acid determination, an extraction was performed with 0.5 g of a freeze-dried sample added to 10 ml of meta phosphoric acid (62.5 mM) and sulfuric acid (5 mM) solution. The mixture was vortexed for 2 min and centrifuged at 10,000×g for 10 min at 4 °C. The supernatant was opportunely diluted and filtered through a 0.45 μm nylon filter. Ascorbic acid was determined

according to Odriozola-Serrano, Hernàndez-Jover, and Martìn-Belloso method (2007). The HPLC system (Jasco LC-1500, Carpi, MO, Italy) was equipped with a diode array UV/Vis detector. A reverse-phase C18 Kinetex (Phenomenex Inc., Torrance, CA, USA) stainless steel column (4.6 × 150 mm) was used as the stationary phase. Samples were introduced in the column through an autosampler (Jasco AS-2055 Plus). The mobile phase was a 0.01% solution of sulfuric acid adjusted to a pH of 2.6. The flow rate was fixed at 1.0 mL/min at room temperature. Data were processed by the software ChromNAV (ver. 1.16.02) from Jasco. The ascorbic acid content was quantified at 245 nm through a standard calibration curve.

2.3.2. Calcium

The calcium concentration was determined using a flame atomic absorption spectrophotometer (Model A Analyst 400, Perkin Elmer, Santa Clara, California, USA), using a lumina hollow cathode lamp (Perkin Elmer) based on the adapted methodology of AOAC – Association of Official Analytical Chemists. (1995). Approximately 6 g of fresh samples (without treatment) and 2 g of treated samples, i.e., freeze dried and previously ground, were weighed in a 50 ml glazed, porcelain crucible placed in a muffle furnace and heated up to 550 °C until complete ignition. Then, the porcelain crucibles were cooled in desiccators, where 20 ml of chloride acid (0.1 M) was added to the capsules with fresh samples and 30 ml was added to the treated samples. The ash was dissolved, and then, an aliquot of this solution was quantitatively taken and diluted 8 times (fresh samples) or 100 times (treated samples) with 0.1 M chloride acid. Standard calcium solutions between 2 and 20 ppm were used to determine a calibration curve of absorbance versus ppm of calcium.

2.4. Mass transfer of osmotic dehydration

Mass transfer during osmotic dehydration was evaluated on the basis of mass balances. The total mass variation in relation to the initial mass during osmotic dehydration was calculated from experimental data according to Eq. (1):

$$\Delta M = \frac{(m - m_0)}{m_0} \times 100 \tag{1}$$

where m = mass and 0 = initial time ($t = 0$).

Water loss (WL), calcium lactate gain (ΔCaLac), ascorbic acid gain (ΔAA) and sucrose gain (ΔSuc), all calculated in relation to initial mass, are shown in the following equations:

$$WL = \frac{(w_w \cdot m) - (w_{w_0} \cdot m_0)}{m_0} \times 100 \tag{2}$$

$$\Delta CaLac = \frac{w_{CaLac} \cdot m - w_{CaLac_0} \cdot m_0}{m_0} \times 100 \tag{3}$$

$$\Delta AA = \frac{w_{AA} \cdot m - w_{AA_0} \cdot m_0}{m_0} \times 100 \tag{4}$$

$$\Delta Suc = (\Delta M - \Delta W - \Delta CaLac - \Delta AA) \times 100 \tag{5}$$

where m = mass; w = mass fraction (w/w); w = water; CaLac = calcium lactate; AA = ascorbic acid; and 0 = initial time ($t = 0$).

In addition, the calcium gain (ΔCa) can be calculated by:

$$\Delta Ca = \frac{w_{Ca} \cdot m - w_{Ca_0} \cdot m_0}{m_0} \tag{6}$$

To evaluate the influence of the OD parameters on the efficiency of the water removal in relation to sugar impregnation of the apples, the efficiency was defined by the following equation:

$$\text{Efficiency} = \left| \frac{WL}{\Delta Suc} \right| \tag{7}$$

2.5. Microscopic analysis

Histological techniques with vital stains, which do not cause a short-term effect on the cell physiology, were used to evaluate the influence of the osmotic dehydration on cell viability using fluorescence intensity and neutral red accumulation for vacuole integrity in preserved vacuoles. Microscopic analysis was performed on osmotic solutions in the following concentrations: Suc (20%), Suc (30%), Suc (40%), Suc–CaLac (20–2%), Suc–CaLac (30–3%), Suc–CaLac (40–4%), Suc–AA (20–1%), Suc–AA (30–1.5%), Suc–AA (40–2%), Suc–CaLac–AA (20%, 2%, 1%), Suc–CaLac–AA (30%, 3%, 1.5%) and Suc–CaLac–AA (40%, 4%, 2%).

2.5.1. Fluorescein diacetate (FDA) staining

1 mm-thick apple slices were obtained using a sharp scalpel and then treated in the osmotic solutions mentioned above for 2 h. The cell viability test was performed using fluorescein diacetate (FDA, Sigma–Aldrich, USA, λ_{ex} = 495 nm, λ_{em} = 518 nm), as described by Tylewicz, Romani, Widell, and Gómez Galindo (2013) with some modifications. Apple slices were incubated for 30 min in a 10^{-4} M FDA in an isotonic sucrose solution (13%, w/w) in the darkness at room temperature. Fluorescein diacetate is known for its ability to passively penetrate protoplast and to be hydrolyzed by cytoplasmic esterases that produce the polar product fluorescein. This charged form is accumulated intracellularly in viable cells because it is unable to cross cellular membranes that remain intact (Saruyama et al., 2013). Viable cells could be easily identified by a bright fluorescence. Observations were performed under a fluorescent light in a Nikon upright microscope (Eclipse Ti-U, Nikon Co., Japan) equipped with a Nikon digital video camera (digital sight DS-Qi1Mc, Nikon Co., Japan) at a magnification of 20×.

2.5.2. Neutral red staining

Apple tissues were stained using a neutral red dye. Neutral red is a vital stain with a relatively low molecular weight and no electric charge that penetrates the vacuole of the intact protoplast of plant cells. In vacuoles, the neutral red is transformed to an ionic state because of the low pH inside the vacuoles; in this form, neutral red is incapable of penetrating the tonoplast, so the neutral red accumulates in the vacuole. Neutral red stain has been prepared in a concentration of 0.05% (Mauro, Tavares, & Menegalli, 2003; Panarese et al., 2012) in an isotonic sucrose solution at 13% (w/w). Slices (~0.5 mm) cut manually with a sharp scalpel were stained with neutral red for 20 min. Each stained slice was immersed in an osmotic solution for a minimum of 120 min. Slices were placed on a microscopic slide accompanied by a drop of solution and covered with the slide cover. The control slices were solely washed in the isotonic solution. Slides were immediately observed under a light microscope (Optech – Optical Technology, Germany) and recorded at a magnification of 10×. RGB images were acquired using a digital camera (Camedia C-4040-ZOOM, Tokyo, Japan) and stored in JPEG format.

Additionally, slides were recorded at a higher resolution in black and white using a Nikon upright microscope (Eclipse Ti-U, Nikon Co., Japan) without a fluorescent light at a magnification of 20×.

2.6. Time domain nuclear magnetic resonance (TD-NMR)

The proton transverse relaxation time (T_2) of the samples was measured for six replicas in a Bruker The Minispec spectrometer

(Bruker Corporation, Germany), operating at 20 MHz and 24 °C, using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence. Fresh or osmotic dehydrated apple cylinders with an 8 mm initial diameter were cut (approximately 10-mm height, 250 mg) to not exceed the active region of the radio frequency coil and placed inside the 10-mm outer diameter NMR tubes. Each measurement comprised 16,000 echoes with a 90–180 interpulse spacing of 100 μs, with 32 scans and a recycle delay of 5 s. The specified instrumental parameters avoided heating the samples and allowed the measurement of the protons with a T_2 between 1 and 2000 ms.

The acquired CPMG curves were normalized by the sample weights and analyzed with the UPEN (uniform penalty inversion of multiexponential decay data) algorithm (Borgia, Brown, & Fantazzini, 1998) to give quasi-continuous distributions of relaxation time. The UPEN default fitting parameters were adjusted to obtain better resolved and more detailed peaks. The number of output relaxation times sampled logarithmically in the 1–2000-ms interval T_2 was set to 200, and the smoothing coefficient beta was increased to 2. However, the resulting T_2 distributions showed partially overlapped peaks. Three proton populations were found in each sample and were ascribed to cell compartment proton pools according to their T_2 and intensity values (Panarese et al., 2012): vacuole, cytoplasm-free space and cell wall. Free spaces are the spaces where the osmotic solution could interpenetrate, i.e., outside the protoplast boundaries.

To obtain quantitative information from the CMPG decay curves, sample signals were fitted using a discrete multi-exponential curve in Eq. (8):

$$S_{(\tau)} = \sum_{i=1}^{N} I_n \exp\left(\frac{-t}{T_{2,n}}\right) + E_{(\tau)} \quad (8)$$

where N is the number of the found protons populations (based on UPEN results, it was set to 3); and I and T_2 are the intensity value and average relaxation time, respectively, of the n proton pool.

The fitting was performed using the "Nonlinear Least Squared" function based on the Gauss–Newton algorithm and implemented in the "R" software (R Foundation for Statistical Computing, Austria), while the I and T_2 starting values were chosen based on the UPEN results.

2.7. Statistical analysis

The significance of the effects of the different osmotic solutions on water loss, sucrose gain, efficiency, transverse relaxation times (T_2) and intensity was evaluated by analysis of variance (ANOVA) and comparison of means using the Duncan test at a 5% probability level. The data were expressed as the mean ± standard deviation.

3. Results and discussion

3.1. Mass transfer: water loss and solid gain

Water loss (Eq. (2)) and sucrose gain (Eq. (5)) during osmotic dehydration of apples treated in different osmotic solutions are shown in Table 1. Samples treated in the sucrose solution show the smallest water loss and high sucrose uptake compared to the other treatments. When AA is added to the sucrose solution, the water loss increases but the sucrose gain also increases, especially during the first 30 min. A consequence of this relationship between the water loss and sugar gain is both the Suc and Suc–AA treatments have a lower process efficiency (Table 1) compared to the Suc–CaLac and Suc–CaLac–AA treatments.

As for the water chemical potential of these solutions, the water activity measured was 0.962 in the sucrose solution (40%), 0.953 in the Suc–CaLac (40–4%), 0.954 in the Suc–AA (40–2%) and 0.944 in the Suc–CaLac–AA (40–4–2%). Consequently, the highest water loss is expected from the Suc–CaLac–AA solution followed by the Suc–CaLac and Suc–AA solutions. Indeed, both the Suc–CaLac and Suc–CaLac–AA solutions promoted greater water loss and did not have significant differences between them. However, when comparing the sucrose gain values between these two treatments, differences were found at 30 and 240 min of the process, as seen in Table 1, which were reflected in the efficiency of these processes. CaLac in solution enhances the efficiency because it is able to promote high water loss and restricts sucrose impregnation, which has been verified by other authors (Mavroudis et al., 2012; Silva et al., 2014a). However, the inhibition in sugar gain is sometimes accompanied by water loss reduction and, hence, a good efficiency is not reached, as verified by Silva et al. (2014a) who exposed pineapple tissue to high concentrations (sucrose 50% solution with 4% CaLac) for 2 h. Barrera et al. (2009) observed that for apples, osmotic dehydration assisted by vacuum impregnation favors solid gain but also reduces water removal. Restriction of solute transport has been attributed to calcium pectate formation, which decreases the cell wall porosity and limits the transport of larger molecules. However, a decrease in the water loss could also be explained by changes in the cellular membranes because calcium can affect the water channel activity (Maurel, 2007). Nevertheless, the significance of the inhibition of plant aquaporins by calcium is complex and has not been clarified, as noted by Maurel (2007), who compared the water permeability of the *Arabidopsis* plasma membrane (Gerbeau et al., 2002) and *Beta vulgaris* roots (Alleva et al., 2006). A low sensitivity to Ca^{2+} was detected in the *Arabidopsis* plasma membrane, but a higher sensitivity was detected in the *B. vulgaris* roots. In the present work, inhibition of Ca^{2+} on water loss was not evident.

In contrast, effect of the addition of AA seems to increase the impregnation of solutes, which is the opposite effect of those promoted by calcium. This was verified by Silva et al. (2014b) and attributed to wall porosity increasing because of acidification (Zemke-White et al., 2000).

During the first 60 min of the Suc–CaLac–AA treatment, the efficiency was high probably because the calcium affected the restriction of the sucrose gain in a similar way to the behavior observed for the Suc–CaLac treatment (Table 1). Then, the efficiency decreased, which suggests that after 1 h of the process, the AA exerted an opposite influence on the sucrose transport. Silva et al. (2014b) also observed that AA positively influenced sucrose and calcium gain in pineapples treated in solutions composed of sucrose, calcium lactate and ascorbic acid. These results suggest that synergetic effects should not be ignored. Genevois, Flores, and De Escalada Pla (2014) fortified pumpkin with vitamin C and iron through a dry infusion process by sprinkling powdered sucrose on the vegetable to form a solution with the lost water from the food. The authors concluded that the addition of Fe or AA to the liquid solution increases the incorporation of sucrose into the pumpkin tissue, but the presence of both additives simultaneously produces an antagonistic effect that diminishes the solid gain.

Good impregnation of Ca and AA contents were observed during the treatments in the Suc–CaLac, Suc–AA and Suc–CaLac–AA solutions. The last solution slightly enhanced the AA and Ca impregnation; the AA content increased after 2 h of process while the Ca content increased after 4 h (Table 2). AA was not detected in the fresh samples. Indeed, very low ascorbic acid content has been previously found in the Pink Lady apples (2.3–3.0 mg/100 g, Castro et al., 2008).

In conclusion, according to mass transport evaluation, the OD efficiency was improved by CaLac, while AA presence exerted an opposite effect; when both additives were present simultaneously,

Table 1
Mean and standard deviation of water loss, sucrose gain and efficiency.

Osmotic solution	30 min	60 min	120 min	240 min
Water loss				
Suc	$-9.36^{a} \pm 0.50$	-13.00^{*}	$-15.94^{a} \pm 0.85$	$-24.66^{a} \pm 0.13$
Suc–CaLac	$-12.49^{b} \pm 0.86$	$-14.96^{a} \pm 1.00$	$-22.30^{b} \pm 0.68$	$-28.96^{b} \pm 0.63$
Suc–AA	$-10.45^{a} \pm 0.04$	$-13.61^{a} \pm 0.86$	$-18.99^{ab} \pm 0.19$	$-26.18^{a} \pm 1.25$
Suc–CaLac–AA	$-12.87^{b} \pm 0.52$	$-16.04^{a} \pm 1.48$	$-20.58^{b} \pm 2.21$	$-28.42^{b} \pm 0.02$
Sucrose gain				
Suc	$2.28^{ab} \pm 0.12$	4.11^{*}	$5.51^{a} \pm 0.26$	$6.70^{a} \pm 0.05$
Suc–CaLac	$2.19^{a} \pm 0.20$	$3.39^{a} \pm 0.27$	$4.86^{a} \pm 0.22$	$5.69^{b} \pm 0.23$
Suc–AA	$3.18^{c} \pm 0.01$	$4.11^{a} \pm 0.23$	$4.93^{a} \pm 0.06$	$6.75^{a} \pm 0.45$
Suc–CaLac–AA	$2.61^{b} \pm 0.12$	$3.20^{a} \pm 0.41$	$4.76^{a} \pm 0.69$	$7.41^{a} \pm 0.01$
Efficiency				
Suc	$4.11^{ab} \pm 0.43$	3.90^{*}	$2.90^{a} \pm 0.29$	$3.68^{a} \pm 0.05$
Suc–CaLac	$5.75^{c} \pm 0.93$	$4.44^{a} \pm 0.65$	$4.60^{a} \pm 0.35$	$5.10^{b} \pm 0.32$
Suc–AA	$3.29^{a} \pm 0.02$	$3.32^{a} \pm 0.39$	$3.85^{a} \pm 0.09$	$3.89^{a} \pm 0.44$
Suc–CaLac–AA	$4.94^{bc} \pm 0.43$	$5.08^{a} \pm 1.11$	$4.41^{a} \pm 1.10$	$3.98^{a} \pm 0.01$

The same letter on the same column means no significant difference by the Duncan test ($p < 0.05$).
* Replica not determined.

Table 2
Mean and standard deviation of calcium and ascorbic acid contents at different osmotic dehydration times and corresponding fresh apple (mg/100 g).

Osmotic solution	0 min (fresh)	30 min	60 min	120 min	240 min
Calcium content					
Suc–CaLac	$2.78^{a} \pm 0.03$	$79.80^{b} \pm 0.55$	$110.70^{c} \pm 1.88$	$142.44^{d} \pm 0.21$	$163.45^{e} \pm 5.35$
Suc–CaLac–AA	$2.78^{a} \pm 0.03$	$81.05^{b} \pm 4.00$	$108.57^{c} \pm 2.18$	$140.01^{d} \pm 12.75$	$195.20^{f} \pm 8.30$
Ascorbic acid content					
Suc–AA	Nd	$429.02^{a} \pm 13.82$	$608.88^{b} \pm 13.47$	$733.32^{c} \pm 54.61$	$1012.45^{e} \pm 2.87$
Suc–CaLac–AA	Nd	$393.09^{a} \pm 10.13$	$576.85^{b} \pm 8.20$	$779.50^{d} \pm 15.87$	$1076.53^{f} \pm 33.50$

The same letter on the same column for each component means no significant difference by the Duncan test ($p < 0.05$);
Nd: not detectable.

AA counterbalanced an initial increase of efficiency caused by calcium, as the OD proceeded. High levels of Ca and AA contents were reached in all treatments and the impregnation of both components was slightly enhanced when they were together in the solution.

3.2. Microscopic analysis

3.2.1. Microphotographs of tissues stained with fluorescein diacetate

Fig. 1 presents slides of apple tissue before and after 2 h of osmotic dehydration in different solutions followed by staining with FDA. For the 20%, 30% and 40% Suc solutions (Fig. 1b.1–b.3), all slides show cell viability with an intensity that was comparable to the fresh tissues (Fig. 1a). Tissues treated in the CaLac–Suc solution presented a higher intensity for the 20% Suc–2% CaLac (Fig. 1c.1) solution. However, as the concentrations of both components increased, the viability decreased, which suggests that the solution with 40% Suc + 4% CaLac (Fig. 1c.3) affected the viability of the cells. A low fluorescence intensity was detected in tissues treated with the Suc–AA treatments in the Suc 20%, AA 1% concentrations (Fig. 1d.1) and no viability at higher concentrations was found (Fig. 1d.2 and d.3). For treatments in the Suc–CaLac–AA solutions, the apple cells did not show any viability (Fig. 1e.1–e.3). If the protoplasts did not retain the FDA, this means disruption of the plasma membrane (cell lysis) or loss of membrane semipermeability (Halperin & Koster, 2006; Koster et al., 2003). The type of membrane injury could be verified by the number of intact protoplasts without ability to retain FDA. However, apples have a poor cytosol content, which makes it difficult to distinguish the protoplasts and vacuoles using light microscopy.

Cellular injury can be caused by low water activity, but all solutions used in these experiments had a relatively high a_w (in a range of 0.944–0.986). Another cause for cellular damage could be the low pH of the osmotic solutions with AA (Zemke-White et al., 2000). For instance, Suc–AA solutions have a pH close to 2.4 and Suc–CaLac–AA solutions near 4.0. Furthermore, it has been demonstrated that AA can cause severe damage in the cellular structure (Qian et al., 2014). Hence, the AA presence in high concentrations certainly affects the cellular membrane structure of plant tissues, but the mechanisms are still not clearly delineated.

3.2.2. Microphotographs of tissues stained with neutral red

Fig. 2 presents slides of apple tissue stained with neutral red, followed by 2 h of osmotic dehydration in different solutions. Fig. 2a shows the control with no osmotic treatment that appeared completely stained. Fig. 2b.1–b.3 represent tissues treated in Suc solutions and show a broad presence of preserved vacuoles and red-stained tissue, probably because neutral red can also provide some contrast to cytoplasm (Carpita et al., 1979). Plasmolysis can be identified by the arrows.

These results agree with cell viability verified by FDA experiments with Suc solution, since, if plasma membranes remain preserved, intact vacuoles must be found.

In Fig. 2c.1–c.3, with tissues treated in Suc–CaLac solutions, vacuoles are well defined. However, the color is not spread out like it was in cells exposed to Suc solutions alone, which suggests that the cytoplasm did not retain the color despite some protoplast viability remaining even in the 30% Suc + 3% CaLac solution (Fig. 1c.2). The possibility that some plasma membranes or tonoplasts have been disrupted is based on the high calcium concentration, which can damage membranes (Wang, Xie, & Long, 2014).

Interestingly, during osmotic dehydration of thin slices previously stained with neutral red, sucrose solutions remained without color but Suc–CaLac solutions changed to red with similar tonality

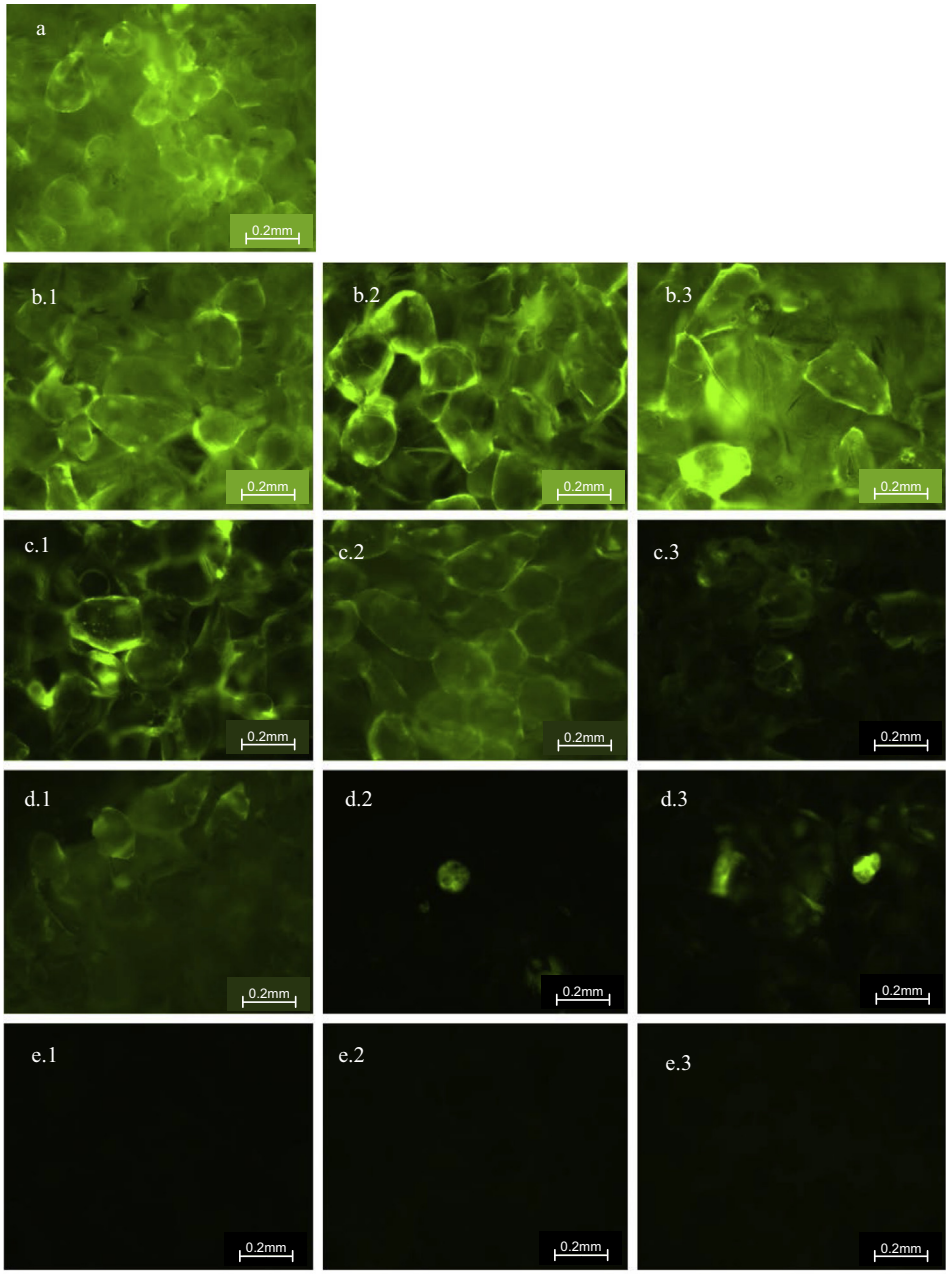


Fig. 1. Slides of parenchyma apple tissue stained with FDA after immersion in osmotic solutions for 120 min: (a) control; (b–e) osmotically dehydrated in osmotic solutions; (b.1) 20% Suc; (b.2) 30% Suc; (b.3) 40% Suc; (c.1) 20% Suc + 2% CaLac; (c.2) 30% Suc + 3% CaLac; (c.3) 40% Suc + 4% CaLac; (d.1) 20% Suc + 1% AA; (d.2) 30% Suc + 1.5% AA; (d.3) 40% Suc + 2% AA; (e.1) 20% Suc + 2% CaLac + 1% AA; (e.2) 30% Suc + 3% CaLac + 1.5% AA; (e.3) 40% Suc + 4% CaLac + 2% AA.

of the neutral red aqueous solution, Suc–AA solutions changed to pink and Suc–CaLac–AA solution changed to intense pink (registers are not shown). This confirms the loss of plasma and/or vacuole membrane permeability, thereby permitting neutral red to leave the tissue.

Effects of the pH of the Suc–CaLac solutions did not seem plausible because the pH of the solutions is nearly neutral. Conversely, tissues treated in the Suc–AA solutions had a complete absence of color, as shown in Fig. 2d.1–d.3. Very low protoplast viability and no stained vacuoles suggest that high AA concentrations and/or very low pH affect the membrane integrity and permeability. Nevertheless, it was a surprise to be able to distinguish some vacuoles without dye (see arrows in Fig. 2d.2 and d.3), which were more visible in images captured at high resolution (Appendix A).

Whether the vacuoles contours are still visible, the membranes exist, but impermeability to a charged form of neutral red must have been lost and the stain left the vacuoles because red contrast was not observed. Moreover, it is known that the loss of plasma membrane semipermeability does not necessary mean cell lyses even though it concerns plasma membranes (Halperin & Koster, 2006), but suggests that tonoplast selectivity must have been modified without complete disruption of the vacuoles.

Finally, treatments in Suc–CaLac–AA solutions showed unexpected results. Although it is possible to visualize vacuoles in Fig. 2e.1–e.3, the cell viability was completely lost in cells that underwent this treatment (Fig. 1e.1–e.3). Because the CaLac addition elevated the pH in comparison to the AA solutions, from 2.4 (Suc–AA) to 4 (Suc–CaLac–AA), it is possible that the tonoplast

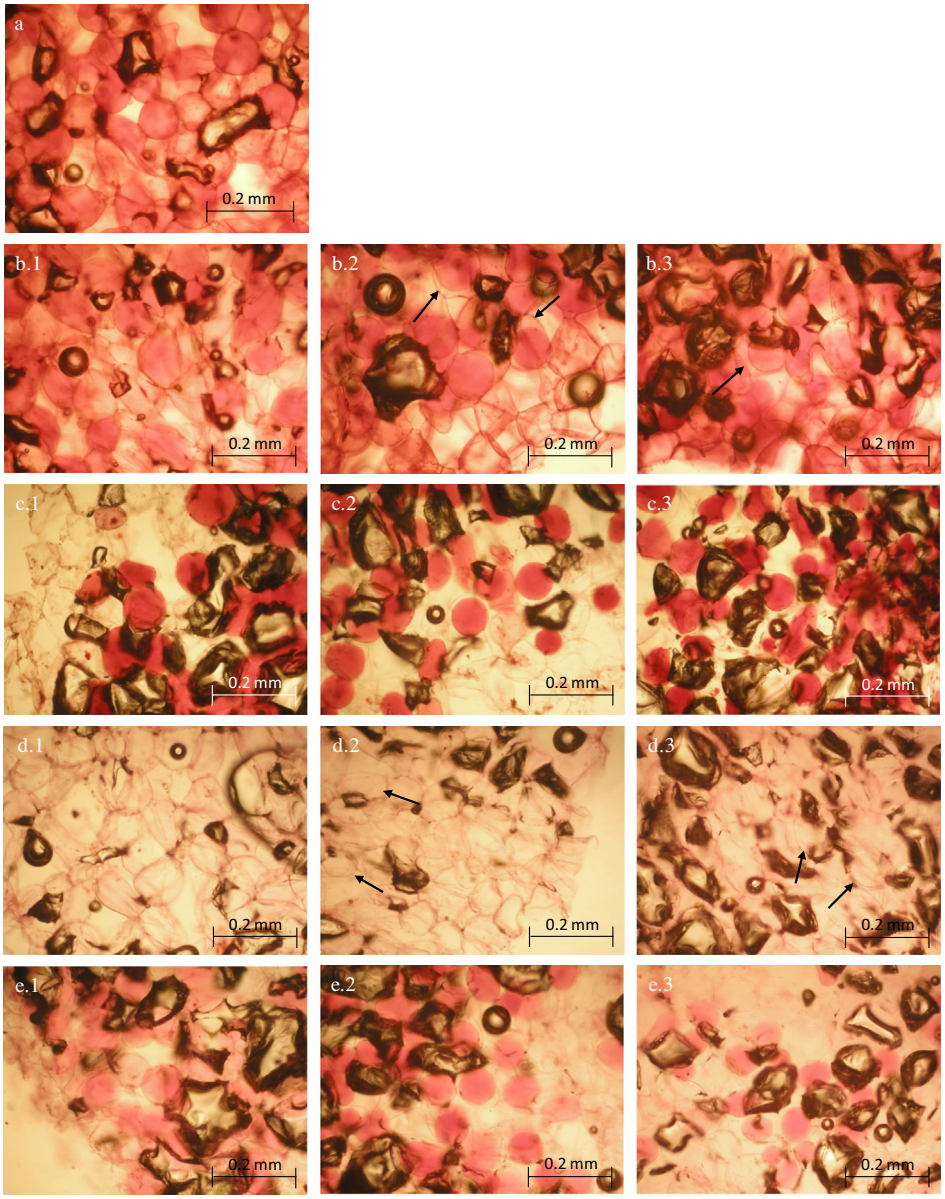


Fig. 2. Slides of apple tissue stained with neutral-red before immersion in osmotic solutions for 120 min: (a) control; (b–e) osmotically dehydrated in osmotic solutions; (b.1) 20% Suc; (b.2) 30% Suc; (b.3) 40% Suc; (c.1) 20% Suc + 2% CaLac; (c.2) 30% Suc + 3% CaLac; (c.3) 40% Suc + 4% CaLac; (d.1) 20% Suc + 1% AA; (d.2) 30% Suc + 1.5% AA; (d.3) 40% Suc + 2% AA; (e.1) 20% Suc + 2% CaLac + 1% AA; (e.2) 30% Suc + 3% CaLac + 1.5% AA; (e.3) 40% Suc + 4% CaLac + 2% AA.

semi permeability was better preserved, so the neutral red remained in some vacuoles. Conversely, plasmalemma was probably damaged due to the low pH and high CaLac and AA concentrations because no viability was detected.

These results show that plasmalemma was more sensitive to Suc–CaLac–AA solutions than tonoplast. AA caused red color absence in the vacuoles but they were visualized in images captured at high resolution, which led to the conclusion that tonoplasts maintained the vacuole content but its permeability was changed. The same inference could not be made for plasmalemma because the low cytoplasm content does not permit one to distinguish this phase.

3.3. Time domain nuclear magnetic resonance (TD-NMR): water mobility

Osmotic dehydration promotes important changes in cellular structure that can affect tissue selectivity and modify water mobility and its distribution through different parts of the cellular tissue.

Water mobility is related to the availability of water and, in this osmo-cellular system, could be modified by concentration of solids or by changes related to sites for hydrogen bonds because of macromolecule structure alteration.

T_2 and relative intensity results are shown in Table 3 and Fig. 3, respectively. Three protons populations were found in each sample at approximately 10, 200 and 1200 ms and were ascribed to cell compartment proton pools based on their T_2 and intensity values: cell wall, cytoplasm-free space and vacuole (Panarese et al., 2012). The free space comprises the plasmolysis space, which forms between the cell wall and plasmalemma, intra- and inter-cellular spaces and interstices in the cell walls (Mauro et al., 2003).

The total signal of raw apples was considered as a reference and set at 100. The intensities of cell wall, cytoplasm-free space and vacuole signals thus corresponded to 2.8 ± 0.4, 20.5 ± 2.3 and 76.7 ± 2.5, respectively. Results related to the water distribution showed a release of water from vacuoles to the cytoplasm-free spaces (Cyt/FS), so that the vacuoles shrank while the Cyt/FS water pools swelled. A more pronounced effect was observed for the

Table 3
Mean and standard deviation of the transverse relaxation time (T_2).

	T_2 (ms)			
	30 min	60 min	120 min	240 min
Vacuole				
Raw (fresh)	$1215.29^{a} \pm 39.78$	$1215.29^{a} \pm 39.78$	$1215.29^{a} \pm 39.78$	$1215.29^{a} \pm 39.78$
Suc	$1134.43^{b} \pm 43.64$	$1115.06^{b} \pm 61.10$	$1091.32^{b} \pm 19.86$	$995.99^{b} \pm 15.66$
Suc–CaLac	$1203.05^{a} \pm 27.62$	$1206.24^{a} \pm 59.40$	$1124.74^{b} \pm 28.35$	$1075.15^{c} \pm 43.15$
Suc–AA	$1147.25^{bc} \pm 24.42$	$1098.85^{b} \pm 24.42$	$1086.37^{b} \pm 19.14$	$1052.85^{c} \pm 44.34$
Suc–CaLac–AA	$1183.82^{ac} \pm 52.40$	$1138.28^{b} \pm 59.85$	$1090.47^{b} \pm 38.97$	$1003.12^{b} \pm 84.20$
Cytoplasm/free space				
Raw (fresh)	$209.19^{a} \pm 23.13$	$209.19^{ab} \pm 23.13$	$209.19^{ab} \pm 23.13$	$209.19^{a} \pm 23.13$
Suc	$211.71^{a} \pm 13.89$	$196.42^{a} \pm 19.73$	$188.18^{a} \pm 15.90$	$193.85^{a} \pm 11.72$
Suc–CaLac	$206.43^{a} \pm 15.90$	$231.98^{b} \pm 21.08$	$229.91^{b} \pm 13.60$	$212.12^{a} \pm 17.92$
Suc–AA	$211.86^{a} \pm 16.19$	$210.36^{ab} \pm 22.63$	$209.23^{ab} \pm 6.96$	$197.26^{a} \pm 12.32$
Suc–CaLac–AA	$210.26^{a} \pm 12.16$	$212.01^{ab} \pm 15.43$	$208.54^{ab} \pm 17.32$	$203.61^{a} \pm 15.69$
Cell wall				
Raw (fresh)	$9.81^{a} \pm 2.42$	$9.81^{a} \pm 2.42$	$9.81^{a} \pm 2.42$	$9.81^{a} \pm 2.42$
Suc	$8.93^{a} \pm 2.41$	$12.91^{b} \pm 4.10$	$10.47^{a} \pm 1.53$	$12.98^{abc} \pm 3.44$
Suc–CaLac	$9.68^{a} \pm 5.00$	$10.90^{ab} \pm 3.09$	$9.81^{a} \pm 6.40$	$15.22^{c} \pm 6.50$
Suc–AA	$8.90^{a} \pm 0.98$	$8.95^{a} \pm 0.85$	$11.05^{a} \pm 2.22$	$10.76^{abc} \pm 3.08$
Suc–CaLac–AA	$11.57^{a} \pm 2.41$	$11.15^{ab} \pm 1.69$	$15.12^{b} \pm 1.81$	$13.52^{bc} \pm 3.22$

The same letter on the same column means no significant difference by the Duncan test ($p < 0.05$).

osmotic treatment with the lowest a_w solution, Sac–CaLac–AA (0.944), than by the Suc–AA (0.954) and Suc–CaLac (0.953) treatments both with similar a_w solution and, finally, by the Suc treatment with the highest a_w solution (0.962) (Fig. 3).

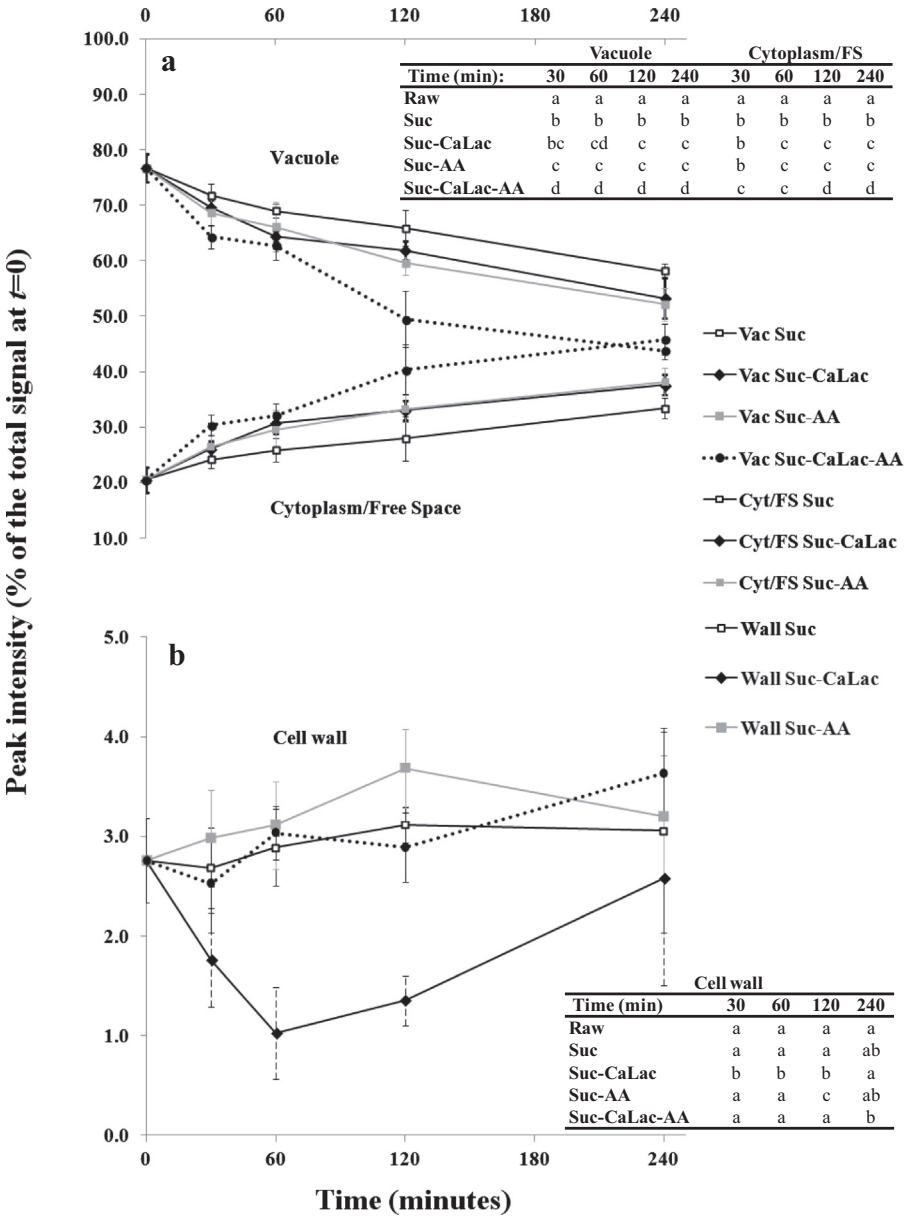
Regarding T_2, while focusing on specific time points, most differences between treatments were insignificant. On the other hand, when T_2 was observed during the redistribution of water proton compartments, trends similar to those observed for signal intensities were registered. In this respect, some aspects should be emphasized. In the treated tissues, the transverse relaxation times T_2 assigned to cytoplasm and free spaces were, in general, very similar to those of raw apples (Table 3). During the first two hours of process in the Suc–CaLac solution, T_2 assigned to vacuoles was greater than those measured in other treatments and close to the raw value. If the water losses are the greatest for this condition (Fig. 1), it is not clear why vacuoles have the highest water mobility once concentration of the vacuole solute content would be expected. A likely explanation is that channels selectivity of the plasma and vacuole membranes for several original cell substances would be different for each osmotic treatment (Maurel, 2007; Peiter et al., 2005; Tapken et al., 2013). In addition, osmotic solutions as well as contact time can affect membrane integrity. Thus, the vacuoles and cytoplasm solute composition and consequent water interactions in these compartments could be different between treatments.

The fact that calcium can traverse both tonoplast and plasmalemma membranes is not ignored. According to Peiter et al. (2005), several classes of Ca^{2+} recently have been identified in plant cells even though not all of the ion channels that underlie these currents have been identified. These authors showed that the TPC1 ('two-pore channel 1') protein, a non-selective channel for Ca^{2+}, encodes a class of Ca^{2+}-dependent Ca^{2+}-release channel known as the slow vacuolar (SV) channel, and they demonstrated that the TPC1 protein is relatively abundant in plant vacuoles. In turn, the plasma membrane cation channels in plant cells have been related to AtGLRs (*A. thaliana* glutamate receptors), proteins that are members of an amino acid receptors family (Tapken et al., 2013). The authors showed that they function as ligand-gated and non-selective cation channels permeable to Ca^{2+}. Consequently, compositional changes involving calcium could influence but not explain the higher water mobility because molecules with low molecular weight have a high capacity to drop water activity.

Still for Suc–CaLac treatments, T_2 assigned to cytoplasm and free spaces increased in relation to the raw material, though not significantly (Table 3). This could mean once again slower compositional changes and modifications of water interactions because calcium limits sucrose entry into the Cyt/FS, as observed by the efficiency obtained from the Suc–CaLac treatment (Table 1). Then, it would be expected that this compartment would have a greater proportion of solutes from the original cell than sucrose arising from the osmotic solution compared to other treatments and, consequently, weaker water interactions than with sucrose during early osmosis. Of course, as osmotic dehydration proceeds, the water chemical potential in each compartment tends to equal those of the osmotic solution.

For cell walls, water mobility practically did not change. However, regarding intensity, the Suc–CaLac treatment promoted a significant reduction in the water amount associated with the wall biopolymers. Roy et al. (1994), investigating changes in the distribution of the anionic binding sites in the cell walls of apples, concluded that calcium could reduce fruit softening by strengthening the cell wall and limiting cell separation through a greater degree of cross-links with pectic acid polymers. An important observation of these authors is that these calcium bindings can restrict access of hydrolytic enzymes or the resulting increase in pH due to Ca could inhibit activity of the wall loosening enzymes that possess acidic pH optima. Nevertheless, T_2 times for the cell wall did not present a pattern, so it would be necessary for more registers because there were great variations between cells (Table 3).

In conclusion, according to TD-NMR results, the Suc treatment seemed to have a lower influence on the cellular compartmentation and functionality, so that a higher vacuole water population and lower cytoplasm-extracellular spaces were observed in comparison with the other treatments. The Suc–CaLac and Suc–AA treatments resulted in similar water populations of vacuoles and cytoplasm. This highlights the presence of the vacuole compartmentation in tissues treated with Suc–AA (Appendix A), although it was not visualized by neutral red staining (Fig. 2d.1–d.3).



	Vacuole				Cytoplasm/FS			
Time (min):	30	60	120	240	30	60	120	240
Raw	a	a	a	a	a	a	a	a
Suc	b	b	b	b	b	b	b	b
Suc-CaLac	bc	cd	c	c	b	c	c	c
Suc-AA	c	c	c	c	b	c	c	c
Suc-CaLac-AA	d	d	d	d	c	c	d	d

Cell wall				
Time (min)	30	60	120	240
Raw	a	a	a	a
Suc	a	a	a	ab
Suc-CaLac	b	b	b	a
Suc-AA	a	a	c	ab
Suc-CaLac-AA	a	a	a	b

Fig. 3. Peak intensity of the proton pools in different cellular compartments as a function of the osmotic dehydration time, in different osmotic solutions. All the intensities were scaled so that the total signal from fresh samples ($t = 0$) equals 100. (a) Vacuole and cytoplasm plus free space; (b) cell wall. The same letter on the same column in the auxiliary tables means no significant difference by the Duncan test ($p < 0.05$).

4. Conclusions

Sucrose treatments preserved the viability and slightly affected the apple cell structure during OD, as shown by a fluorescence intensity which was comparable to fresh tissue, by a broad presence of red-stained vacuoles and by moderate changes in the water distribution within the cells, according to TD-NMR.

CaLac in the sucrose solution contributed to extended cell viability, and TD-NMR allowed detection of its influence on the cell wall as the proton intensity reduced during the first two hours of the process. In contrast, proton pools related to cell walls expanded in all other treatments. CaLac also enhanced the OD efficiency, which is associated with cell wall pore reduction due to calcium pectate formation.

Only adding AA into the sucrose solution visibly affected the cell membrane permeability by revealing the loss of viability of protoplasts and capacity of retaining vital stain in vacuoles and, simultaneously, the presence of vacuole compartmentation, which was detected by TD-NMR and also by images captured in a high resolution.

AA together with calcium strongly affect the tissue functionality, showing no viability but still some stain retention by vacuoles, and a remarkable water redistribution by vacuole shrinkage and Cyt/FS swelling verified by TD-NMR. Plasmalemma was more sensitive to Suc–CaLac–AA solutions than tonoplast. The presence of AA reduced the process efficiency and enhanced Ca impregnation in the four process hours, which were related to increase the cell wall porosity and change the membrane permeability.

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qualitative, metabolic and functional aspects). Research work supported by POR-FESR 2007–2013 Emilia-Romagna Region.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.04.096.

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Paper X

Effects of calcium and ascorbic acid on osmotic dehydration kinetics and metabolic profile of apples

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Effects of calcium and ascorbic acid on osmotic dehydration kinetics and metabolic profile of apples

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Abstract

The influence of the addition of calcium lactate (CaLac) and ascorbic acid (AA) to sucrose (Suc) osmotic solutions on osmotic dehydration kinetics and endogenous metabolic heat production of apple tissue was evaluated. The research activity was aimed at increase the understanding of mass transfer and endogenous metabolic phenomena of the tissue, in order to obtain minimally processed apples. The presence of calcium and ascorbic acid in solution affected the effective diffusivities attributed to the changes in cellular spaces, increasing spaces viable to solute transport. Metabolic heat production in samples treated in sucrose (Suc) solutions was slightly lower than in untreated samples and it was further reduced with calcium lactate (CaLac) addition. However, samples impregnated with ascorbic acid (AA) showed a higher heat production, as there was a metabolic response of the apple tissue to AA treatment. When combined with Ca, heat production decreased sharply to a level lower than untreated samples, except for those treated for 120 and 240 min (higher impregnation), achieving the highest heat production values. These results confirm previous findings, suggesting that AA solution can promote a stress response on specific fresh-cut vegetable tissues, and an increase of their endogenous metabolic activity, confirmed by a higher O_2 consumption observed by head space gas determination.

Keywords: Minimally processed apples, sucrose, ascorbic acid, calcium lactate, diffusion coefficients, endogenous metabolic activity

1. Introduction

Osmotic dehydration (OD) can be used as further processing for many purposes aiming to improve quality and stability of fruit previously subjected to peeling and cutting or for the production of semi-finished food destined to drying, freezing etc. Despite these applications, OD can present several advantages also for the production of minimally processed fruit.

The dehydration or water removal from fresh tissue is usually flanked by its gain in solute (or solutes) that are present in the osmotic medium. This process can lead to a system of efficient water removal and at the same time to a modification of the soluble components of the food itself modifying/improving its sensorial characteristics.

The type of solute used in the osmotic solution is a fundamental issue because it affects not only the dehydration kinetics and the process cost, but also the organoleptic and nutritional properties of the final product. Sucrose is considered by many authors as the optimal osmotic agent as it is associated with higher efficiency if compared to glucose (Saputra 2001), reducing enzymatic browning and loss of aroma (Cortellino et l. 2011; Lenart 1996; Qi et al.1998).

OD with calcium in solution has been used in an attempt to increase firmness of plant tissue and enhance the process efficiency, restricting the sugar gain and increasing the water loss (Ferrari et al. 2010; Nikolaos et al. 2012; Pereira et al. 2006). Calcium has the ability of reinforcing cell walls by the cross linking of pectic polymers and hence, is able to reduce damages due to dehydration (Pereira et al. 2006). At the same time, when the concentration increases or as the treatment proceeds, a damage to cell membranes may occur, as reported by (Aninoet al. 2006). Moreover, calcium has been used in the osmotic solution as a method for obtaining nutritionally fortified products to increase consumer intake (Barrera et al. 2004; Silva et al. 2014b).

The addition of ascorbic acid to the osmotic solution has been used for reducing enzymatic browning (Lenart et al. 1997) and for compensate the loss of ascorbic acid in the fruits during the dehydration process (Ramallo and Mascheroni 2010).

When adding solutes to the osmotic medium, with the aim to obtain a minimally processed product to be stored at refrigeration temperature, it is important to consider that they may not affects only the compositional and nutritional profile, but also the tissue metabolism with consequences on the final product stability and shelf-life.

Various authors have observed a reduction in the respiration rate of osmotically dehydrated mangoes, strawberries, pineapples and kiwifruit (Castelló et al. 2010; Moraga et al. 2009; Torres et al. 2008). Nevertheless, after few days of storage, the respiratory quotient was generally observed to increase, as a consequence of the development of fermentative routes, as an optional metabolic pathway trigger by osmotic stress.

Salvatori and Alzamora (2000) found that a 25% w/w sucrose solution can cause vesciculation and rupture of cell membranes in apple tissue. According to (Mavroudis et al. 2004) only few layers of cells on the surface are expected to die upon an osmotic treatment while plasmolysis and shrinkage occur in the rest of the tissue. In a previous research, the authors found that a 40% w/w sucrose treatment generally preserved the viability of apple cells, affecting only slightly the cell structure observed by fluorescence microscopy and the water distribution within the cells, as observed by time domain nuclear magnetic resonance (TD-NMR) (Mauro et al. 2016).

For different kind of fruit species, calcium has shown the ability to decrease the tissue metabolic activity and respiration rate (Castelló et al. 2010; Lester 1996; Luna-Guzmán et al. 1999), potentially enhancing the product stability during storage considering that a lower respiration rate may lead to a longer shelf life. In addition, Ca can affect membrane and cell wall structure and functioning.

On the other hand, the presence of AA can cause serious injury to cellular structure, as it has been previously reported by Mauro et al. (2016) that observed a loss of the capacity to retain FDA

colorant due to damage to cell membranes, after exposition to OD in a sucrose-ascorbic acid solution. As the AA concentration increased a loss of vitality was detected.

Rocculi et al. (2005) found a higher metabolic activity in potato tissue upon dipping treatments with citric and ascorbic acid, suggesting that AA solution can promote a stress response on specific fresh-cut vegetable tissues, and an acceleration of their endogenous metabolic activity, confirmed by a higher O_2 consumption which was observed by head space gas determination. Limbo and Piergiovanni (2007) detected an increase in the respiration rate of sliced potatoes subjected to dipping treatment with 2.5% of AA. Actually, when AA concentration increased, respiration rate decreased.

Isothermal calorimetry has been recognized as a useful tool to assess metabolic responses of various plant tissues to wounding stress (Wadsö et al. 2004), dipping treatment (Rocculi et al. 2005), thermal treatments (Gómez et al. 2004) and OD (Panarese et al. 2012).

Generally, when a tissue is wounded, it 'sends a signal' and the plant starts a number of protective processes that lead to an increase of the produced metabolic heat (Wadsö et al. 2004). As reported by Gómez et al. (2004), after wounding, the energy released by the cell is due to the sum of the 'basic' metabolic activity and that originating from wounding stress produced by the cells near the cut surface. Part of the processes that occur after wounding are design to membrane restoration and strengthening of cell walls by cells close to the site of injury (Rolle and Chism 1987). A progressive reduction in the metabolic heat production during OD in kiwifruit slices was observed by Panarese et al. (2012) using isothermal calorimetry. The authors suggested that the decrease was due to a reduction of cell viability induced by osmotic stress. Finally the metabolic response of fruit tissues to OD was found to depend on the botanical origin, on the osmotic pressure exerted (Ferrando and Spiess 2001; Mavroudis et al. 2004) and also on its physiological state, as a loss of membrane integrity upon ripening leading to a higher permeability made the tissue more sensitive to osmotic stress.

This work evaluated the effects of the addition of calcium lactate (CaLac) and ascorbic acid (AA) to sucrose (Suc) osmotic solutions, on Ca and AA diffusivities and on raw endogenous metabolic response (respiration and heat production) of the tissue. Particularly obtained information can be very useful in order to investigate the potential stability of minimally processed apples.

2. Materials and methods

2.1. Raw materials

30 kg of apples (*Malus domestica* Borkh) of the Cripps Pink variety, popularly known by the brand name Pink Lady (de Castro et al. 2008), were bought at the local market and stored at 5±1°C for 2

weeks, during which the experimental research was carried out. Apples were characterized by an average weight of 233.5±17.7 g and soluble solids content of 13.4±0.3 g/100g. From the central part of the fruits, cylindrical samples (8-mm diameter, 40-mm length) were cut with a manual cork borer and a manual cutter designed for the purpose. For osmotic treatments, commercial sucrose (refined sugar, Eridania, Italy), L-ascorbic acid (Shandong Luwei Pharmaceutical Co., China) and calcium lactate (calcium-L-lactate 5-hydrate powder, PURACAL® PP Food, Corbion PURAC, Netherlands) were used.

2.2. Osmotic dehydration

OD was carried out at 25°C using four different osmotic solutions (w/w): 40% sucrose (Suc), 40% sucrose + 4% calcium lactate (Suc-CaLac), 40% sucrose + 2% ascorbic acid (Suc-AA) and 40% sucrose + 4% calcium lactate + 2% ascorbic acid (Suc-CaLac-AA).

Approximately 100 g of apple cylinders were weighed for each treatment time (0.5, 1, 2 and 4 h) and placed in mesh baskets that were immersed in 4.5 kg of aqueous osmotic solution with a syrup-to-fruit ratio of about 15:1 (w/w), to avoid changes in the concentration of the solution during the treatment. Through an impeller of a mechanical stirrer, the cylindrical baskets were continuously rotated. The rotational speed was experimentally determined to assure negligible external resistance to mass transfer. Two baskets were prepared for each process time.

After each treatment time, samples were removed from the solution, rinsed with distilled water, blotted with absorbing paper, and weighed.

Total and soluble solids contents were determined in triplicate immediately after treatment. Samples for calcium and ascorbic acid analyses were freeze-dried.

After OD, cylinders were placed in glass sealed ampoules for the measurement of endogenous metabolic heat production with isothermal calorimetry during 16 hours, followed by the determination of O_2 and CO_2 on ampoule headspaces.

2.3. Analytical methods

Moisture content of fresh and osmotically dehydrated samples was determined gravimetrically, in triplicate, by drying cylindrical apple samples at 70°C until a constant weight was reached.

Soluble solids content was determined at 20°C by measuring the refractive index with a digital refractometer (PR1, Atago, Japan).

2.3.1 Ascorbic acid

Ascorbic acid was determined by HPLC analysis according to the method described by (Odriozola-Serrano et al. 2007). Briefly, approximately 0.5 g of freeze-dried sample were added to 10 ml of meta-phosphoric acid (62.5 mM) and sulfuric acid (5 mM) solution, vortexed for 2 minutes and centrifuged at 10000*g* for 10 minutes at 4°C. The supernatant was opportunely diluted and filtered

through a 0.45 μm nylon filter. The HPLC system LC-1500 (Jasco, Carpi, MO, Italy) was equipped with a diode array UV/Vis detector. A reverse-phase C18 Kinetex (Phenomenex Inc., Torrance, CA, USA) stainless steel column (4.6 mm x 150 mm) was used as the stationary phase. A Jasco AS-2055 Plus autosampler was used to introduce samples in the column. The mobile phase was a 0.01% solution of sulfuric acid adjusted to a pH of 2.6. The flow rate was fixed at 1.0 mL/min at room temperature. Data were processed by the software ChromNAV (ver. 1.16.02) from Jasco. The ascorbic acid content was quantified at 245 nm through a standard calibration curve, set up using ascorbic acid solution between 0.5 to 30 ppm. The determination was carried out in triplicate.

2.3.2. Calcium

The calcium concentration was determined using a flame atomic absorption spectrophotometer (Model A Analyst 400, Perkin Elmer, Santa Clara, California, USA), using a lumina hollow cathode lamp (Perkin Elmer) based on the adapted methodology of AOAC (1995). Briefly, approximately 6 g of freeze-dried untreated samples and 2 g of freeze-dried treated samples, were weighed in a 50 ml glazed, porcelain crucible, placed in a muffle furnace and heated up to 550 °C until complete ignition. After cooling in desiccators, the ash were dissolved in 20 ml for fresh samples or 30 ml for treated samples of HCl (0.1 M). The ash was dissolved, and then the solution was opportunely diluted with 0.1 M HCl. A calibration curve of absorbance versus ppm of calcium was set up using standard calcium solutions between 2 to 20 ppm. The determination was carried out in triplicate

2.3.3. Metabolic heat production

Two fresh cylindrical samples (8-mm diameter, 40-mm length) and three osmotically dehydrated samples were placed in 20 ml glass ampoule and sealed with a teflon coated rubber seals and an aluminum crimp cap. Three replicates for each sample were performed. The rate of heat production was continuously measured in a TAM air isothermal calorimeter (Thermometric AB, Järfälla, Sweden), with a sensitivity (precision) of ±10 μW (Wadsö et al. 2009). This instrument contains eight twin calorimeters in which each sample is inserted with its own reference, being that the measured signal is the difference between the sample signal and the reference signal. The reference has to be a material that does not produce any heat but is characterized by thermal properties similar to the sample. For this, water was chosen as the reference material and its quantity in each reference ampoule (m_w^o) was previously determined based on the average composition of the samples and on the heat capacities ($J \cdot g^{-1} \cdot K^{-1}$) of the water ($C_w$) and the total solids ($C_{TS}$), as the following equation:

$$m_w^o = \frac{C_{TS} \cdot m_{TS} + C_w \cdot m_w}{C_w} \quad (1)$$

where m_{TS} is the dry matter content (g), m_w is the water content of the fruit sample (g) and the average heat capacity of the total solids of the apple samples was assumed to be 1 J g^{-1} K^{-1}. The analysis was carried out at 10 °C for 16 h.

2.3.4. Respiration rate

Immediately after the ampoules discharging from the calorimeters, the O_2 and CO_2 percentages were measured in the ampoule headspaces by a check point gas analyzer O_2/CO_2 mod. MFA III S/L (Witt-Gasetechnik, Witten, Germany). The apparatus has a paramagnetic sensor for O_2 and a mini-IR spectrophotometer for CO_2 detection. The instrument was calibrated with O_2 and CO_2 air percentages.

Respiration rate was calculated as mg of consumed O_2 (RR_{O_2}) or produced CO_2 (RR_{CO_2}) h^{-1} kg fw^{-1} according to the following equations:

$$RR_{O_2} = \frac{M_{O_2} \cdot V_{head} \cdot \frac{(20.8 - \%O_{2,head})}{100} \cdot P}{t \cdot m \cdot R \cdot T} \quad (2)$$

$$RR_{CO_2} = \frac{M_{CO_2} \cdot V_{head} \cdot \frac{\%CO_{2,head}}{100} \cdot P}{t \cdot m \cdot R \cdot T} \quad (3)$$

where M_{O_2} and M_{CO_2} refer to gases molar mass (g·mol^{-1}), V_{head} represents the ampoule headspace volume (dm^3), $\%O_{2,head}$ and $\%CO_{2,head}$ refer to molar gases percentages in the ampoule headspace at time *t* (h); *m* is the sample mass (g); *R* is the gas constant (8.314472 dm^3 kPa K^{-1} mol^{-1}), *P* is the pressure (101.325 kPa) and *T* is the absolute temperature (283.15K).

2.4. Osmotic dehydration kinetics

Mass transfer of water, sucrose, calcium and ascorbic acid during the osmotic process were modeled according to the empirical model proposed by Peleg (1988) and redefined by Palou et al. (1994), as follows:

$$\Delta w_k = w_{k,t} - w_{k,0} = -\frac{t}{k_1 + k_2 t} \quad (4)$$

where w_k is the mass fraction (g·g^{-1} total mass) of the *k* species: water, sucrose, calcium or ascorbic acid. The constants of the Peleg's model are k_1 [s·(g·g^{-1} total mass)$^{-1}$] and k_2 [1·(g·g^{-1} total mass)$^{-1}$]. This kinetic model permits, through the inverse of the two constants, to define the initial (*t*=0) rate of mass transfer $(1/k_1)$ and the concentration value at equilibrium ($t \rightarrow \infty$) conditions $(w_{eq} = x_0 \pm 1/k_2)$ (Sacchetti et al. 2001).

Diffusivities of water, sucrose, calcium and ascorbic acid were also calculated based on the second Fick's Law, considering diffusion coefficients and global densities approximately constants. For an infinite cylinder with radial component r, the mass transfer is described by:

$$\frac{\partial w_k}{\partial t} = D_{km}\left(\frac{\partial^2 w_k}{\partial r^2} + \frac{1}{r}\frac{\partial w_k}{\partial r}\right) \quad (5)$$

where D_{km} is the effective diffusion coefficient ($m^2 \cdot s^{-1}$) of species *k* diffusing through the tissue medium, being the diffusivity of each component treated as the binary form of Fick's Law (Cussler 1984). The correspondent analytical solution, integrated along the cylinder ratio (a = 4×10^{-3} m) assumed as constant throughout the process is (Crank, 1975):

$$\frac{\overline{w}_k - w_{k,eq}}{w_k^0 - w_{k,eq}} = 4\left[\frac{1}{5{,}783}e^{\left(-5{,}783\frac{D_{km}t}{a}\right)} + \frac{1}{30{,}472}e^{\left(-30{,}472\frac{D_{km}t}{a}\right)} + \frac{1}{74{,}887}e^{\left(-74{,}887\frac{D_{km}t}{a}\right)} + \frac{1}{139{,}039}e^{\left(-139{,}039\frac{D_{km}t}{a}\right)} + ..\right]$$

(6)

where $\overline{w}_k$ is the average mass fraction of species k, $w_{k,0}$ is the mass fraction at initial time (t=0) and $w_{k,eq}$ represents the equilibrium concentration (on the surface) of each *k* component.

2.5 Statistical Analysis and Fitting

The results were statistically evaluated using the analysis of variance (ANOVA) for each treatment and for each process time, with the sources of variation being the sample type and number of samples, and the Tukey post-hoc test being applied at the 5 % level of significance.

The Peleg's model and the analytical solution of the Fick's Law were fitted to the experimental data by using the Levenberg-Marquardt algorithm (Marquardt 1963) for the least-square estimation of the non-linear parameters. This is a search method to minimize the sum of the squares of the difference between predicted and measured values. The algorithm calculates the set of parameters with the lowest residual sum of squares (RSS) and their 95% confidence interval.

The fitting efficiency was evaluated by the determination coefficient (R^2) and the relative root mean square error (*RRMSE*), which was calculated as equation (7):

$$RRMSE(\%) = \sqrt{\frac{1}{N}\sum_{n=1}^{N}\left(\frac{y_{obs} - y_{calc}}{y_{calc}}\right)^2} \cdot 100 \quad (7)$$

3. Results and discussion

3.1 Osmotic dehydration kinetics

The Peleg kinetic model (1988) was used as an empirical model to fit mass transfer parameter data of sucrose gain and water loss, over processing time. Constants of equation (k_1 and k_2), their inverse and equilibrium concentrations obtained through Eq. 4 are reported in **Table 1**.

Table 1. Kinetic model of water, sucrose, calcium and ascorbic acid transfer in each osmotic solution according to Peleg's model (eq. 4) and equilibrium content (g·g^{-1} total mass).

Solution	k_1 (s)	$1/k_1 \times 10^3$ (s^{-1})	k_2 (g·g^{-1})	$1/k_2 \times 10^3$ (g·g^{-1})$^{-1}$	R^2	*RRMSE* (%)	$w_{k,eq}$ (g·g^{-1})
			Water				
Suc	11.18	89.45	6.10	163.90	0.997	3.8	0.6802
Suc-CaLac	9.12	109.70	5.68	176.09	0.997	3.1	0.6708
Suc-AA	8.93	112.00	5.98	167.26	0.998	3.1	0.6832
Suc-CaLac-AA	9.10	109.92	4.83	206.88	0.992	5.3	0.6428
			Sucrose				
Suc	14.51	68.92	6.92	144.54	0.998	3.8	0.1967
Suc-CaLac	14.59	68.53	8.08	123.79	0.998	3.2	0.1759
Suc-AA	11.49	87.05	8.13	123.03	0.980	8.60	0.1752
Suc-CaLac-AA	17.36	57.62	6.25	160.02	0.979	12.0	0.2122
			Calcium				
Suc-CaLac	392.99	2.54	523.45	1.91	1.000	0.7	0.0019
Suc-AA-CaLac	524.34	1.91	408.00	2.45	0.986	7.0	0.0025
			Ascorbic acid				
Suc-AA	85.44	11.70	81.48	12.27	0.986	5.9	0.0123
Suc-AA-CaLac	104.72	9.55	68.83	15.53	0.994	4.8	0.0145

The predictive capability of the model can be observed in **Fig. 1**, where eq. 7 was used to model mass transfer parameters for water (a), sucrose (b), calcium (c) and ascorbic acid (d). In all samples, water removal and solute uptakes followed the typical behaviour of osmotic processes characterized by a higher initial rate followed by a slower one (Sacchetti et al. 2001).

In general, the model showed a good fit to experimental data, as high R^2 values and low RRMSE were found (**Table 1**), confirming its suitability for describing mass transfer phenomena as already reported by Peleg (1988) and on other studies successively (Palou et al. 1994; Sacchetti et al. 2001). The initial rate of dehydration was increased by the presence of solutes in the solution compared to the only sucrose (higher $1/k_1$ values), but the equilibrium values showed contrasting behaviour, as the water equilibrium concentrations for Suc and Suc-AA osmotic solutions were very similar. As reported in Fig. 1a and 1b, the presence of calcium in both CaLac-Suc and CaLac-AA-Suc solutions promoted a higher water reduction compared to the Suc or Suc-AA solutions. With the combination of both solutes, water content was further reduced to 0.6428 g·g^{-1}, that is related to a higher osmotic

potential due to the lower water activity of the ternary solution, as reported by Mauro et al. (2016). Actually, the a_w of the OD solutions were 0.962 for Suc, 0.953 for Suc-CaLac, 0.954 for Suc-AA and 0.944 for Suc-CaLac-AA solutions.

Conversely, although the AA solution showed a higher initial rate of water removal, the water concentration tends to be similar to those in Suc treatment. As OD proceeds and, the water equilibrium concentration of both treatments resulted very similar.

Regarding sucrose content, the highest value of $1/k_1$ corresponds to the Suc-AA treatment, while $1/k_2$ was maximised by the Suc-CaLac-AA solution, with the highest equilibrium value of 0.2122 $g \cdot g^{-1}$. These trends can be observed in **Fig 1 b**, where only the Suc-CaLac treatment seems to inhibit the sucrose mass transfer during OD. In a previous work, calcium proved to enhance the efficiency of this process, increasing water loss and limiting sugar gain (Mauro et al., 2016). Calcium effect has been already observed in various fruit tissue subjected to OD in the presence of calcium salts, and it has been attributed to the reduction in cell wall porosity and to the formation of calcium pectate due to the interaction of the ion with pectic carboxylic groups (Mavroudis et al. 2012; Pereira et al. 2006; Silva et al. 2014a, b).

Even though the sucrose impregnation has been the lowest in the Suc-CaLac treatment, the equilibrium content was similar to Suc-AA treatment, as reported in **Fig 1 b**, where the tendency of the two curves is to join in.

Good impregnation of both Ca and AA were obtained, as shown by **Fig. 1 c and d**. When both solutes were present in the solution, the initial rates ($1/k_1$) of CaLac and AA impregnation were lower but after two hours of process, the impregnation tended to rise. It can also be observed in Table 1, where the equilibrium concentration calculated on the basis of the parameter k_2 were higher, showing how the quaternary solution enhanced the impregnation of those solutes.

The addition of ascorbic acid may enhance the impregnation of other solutes by acidifying the solution and hence increasing the porosity of cell wall, as observed by Zemke-White et al. (2000). In addition in a previous work, Mauro et al. (2016), ascorbic acid added to sucrose osmotic solution caused severe damage on cellular membranes of apple cells, that lost its selectivity. Regarding **Table 1**, the worst fittings were always found when the osmotic solution contained AA, probably because the damages on the structure of cell walls and membranes promoted changes on the transport phenomena during the process.

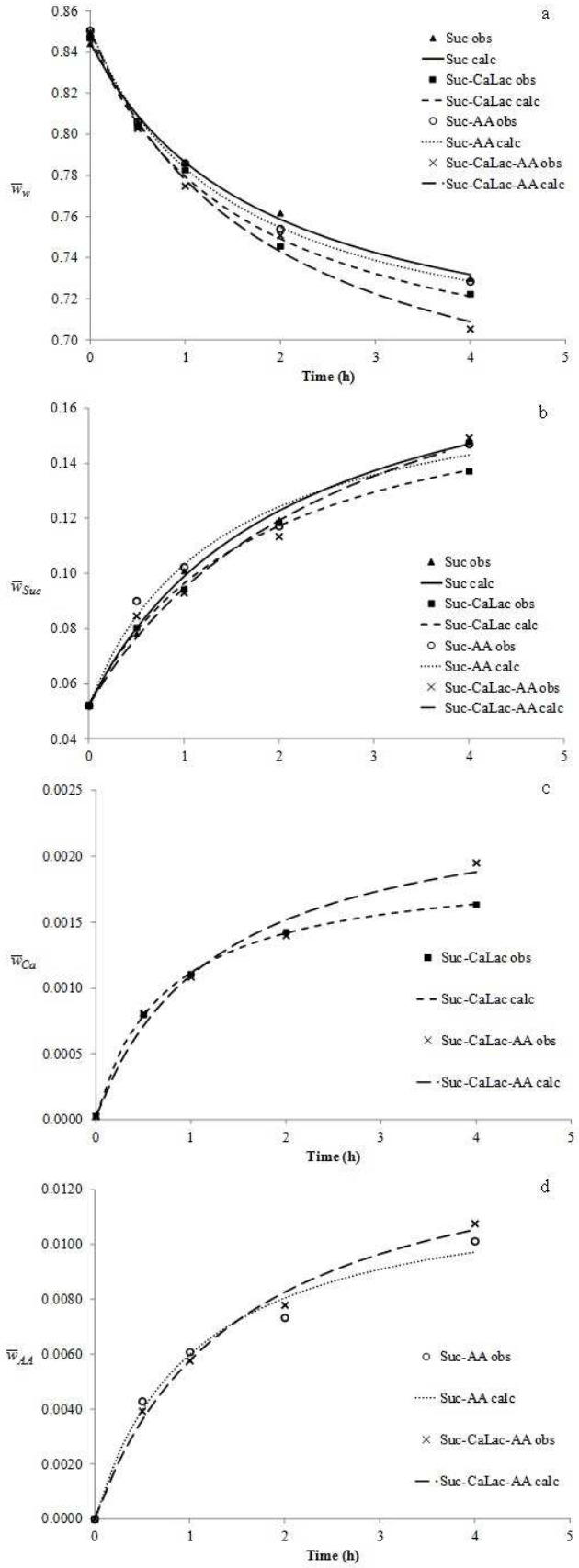

Fig 1: Comparison between observed (obs) and calculated (calc) mass fraction of water (a), sucrose (b), calcium (c) and ascorbic acid (d) according to the Peleg's model (eq. 4), in $g \cdot g^{-1}$ total mass, for the different treatments.

To better understand the influence of the solutes on the mass transfer kinetics, effective diffusion coefficients were calculated and are reported in **Table 2**.

Table 2: Effective diffusion coefficients for water, sucrose (Suc), ascorbic acid (AA) and calcium (CaLac) calculated for the four osmotic solutions.

	$D_{w,m}$ $\times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)	$D_{Suc,m}$ $\times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)	$D_{Ca,m}$ $\times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)	$D_{AA,m}$ $\times 10^{10}$ [$m^2 \cdot s^{-1}$]	R^2	*RRMSE* (%)
Suc	1.4	0.976	5.8	1.2	0.961	6.8	-	-		-	-	
Suc-CaLac	1.6	0.972	6.3	1.4	0.971	5.7	3.4	0.985	12.8	-	-	
Suc-AA	1.8	0.980	4.9	1.9	0.979	8.1	-	-		2.5	0.984	6.6
Suc-CaLac-AA	1.4	0.973	6.9	0.9	0.951	7.5	2.0	0.984	6.5	1.7	0.982	5.4

In the treatment with only sucrose, the effective water diffusion coefficients were slightly higher than the sucrose ones. This behaviour can be expected in a plant tissue in which the selective permeability of the membranes is preserved, because they reduce the transport of larger molecules such as sucrose through the cell tissue. Consecutively not all the space is available to sucrose transport, while water can diffuses throughout membranes and occupy all liquid phases of the plant cell (Mauro and Menegalli 2003).

The addition of CaLac and AA had a variable and unexpected influence on diffusion coefficients. The osmotic solution containing 4% of CaLac promoted a slight increase of both water and sucrose diffusivity in comparison to Suc solution. The water diffusion coefficient resulted still slightly higher than those found for sucrose, following the expected behaviour for preserved plant tissue. However, when Ca is present, the sucrose diffusion is limited and, thus, lower sucrose contents is observed during the process (**Fig 1 b**). However, this result is in disagreement with Silva et al. (2014a) that found a reduction of the diffusion coefficient for both water and sucrose due to the presence of 2% CaLac in the osmotic solution used for pineapple treatment. On the other side, increasing the concentration of CaLac in the solution from 2 to 4% promoted an increase of water and sucrose diffusivities, but still slightly lower than those found in treatment with the only sucrose solution. Also the sucrose diffusion coefficients were lower than water coefficients. Authors suggested a partial damage to the pineapple tissue structure because of the calcium effect on cellular membranes, that probably has occurred even in the present experiment. Moreover, the diffusion coefficients in pineapple dehydration were higher than those found in apples, probably because pineapple is characterized by a larger porous structure and a softer tissue compared to apple. It is important to point out that each tissue presents a specific OD response (Fernandez et al. 2004) reported a method based on the water and solute fluxes, for classifying mass transport as a function

of the tissue and observed different behaviours between the studied fruit tissues as a function of their individual internal texture and cellular organization.

In addition, the differences between tissues also appear comparing the calcium diffusivities for apple treatment with those obtained by Silva et al. (2014a) for pineapples, which coefficients were lower than the formers. Observed variations were mainly related to the equilibrium concentration values obtained by the referred authors, which have used a different methodology that consists in analysing the sample composition after its exposure to the osmotic solution for a long equilibrium time. During these experimental essays the authors observed non-equilibrium behaviour of the calcium, evidencing the instability of these systems, attributing it to the activity of the pectin-methyl esterase, that is an important enzyme in pineapple. In the present work, the equilibrium data were obtained based on the four hours of processing. Hence they were not affected by any successive change, even though influence of the enzymes on this system is expected. As OD proceeds, damages on the tissue promoted by the solution components and/or by the dehydration should cause depolymerisation and solubilisation of pectins together with de-methylation by action of enzymes. If calcium ion is present, de-esterified pectin can bind calcium and produce calcium pectates. Consequently, Ca is immobilized by an irreversible reaction and thus equilibrium is not reached. Studies about ripening of apples affirm that the pectin-degrading enzymes such as pectin-methylesterase, endo poly galacturonase and pectate lyase are very low in this fruit (Bonnin and Lahaye 2013). However, although other polysaccharide-degrading enzymes are likely involved in pectinolytic activities, these effects should be less intense in apples.

When calcium is added to the osmotic solution, three phenomena are expected to affect the transport. First, the higher solute concentration increases the water chemical potential difference, increasing the water loss from the tissue to the osmotic solution, but diffusion coefficients should decrease because a more concentrated system is a limiting factor.

The other response is related to the effect on membranes and cell wall of calcium, that can limit the solute transfer, as mentioned above, because it restricts the diffusion to spaces out of the cellular membranes. However, a third effect is related to calcium in excess, which can cause damages on the tissue, affecting firmness and membrane selectivity, as mentioned above in reference to Silva et al. (2014), that reported an increasing of diffusivities due to an increase of calcium concentration.

Consequently, the balance between all these effects will result in concentrations and diffusivities sometimes unexpected because the solution composition and the kind of tissue can exert different effects on the OD efficiency. In addition, structure changes during the process in several ways: shrinkage diminishes the pore size and calcium pectate formation also influences the cell wall porosity. Because of these transformations, it is possible that the driving forces, represented by the

equilibrium concentrations on the solid surfaces, undergo modifications from the initial steps until the final OD.

While Ca seemed to restrict in part the sucrose impregnation, the inverse was observed with AA. The addition of 2% ascorbic acid promoted a more intensive increase of the diffusion coefficients compared to the sucrose solution. However, water and sucrose diffusivities were similar, with the sucrose slightly above the water value. This indicates severe disruption and integrity losses by the tissue, making available all cellular spaces for water and solutes diffusion. Ascorbic acid presented higher diffusion coefficient than water and sucrose. Even though its molecular weight is lower than the sucrose one, the high value can also be related to assumptions. For instance, being this a multicomponent system, interferences between the mass fluxes, including other original solutes from apple, as well as shrinkage because of the water loss, were neglected.

Conversely, diffusion coefficients for Ca and AA, in apple treated in AA-Suc and CaLac-Suc solutions, are higher than in CaLac-AA-Suc as it is expected when the osmotic solution concentration is higher. During the first part of the dehydration, Suc-CaLac-AA treatments shows a behaviour of water and sucrose contents that, after 2 hours, seems to change, with their contents tending to an equilibrium concentration more distant (**Fig 1**). This fact led to smaller diffusion coefficients than if they were calculated considering only 2 hours of process. A hypothesis is that damages on cell walls, after a long treatment time, have caused excessive impregnation. This behaviour was also observed for sucrose contents in tissue treated by Suc-AA, reinforcing the AA role on damages and consequent solute impregnation.

3.2 Metabolic profiles

Results of the total metabolic heat produced during 16 h at 10 °C, measured through isothermal calorimetry after osmotic treatments carried out for 0, 30, 60, 120 and 240 min, are represented in **Fig. 2**. Since the concentration of O_2 and CO_2 can also give useful information about tissue metabolism, after calorimetric analysis, the composition of the headspace of the vials was evaluated. The measured respiration rate (RRO_2 and $RRCO_2$) are presented in **Fig 3**.

As a consequence of Suc treatment, a slight reduction in metabolic heat production (Fig. 2) proportional to treatment time until two hours of process, and a lower respiration rate compared to the fresh samples both in terms of CO_2 produced and O_2 consumed (Fig 3) were observed. A partial loss of cell viability could be expected after OD treatment, even if the osmotic solution concentration is very low (Panarese et al. 2012). In a previous experiment (Mauro et al. 2016), we found that cell viability was preserved in apple tissue subjected to OD treatment with 40% sucrose solution as observed by FDA staining technique that allows to determine plasma membranes

integrity. On the other hand, neutral red staining revealed the incidence of plasmolysis that could give a possible explanation to the decrease of the metabolic heat produced by the tissue. Salvatori and Alzamora (2000) found that a 25% w/w sucrose solution can cause vesciculation and rupture of cell membranes in apple tissue. According to Mavroudis et al. (2004) only few layers of cells on the surface are expected to die upon an osmotic treatment while plasmolysis and shrinkage occur in the rest of the tissue.

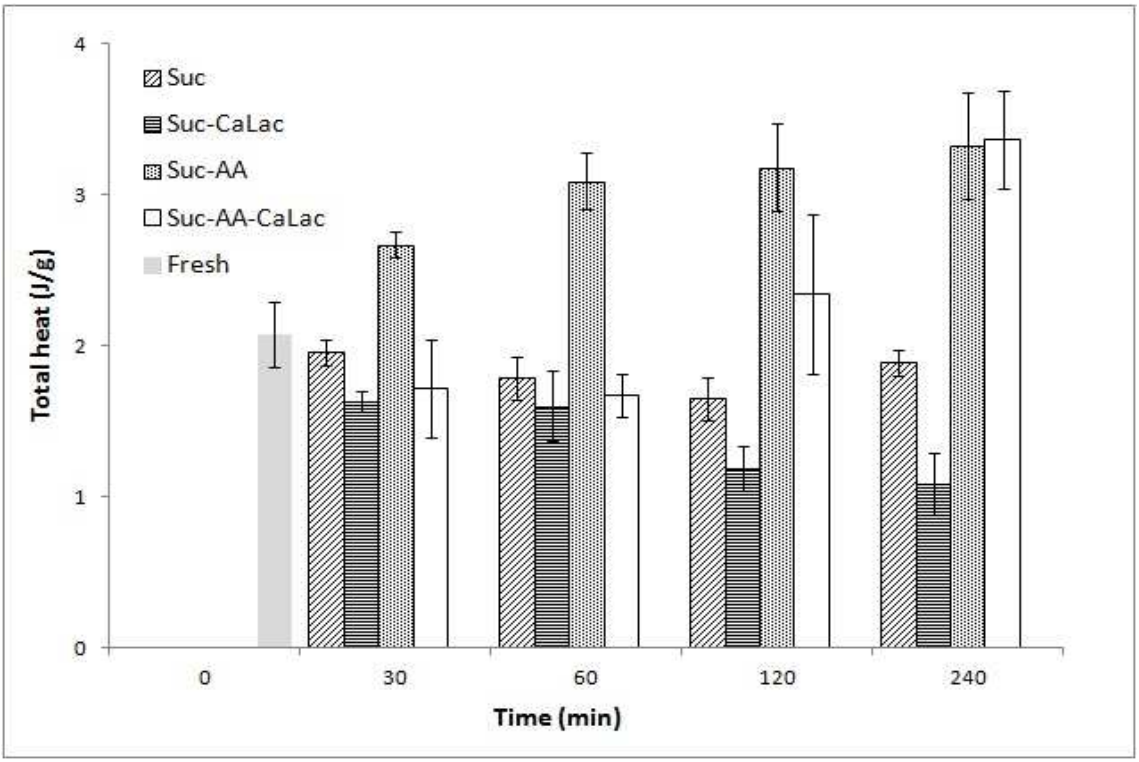

Fig. 2: Total heat production (J/g) of fresh and osmotically dehydrated samples during 16 h at 10°C.

The presence of calcium in the osmotic solution caused a further decrease of metabolic heat production. This result is in accordance with previous literature reports (Castelló et al. 2010; Luna-Guzmán et al. 1999), and confirms the ability of calcium to slow down tissue metabolic activity and thus to enhance the stability of minimally processed fruit. Confirming calorimetric measurement, samples dehydrated Suc-CaLac solutions, showed slightly lower values of respiration rate (mainly in terms of RR_{O_2}), indicating that the reduction of heat produced could be related to the reduction of the respiratory activity, but also to other kind of biochemical phenomena.

Actually the effect of calcium on respiration has not been fully clarified yet but it has indeed been observed in various fruits, both whole and cut, together with a reduction of ethylene production and a general slowing down of ripening and senescence (Lester 1996; Saftner et al. 1999). In particular, different explanations have been put forward for the reduction of the respiration rate, a protective

osmotic effect due to the high salt concentration (Ferguson 1984), the indirect effect on substrate transport due to the alteration of membrane permeability (Bangerth et al. 1972), the formation of a transient barrier between fruit and atmosphere that hinders the gas exchange (Saftner et al. 1999), the inhibition of plant aquaporins, that regulate membrane permeability, causing an increase in the cytoplasmic ATP concentration that in turn remains available for other biochemical routes (Kinoshita et al. 1995) or the delay of senescence-related changes (Lester 1996). At the same time, an excess of calcium has been related to a hastening of senescence because of damages to the plasma membrane structure and functionality.

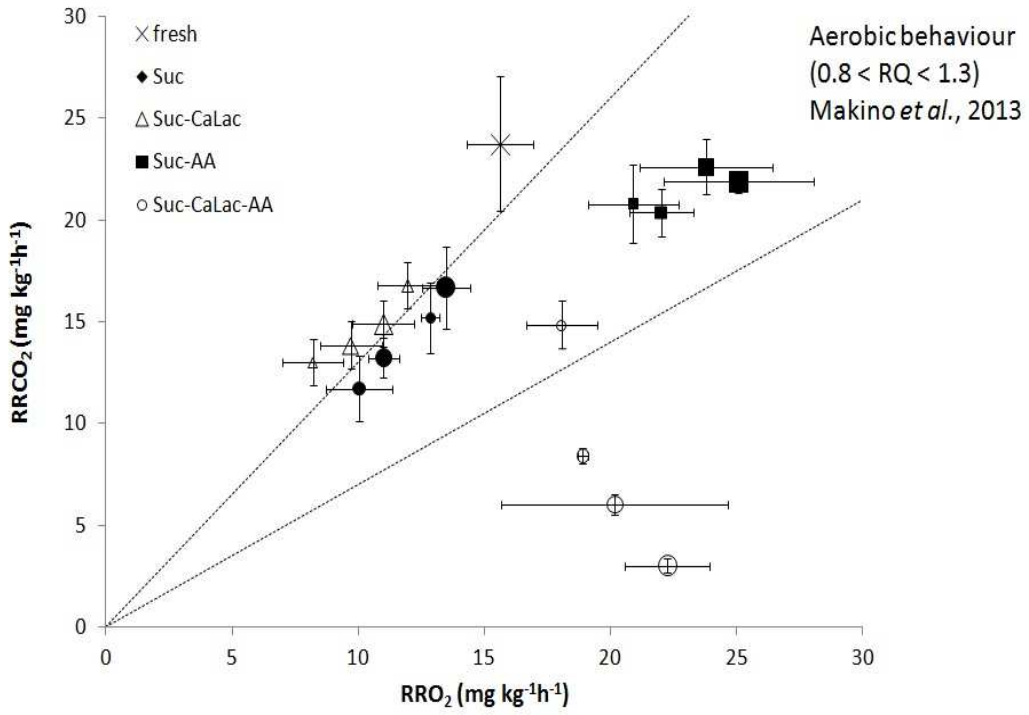

Fig. 3: Respiration rates expressed as oxygen consumed (RRO_2) and carbon dioxide produced ($RRCO_2$), for treatment time of 30 min (smaller size symbol), 60 min, 120 min and 240 min (higher size symbol).

Conversely, the presence of AA in the osmotic solution promoted a drastic increase of metabolic heat production as treatment time increased, up until 50% compared to the fresh sample. This increase can be probably attributed to the physiological stress caused to the tissue, as already observed for sliced potatoes (Limbo and Piergiovanni 2007; Rocculi et al. 2005). The damage to cellular structures promoted by osmotic AA solution can be mainly caused by its lower pH. Actually, at low pH, plasma membrane ATPases in the tissue increase the active H+ pumping to deal with excess of H+ uptake leading to an increase of the demand for respiratory energy. An ulterior pH decreases can cause also a decline in the respiration rates.

AA combined with Ca initially promoted a decrease of the heat production to a level lower than untreated samples but after 2 h, the metabolism rose sharply. This behavior suggests that during the first part of the treatment calcium acted as stabilizer and reduced the metabolic activity of the tissue but, as the treatment proceeded, a progressive damage to cellular structures occurred, probably related to the intake of AA.

Conversely, for sample dehydrated in the presence of AA, both alone or in combination with CaLac, there was a noticeable change in the respiratory pathway, particularly in terms of increase of oxygen consumption in comparison with Suc and Suc-Ca samples.

CO_2 production was quite constant for all treatment times in AA samples, with a reduction of about 25% compared to the fresh tissue, but higher compared to Suc and Suc-CaLac samples. On the other hand, noticeable $RRCO_2$ decrease was verified in Suc-CaLac-AA condition, proportionally to treatment time.

Respiratory quotient is an indicator of the respiration pathway adopted by tissues. The complete oxidation of glucose through the aerobic pathway produces an equal amount of CO_2 as the O_2 consumed, so that the respiratory quotient is 1. Variations in the RQ may depend on a different substrate used for respiration, such as malate or long chain fatty acids, although generally, an increase in RQ indicates the onset of fermentative routes (Taiz and Zeiger 1998). However, according to (Makino 2013), RQ in the range of 0.7 to 1.3 could be considered indicator of aerobic respiration. Roughly, following this indication, in Figure 3 it was possible to identify which samples were characterized by aerobic metabolism. In our experiment, fresh sample had an RQ value of 1.5, while in Suc and Suc-CaLac, RQ values were lower and closer to 1, showing negligible anaerobic metabolism.

Anaerobic metabolism can be prompted by either low oxygen or high carbon dioxide concentration in the environment, respectively lower than 2-5 % and higher than 4-5 % (Cortellino et al. 2015; Iversen et al. 1989). Although this values was never exceeded, in some samples, and in particular in the fresh one, after 20 h CO_2 content was very close to this limit, and may have caused the development of some fermentative pathways leading to an imbalance between CO_2 production and O_2 consumption in the tissue that caused an increase of RQ.

Thus, not considering the Fresh sample and sample Suc-CaLac that is very similar to Suc, only the sample treated with Suc-CaLac-AA seemed to have a non-aerobic response to the treatment, but only if applied for more than 30 min.

As a consequence of OD, an increase of RQ was observed by Torres et al. (2008) and by Castelló et al. (2010) on mango and strawberry tissues. Anaerobic metabolism is often found in plant tissue as a physiological response to stress conditions, such as dehydration, as an optional metabolic pathway

Torres et al. (2008). While oxygen diffusion through the tissue decreases because of structural alteration of the cells as the treatment proceeds, generally an increase of CO_2 production has been observed by these authors. The oxygen consumed was attributed to the effort of some enzymatic systems to react to the stress caused by the osmotic treatment (Lewicki et al. 2001). Conversely, Moraga et al. (2009) did not find changes due to calcium lactate presence in the RQ of osmo-dehydrated grapefruit although the respiration rate generally decreased.

In samples dehydrated in the presence of AA, a lower RQ was calculated and was found to decrease slightly by increasing treatment time between 0.72 and 0.64 (data not shown). The combination of sucrose and ascorbic acid showed to cause cellular damage to the tissue, the effect on plasmalemma and tonoplast was different and not clear but a strong influence on tissue functionality was definitely observed (Mauro et al. 2016). When both AA and CaLac were used, the RQ decreased sharply as the dehydration proceeded, from 0.59 to 0.09. This decrease is mainly due to the higher oxygen consumption observed compared to CO_2 production.

It is important to underline that the variation of the gas composition in the sample headspace could be due not only to the respiratory metabolism of the tissue but also to the presence of other enzymatic reactions. According to Igual et al. (2008) this consumption of O_2 can be considered as ‘‘apparent” respiration rate. Because in plant tissue, molecular oxygen can be used as substrate by many enzymes, it can contribute to the ‘‘apparent” respiration rate if measured in terms of oxygen consumption, but not in terms of production of CO_2 (Taiz and Zeiger 1998). In this direction, the effect of sugar, calcium and ascorbic acid on the complexity of fresh tissue enzymatic activity has to be taken into account.

4. Conclusions

The investigated osmotic dehydration treatments showed different effects on the product, both in terms of mass transfer phenomena during processing and metabolic activity of the apple tissue. The presence of calcium and ascorbic acid affected the effective diffusivities attributed to the changes in cellular spaces, increasing spaces viable to solute transport. Metabolic heat production in samples treated in sucrose solutions was slightly lower than in untreated samples and it was further reduced with calcium lactate (CaLac) addition. However, samples impregnated with ascorbic acid (AA) showed a higher heat production, as there was a metabolic response of the apple tissue to AA treatment. When combined with Ca, heat production decreased sharply to a level lower than untreated samples, except for those treated for 240 min (higher solid gain), which showed the highest heat production values. These results confirm previous findings, suggesting that AA solution can promote a stress response on specific fresh-cut vegetable tissues, and an increase of

their endogenous metabolic activity, confirmed by a higher O_2 consumption observed by head space gas determination.

In order to clarify the effect on enzymatic activity in apples osmotically dehydrated in sucrose, calcium and ascorbic acid osmotic solutions and the real influence of these phenomena on respiration pathways, further studies are needed, coupling the calo-respirometric approach with metabolomic analytical techniques.

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Paper XI

Metabolic response of fresh-cut apples induced by pulsed electric fields

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Metabolic response of fresh-cut apples induced by pulsed electric fields

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Abstract

Pulsed electric field (PEF) treatments can induce metabolic stress responses in plant tissue as function of the applied conditions. This study highlighted the metabolic effects of reversible and irreversible electroporation in fresh-cut apple tissue by adjusting the electric field strength to 100, 250 and 400 V/cm. Metabolic heat, O2 and CO2 gas analysis along with metabolomics were employed to jointly evaluate the gross and specific PEF-induced effects after 24 h at 10 °C.

Each tested electric field strength influenced the metabolic response, however, marked changes were registered when the threshold of electroporation was exceeded at 250 and 400 V/cm. A drop of metabolic heat and respiration rate was observed as a probable consequence of the loss of the cell viability, anaerobic respiration pathways were noticeably lowered while GABA metabolism was activated. Conversely, minimal modifications of the metabolism heat and metabolites concentrations were noticed when 100 V/cm were applied.

Industrial relevance

Metabolic response of fresh-cut fruit and vegetables as function of the manufacturing process is a fundamental aspect directly related to the shelf life of the final products. Pulsed electric fields (PEF), as well as other innovative technologies, can induce undesired effects on tissue metabolism that might limit the industrial application. Furthermore, the analytical methods used in the present work provide useful tools for the optimization of the PEF treatment conditions for fresh-cut manufacturers.

Key words: PEF; Apple tissue; Isothermal calorimetry; Respiration rate; HR-NMR; Metabolomics

1. Introduction

Pulsed electric field (PEF) technology is a non-thermal process which has recently demonstrated an increasing interest in the food field. The application of high electric fields between two electrodes can be exploited for different goals, for instance to enhance mass transfer phenomena (Donsì, Ferrari, & Pataro, 2010; Puértolas, Luengo, Álvarez, & Raso, 2012; Taiwo, Angersbach, & Knorr, 2002) or to inactivate microorganisms (González-Arenzana, et al., 2015; Timmermans, Groot, Nederhoff, van Boekel, Matser, & Mastwijk, 2014). The mechanism of action includes the creation of pores due to the application of electric fields high enough to induce a potential difference of approximately 0.2 V across the cell membrane (Teissie, Eynard, Gabriel, & Rols, 1999). In a second step, pores can expanse and aggregate and, once the external electric field is removed, they can reseal (Vorobiev & Lebovka, 2009). The extent of the process, also known as electroporation,

strongly depends on the applied process parameters such as electric field strength, number and shape of pulses, their width and frequency. Indeed, different goals and industrial applications can be achieved by adjusting the treatment conditions (Barba, et al., 2015).

The effect of PEF in plant tissues has been studied by several techniques according to the desired objective: example are the release of valuable compounds (Carbonell-Capella, Buniowska, Esteve, & Frígola, 2015; Luengo, Álvarez, & Raso, 2013), extraction yield (Bazhal, Lebovka, & Vorobiev, 2001), changes in colour and texture (Lebovka, Praporscic, & Vorobiev, 2004; Wiktor, Schulz, Voigt, Witrowa-Rajchert, & Knorr, 2015). Moreover, methods have been developed to indirectly evaluate the extent of electroporation based on electrical impedance (Angersbach, Heinz, & Knorr, 2002; Ivorra, 2010; Lebovka, Bazhal, & Vorobiev, 2002), microscopy (Fincan & Dejmek, 2002) and nuclear magnetic resonance (Dellarosa, et al., 2016).

Pulsed electric fields, by acting at the level of membranes, can also deeply affect the cell activities. As a consequence, metabolic stress responses of cells can be induced and lead to undesired effects on the quality of the final products. This might limit the application of PEF in fresh-cut products. Generally, fresh cut fruit and vegetables undergo to minimal processing, such as peeling, cutting or pre-treatment with different solutions (Mauro, et al., 2016; Santagapita, et al., 2013) which, nevertheless, provokes metabolic responses (Rocculi, et al., 2012). In this contest, the application of PEF can, from one side, ease the mass exchange between the outer solution and the tissue, anyway, from the other side, lead to trigger further stress responses. To the best of our knowledge, few works have been focused on the metabolic aspects induced by PEF in postharvest fruit and vegetable products (Galindo, et al., 2009; Galindo, Wadsö, Vicente, & Dejmek, 2008).

Fresh-cut products are metabolic active tissues, hence, they produce heat as a function of both the normal cell activities and technological processes applied. Thermal power and heat can be continuously monitored by isothermal calorimetry and this gives rise to gross values of the cell metabolisms (Galindo, Rocculi, Wadsö, & Sjöholm, 2005; Wadsö & Galindo, 2009). Interestingly, whether the sample conditions are standardized, a direct evaluation of the effects of the technologies can be carried out (Panarese, et al., 2012; Tappi, et al., 2014). Moreover, the measurement of the heat is often coupled with the analysis of the consumed O_2 and produced CO_2 which allow clarifying whether other non-aerobic metabolisms are activated (Cortellino, Gobbi, Bianchi, & Rizzolo, 2015).

Beside analytical methods that estimate the overall stress response, the metabolic profiling by means of high-throughput techniques is a comprehensive analysis of the soluble metabolites, i.e. the metabolome (Wishart, 2008). This approach has been successfully applied for food quality control, health and nutritional purposes, fingerprinting, including traceability and authenticity, and, recently,

to assess and backwardly adjust technological processes (Trimigno, Marincola, Dellarosa, Picone, & Laghi, 2015). To the purpose, specific multivariate analytical tools need to be developed and tailored to discriminate the effects of the applied technologies on precursors, intermediates and products of different metabolic pathways (Laghi, Picone, & Capozzi, 2014).

The objective of the present work was to assess the metabolic response of fresh-cut apples upon pulsed electric field treatments. Three different levels of electric field strength, 100, 250 and 400 V/cm were studied because they are known to produce both reversible and irreversible electroporation effects on cell membranes in apple tissue (Dellarosa, et al., 2016). A comprehensive evaluation by means of a multianalytical approach based on calorimetry, gas analysis and metabolomics was chosen to complementary describe gross alteration on metabolic activities and specific fine changes in metabolites

composition. High resolution ^{1}H nuclear magnetic resonance (HR-NMR) was employed for the analysis of the metabolic profiling together with a novel non-targeted statistical tool based on sparse Partial Least Square Discriminant Analysis (sPLSDA) and Linear Discriminant Analysis (LDA).

2. Material and methods

2.1 Raw material

Apples (*Malus domestica,* cv Cripps Pink) were purchased at a local market and stored at 2 ± 1 °C for three weeks, during which all the experiments were conducted. Before experiments, apples were kept at room temperature for 2 h. Raw material had an average moisture content of 83.5 ± 0.5 g and soluble solid content of 13.5 ± 0.5 g per 100 g of fresh product. Cylindrical samples (8 mm diameter and 10 mm length of an average weight of 1 g) were obtained from apple parenchyma by cutting with a manual cork borer and a scalpel. Eight cylinders from each fruits have been used for the experiments.

2.2 Pulsed electric field (PEF) treatments

PEF were applied to apple samples using an in-house developed pulse generator equipment based on capacitors as energy tank and controlled by MOSFET. Briefly, 60 monopolar pulses of near-rectangular shape, fixed pulse width of 100 ± 2 µs and repetition time of 10.0 ± 0.1 ms were chosen, according to the experimental conditions used by Dellarosa et al. (2016). PEF treatments were conducted at 20 °C in a 30 × 20 × 20 mm (length × width × height) chamber equipped with two stainless steel electrodes with an active contact surface of 20 × 20 mm^2. For each trial, 12 apple cylinders were arranged within the two circle sides parallel to the electrodes and the chamber was filled up with tap water (conductivity at 25°C of 328 ± 1 µS cm^{-1}) with a final product-to-water

ratio around 1:1 (v/v). Applied current and voltage values were measured by a digital oscilloscope (PicoScope 2204a, Pico Technology, UK), connected to the equipment and a personal computer.
Four samples groups, including control, were obtained by treating apple cylinders with a voltage of 300, 750 and 1200 V to the electrodes. These conditions led to the average electric field strengths of 100, 250 and 400 V/cm in the chamber. However, the real voltage values, calculated by equivalent circuits on the circle sides of the apple cylinders parallel to the electrodes, were dissimilar due to the simultaneous presence of tap water and apple cylinders between the electrodes. Consequentially, treatments at the average field strengths of 100, 250 and 400 V/cm gave rise to real values of 115, 245 and 275 V/cm, respectively. As commonly accepted throughout the literature, in the present work the treatments and the sample names were referred to the average electric field strengths.

2.3 Metabolic heat

Three fresh cylindrical samples (about 3 g) were placed in 20 mL glass ampoule and sealed with a teflon coated rubber seals and an aluminium crimp cap. For each sample, two replicates for three independent treatments were analysed. A TAM air isothermal calorimeter (TA Instruments, New Castel, USA) with a sensitivity (precision) of ±10 μW was used to measure the heat production. This instrument contains eight twin calorimeters in which each sample is coupled with its own reference (Wadso & Gomez Galindo, 2009). Water was chosen as reference material, the quantity was calculated according to Panarese et al. 2012 The analysis was carried out at 10 °C for 24 h, baseline were recorded before and after each measurement. Specific thermal powers (mW g^{-1}) were calculated according to Galindo, Wadsö, Vicente & Dejmek (2008).

2.4 Respiration rate

The concentration of O_2 and CO_2 (%) were measured in the ampoule headspaces by a gas analyzer (MFA III S/L gas analyzer, Witt-Gasetechnik, Witten, Germany) at the end of the calorimetric measurements, and on other “twin” ampoules with samples treated at the same conditions, stored in the dark for the same period of time (24 h) at the same temperature (10°C), for a total of 18 repetitions for sample. Respiration rate was calculated as mg of consumed O_2 (RRO_2) and produced CO_2 ($RRCO_2$) kg^{-1} h^{-1}, according to the following equations:

$$RRO_2 = \frac{mm_{O_2} \cdot V_{head} \cdot \frac{(20.8 - \%O_{2,head})}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283}$$

$$RRCO_2 = \frac{mm_{CO_2} \cdot V_{head} \cdot \frac{\%CO_{2,head}}{100} \cdot 101.325}{t \cdot m \cdot R \cdot 283}$$

where mmO_2 and $mmCO_2$ refer to gases molar mass (g mol^{-1}), V_{head} represents the ampoule headspace volume (dm^3), % $O_{2,head}$ and % $CO_{2,head}$ refer to gases percentages in the ampoule headspace at time t (h); m is the sample mass (kg); R is the gas constant (8.314472 dm^3 kPa K^{-1} mol^{-1}), P is the pressure (101.325 kPa) and T is the absolute temperature (283 K).

2.5 Metabolomics

2.5.1 High resolution ^{1}H nuclear magnetic resonance (HR-NMR)

Apple cylinders were collected after 24 h at the same experimental conditions applied for metabolic heat and respiration rate analysis (stored in the dark at 10°C). For each sample, three of them (about 3 g) were squeezed until an aliquot of 1 mL was obtained for each sample, for a total of 36 repetition per sample. Afterwards, samples were centrifuged at 21380 ×*g* and 4°C for 20 min in an Eppendorf tube, 700 µL of the supernatant was collected and added to 70 µL of 10 mM TSP (3-TrimethylSilyl-Propanoic-2,2,3,3-d4 acid sodium salt) in deuterium oxide with the addition of sodium azide at the final concentration of 0.04 % to prevent microbial activities. Samples were frozen at -20 °C until analysis when they were thawed and successively centrifuged at 21380 ×*g* and 4°C for 20 min to further remove impurities. Finally, the supernatant was placed in a 5-mm internal diameter NMR tube for metabolomic analysis.

^{1}H spectra were recorded at 298 K with an Avance III spectrometer (Bruker, Italy) operating at a frequency of 600.13 MHz. Residual water signal was suppressed using the NOESY sequence. Each acquisition included 32 K data points over 7796 Hz spectral width and 128 scans while the 90° pulse time was calculated for each acquisition. Spectra were pre-treated using TOP SPIN 3.0 software (Bruker, Italy), by alignment towards TSP signal and the line broadening of 0.3. The principle of reciprocity (Hoult, 2011) was used to normalize each spectrum, so that quantitative results could be obtained. Using citric acid as external standard high linearity (R^2=0.9997) was found in the range of 0.01 - 800 mM. A total of 144 NMR spectra, homogeneously distributed among the four sample groups, were acquired and exported for further data analysis.

2.5.2 Data pre-treatment and analysis

HR-NMR spectra were subjected to a drift and baseline corrections, carried out in R statistical software (R Foundation for Statistical Computing, Vienna, Austria), as reported by De Filippis et al. (2015). Signals of the spectra were manually integrated resulting in 43 protons peaks belonging to alcohols, amino acids, organic acids, sugars and other secondary metabolites. The obtained 144

(samples) × 43 (metabolites) matrix was scaled and centred before undergoing to multivariate statistical analysis.

To gain insight into metabolic changes which occurred upon PEF treatments, two chemometric analysis, the Principal Component Analysis (PCA) and sparse Partial Least Square Discriminant Analysis (sPLSDA) followed by Linear Discriminant Analysis (LDA) were employed. The R packages 'mixOmics' and 'MASS' were used to the purpose (Lê Cao et al., 2011). The approach based on sPLSDA-LDA was chosen because it is an embedded chemometric method which allows to both find correlation between predictors and response classes, while simultaneously sort and selection of the relevant variables (Mehmood et al., 2012). This powerful tool was used to evaluate the changes in the metabolic profiles as a consequence of the different technological treatments and, at the same time, to focus the discussion on the few metabolites significantly affected by PEF.

The analytical process included the splitting of the 144 samples spectra into training and test sets, accounting for 70% and 30 % respectively. The training set was used to build the sPLSDA model, validated through the M-fold validation step (M = 10), which also optimized the selection of latent variables and metabolites, as a function of the error rate. Afterwards, the test set was employed as independent dataset to verify the performance of the built model. A thousand models were iteratively trained and tested by randomly dividing training and test sets to enhance the robustness of the analysis. Finally, the class prediction errors over the repetitions were expresses, scaled to the unit, as incorrect assignments in the confusion matrix. In parallel, the metabolites arisen from the multivariate analysis were considered as important whether their mean VIP (Variable Importance in Projection) value was higher than one and their frequency in the model was higher than 70 % (Chong and Jun, 2005).

2.6 Statistical analysis

Significant differences between control and PEF-treated samples were evaluated by the analysis of variance (ANOVA) and Tukey's multiple comparisons at the significance level of 95 % ($p<0.05$) implemented in R statistical software (R Foundation for Statistical Computing, Vienna, Austria). All the experiments were repeated at least six times and results were expressed as mean ± standard deviation of replications.

3. Results and discussion

3.1 Changes in electrical resistivity: measurement of electroporation reversibility

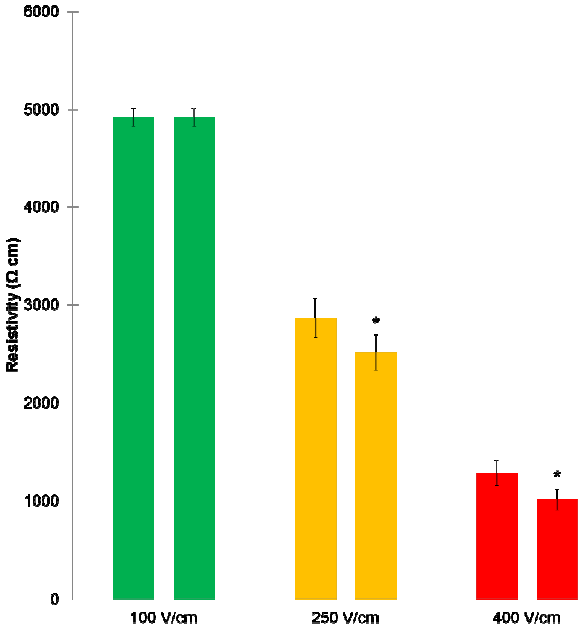

Fig. 1. Resistivity of the apple samples calculated using equivalent circuits on the first (left bar) and the last (right bar) pulse of the 60 pulses train series for each electric field strength. Values are means ± standard deviations (n=16) and differences between means with the same letter are not significant at $p<0.05$.

The effect of electroporation on apple tissue was primarily observed by means of the resistance changes of the material during the treatments. The electrical resistivity of apple tissue, determined by equivalent circuits, was a function of both the average electric field strengths applied between the electrodes and the treatment time, i.e. the considered pulse. **Fig 1** shows the resistivity at the beginning and at the end of the PEF treatment, respectively, calculated, for each field strength, taking into account the first (left bar) and the last pulse (right bar) of the trains. The resistivity of the tissue linearly decreased from about 4900 Ω cm to around 2900 and 1300 Ω cm when the electric field strength was increased from 100 V/cm to 250 and 400 V/cm, respectively, upon the application of the first pulse. Nevertheless, solely the samples treated at 250 and 400 V/cm highlighted a significant decrease of the resistivity when the first pulse was compared to the last one. This changes suggested that the two highest electric field strengths irreversibly altered the structure of the tissue while at 100 V/cm the electroporation effects were reversibly recovered by the cells during the treatment. This confirms a previous work where the structural change of cells, at the same treatment conditions, were studied by time domain nuclear magnetic resonance (Dellarosa et al., 2016). The loss of plasma membrane selectivity at average electric field strength higher than 150 V/cm caused the leakage of the cellular solutions toward the extracellular spaces which

probably contributed to the decrease of the resistivity during the pulsation (Angersbach, Heinz, & Knorr, 2000; Vorobiev & Lebovka, 2009). Similarly, in potato tissue Galindo et al. (2009) found that, although different field strengths led to the electroporation of the cells, only samples irreversibly damaged showed a change in resistivity.

3.2 Heat production and respiration rate: gross metabolic response of the tissue

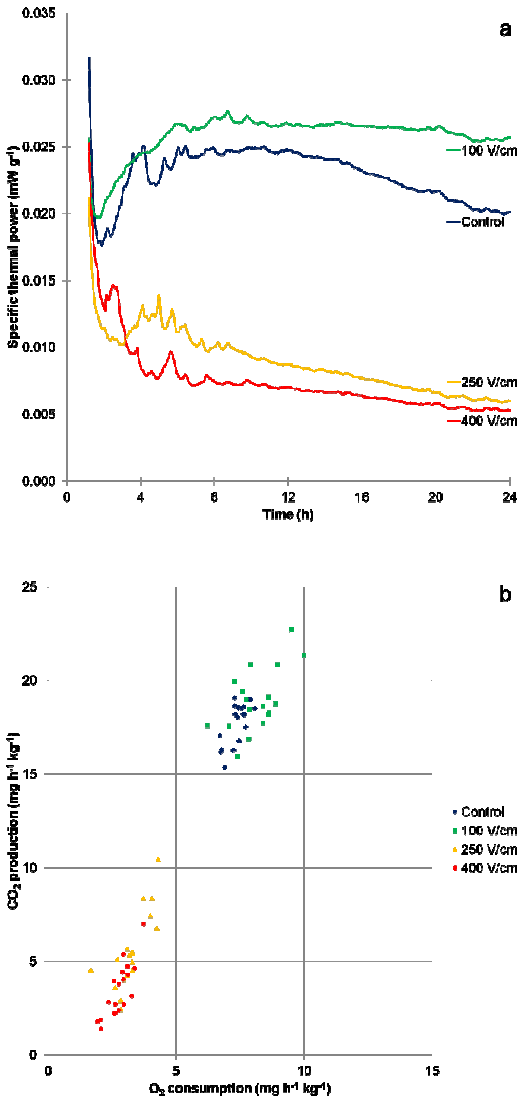

Fig. 2. Specific thermal power of apple samples during 24 h of analysis at 10 °C (A), each thermogram is an average of six replicates. CO2 production (RRCO2) vs. O2 consumption (RRO2) during 24 h at 10 °C (B).

Fresh apple is a metabolic active tissue, which produces heat and CO_2 while consuming O_2, as a consequence of the respiration activities. In the absence of microbial growth on the sample, the metabolic heat production of fresh-cut fruit is mainly due to the sum of the normal respiration activity and wounding response (Rocculi, et al., 2012; Wadsö, et al., 2009; Wadsö, Gomez,

Sjöholm, & Rocculi, 2004) upon cutting and further treatments, for instance PEF (Galindo et al., 2009).

The average thermograms acquired by means of isothermal calorimetry of PEF-treated samples at different field strengths are shown in **Fig. 2a**. A clear difference between samples treated at 250 and 400 V/cm was noticed when compared to both 100 V/cm and control samples. The lowest field strength seemed to induce a stress response in the tissue possibly ascribable to both the recovery activity due to the reversible alteration of plasma membrane (Vorobiev & Lebovka, 2008) and the subcellular changes, in particular, the water migration from vacuole to cytoplasm (Dellarosa et al., 2016).

The average metabolic heat production was calculated by integrating the metabolic heat profiles. The first 4 h of analysis were excluded in order to prevent the influence of the initial disturbance due to sample loading and conditioning, hence values reported in Table 1 refer to 20 h at 10°C.

Table 1. Means values of the metabolic heat and respiration rates during 24 h at 10 °C.

	Metabolic heat ($J\ kg^{-1}\ h^{-1}$)	RRO_2 ($mg\ kg^{-1}\ h^{-1}$)	$RRCO_2$ ($mg\ kg^{-1}\ h^{-1}$)
Control	82.79 ± 14.69^{a}	7.39 ± 0.39^{a}	16.93 ± 3.60^{a}
100 V/cm	94.46 ± 22.35^{a}	8.15 ± 0.88^{a}	18.83 ± 1.65^{a}
250 V/cm	30.22 ± 6.73^{b}	3.17 ± 0.70^{b}	5.77 ± 2.25^{b}
400 V/cm	23.78 ± 5.21^{b}	2.76 ± 0.45^{b}	3.27 ± 1.59^{c}

RRO_2: O_2 respiration rate (O_2 production). $RRCO_2$: CO_2 respiration rate (CO_2 consumption). Values are means ± standard deviations (n=6 for metabolic heat, n=18 for O_2 and CO_2 respiration rates) and differences between means with the same letter are not significant at p=0.05.

It is worth observing that significant differences were only found when irreversible electroporation took place. At 250 and 400 V/cm the metabolic heat dropped 2.5-3.5 times in comparison to the control as a consequence of the likely loss of the cell viability due to the irreversible membrane poration. In this direction, PEF led to a similar effect on heat production to that previously observed as a consequence of different stabilizing treatment on fresh vegetable tissue. Indeed, Tappi et al. (2014) found a significant decrease of metabolic heat when fresh-cut apple were treated by cold gas plasma and a direct correlation of the effect with the treatment intensity. Nevertheless, in contrast to atmospheric gas plasma, PEF is known to produce more homogeneously distributed effects through the material (Parniakov, Lebovka, Bals, & Vorobiev, 2015), not only limited to the surface, explaining the higher inhibition of heat production. Similarly, other studies regarding different fresh-cut vegetable subjected to more traditional treatments such as blanching (Gómez, Toledo, Wadsö, Gekas, & Sjöholm, 2004) and osmotic dehydration (Panarese et al., 2012) showed a partial

reduction of metabolic activity proportionally to the treatment parameters. Conversely to other treatments, the main effect of PEF on cell processes, at the present treatment conditions, is only ascribable to the alteration of the membrane permeability and functionality, while the direct enzyme inactivation is negligible. Indeed, according to several authors finding, a significant decrease of enzymes activities only occurred when electric field strength higher than 5 kV/cm was applied (Giner, Gimeno, Barbosa-Cánovas, & Martín, 2001; Zhong, et al., 2007).

Besides, the respiration rate was measured using a static method after 24 h at the same experimental conditions applied for the isothermal calorimetry measurements. **Fig 2b** and **Table 1** show the results, including the statistical analysis. Accordingly, a marked decrease of both O_2 consumption and CO_2 production was observed upon PEF treatments at 250 and 400 V/cm. This confirmed the severe loss of viability of apple tissue caused by the irreversible electroporation. A significant difference was also noticed in the $RRCO_2$ between the two highest field strengths, leading to the conclusion that the metabolic response was affected by the field strength even over the threshold of the irreversible electroporation.

The aerobic cell respiration of fresh fruit produces 455 kJ per mol of O_2 consumed, hence results obtained by calo-respirometric analysis could be compared (Wadsö et al. ,2009). However, because for metabolic heat production the first 4 h of analysis were excluded, in order to obtain comparable data, the O_2 consumed during that interval was measured in a parallel experiment and RRO_2 data adjusted consequentially. Results showed that the first 4 h accounted for the 27.0 ±0.8 % of the total O2 confirming the non-linear consumption throughout 24 h highlighted in previous works (Tappi, et al., 2014; Tappi, et al., 2016; Torrieri, Cavella, & Masi, 2009). Metabolic heat and RRO_2 were found linearly correlated (R^2 = 0.9994), nevertheless, O_2 consumption calculated from metabolic heat was found to be lower, in all the samples, with a bias spanning from 0.50 to 0.75 mg kg^{-1} h^{-1}. The achieved difference was attributed to the wounding response as consequence of both cutting (Wadsö, et al., 2004) and PEF treatments (Galindo, et al., 2008).

Taking into account the respiration quotient, i.e. the ratio between $RRCO_2$ and RRO_2, all the samples showed values higher than 1. In particular, those observed for control and treated samples at 100 V/cm were the highest, with similar scores around 2.3. Several authors pointed out that the anaerobic processes were prompted by either low oxygen or high carbon dioxide conditions, respectively lower than 2-5 % and higher than 4-5 % (Cortellino, et al., 2015; Iversen, Wilhelmsen, & Criddle, 1989; Yearsley et al., 1996). Even though the recorded O_2 values around 18 % and the highest CO_2 level around 4 % stood below the anaerobic threshold reported in literature, the high respiration quotients suggested that metabolic pathways different from the aerobic respiration were

triggered. In order to clarify the metabolic response of fresh-cut apples upon PEF treatments, samples were collected and analysed by means of NMR-based metabolomic approach.

3.3 Metabolic profiling by HD-NMR: specific metabolic response of the tissue

The metabolic profiles of the 4 sample groups, acquired by HR-NMR analysis, gave rise to 43 quantified signals which are displayed in **Table 2**. Using Chemomx software, available literature (Belton et al., 1997; Tomita et al., 2015; Vandendriessche et al., 2013; Capitani et al., 2012; Monakhova et al.; 2014) along with HMDB (Wishart et al., 2012) and Madison (Cui et al., 2008) public databases, 38 metabolites were identified.

A multivariate non-targeted approach was chosen to investigate the differences in the metabolic profiles upon the application of PEF at different electric field strengths. Initially, an unsupervised statistical method, the principal component analysis, was applied to highlights the main sources of variation among the spectra.

Table 2. Metabolites in NMR profiles underwent to statistical analysis

Alcohols	**Amino acids**	**Organic acids**	**Sugars**	**Other**
Butanol	Alanine	Acetic acid	Fructose	Acetaldehyde
Ethanol	Asparagine	Chlorogenic acid	Maltose	Acetoin
Isopropanol	Asparagine	Citramalic acid	Sucrose	Epicatechin
Methanol	Aspartic acid	Formic acid	Trehalose	myo-Inositol
Propanol	Glutamic acid	Lactic acid	Xylose	Trigonelline
	Leucine	Malic acid	α-Galactose	Unknown at 3,34 ppm
	Phenylalanine	Pyruvic acid	β-Galactose	Unknown at 4,32 ppm
	Valine	Quinic acid	α-Glucose	Unknown at 4,51 ppm
		Succinic acid	β-Glucose	Unknown at 4,98 ppm
		Tartaric acid		Unknown at 5,49 ppm
		γ-Aminobutyric acid		

The first and second principal component, displayed in **Fig 3a**, explained only the 25.65 and 15.47 % of the variance, respectively. A reasonable separation of two main clusters was achieved, so that samples treated above and below the threshold of irreversible electroporation were separately observed. However, to boost the discrimination between the 4 classes a supervised chemometric tool based on sPLSDA-LDA was used.

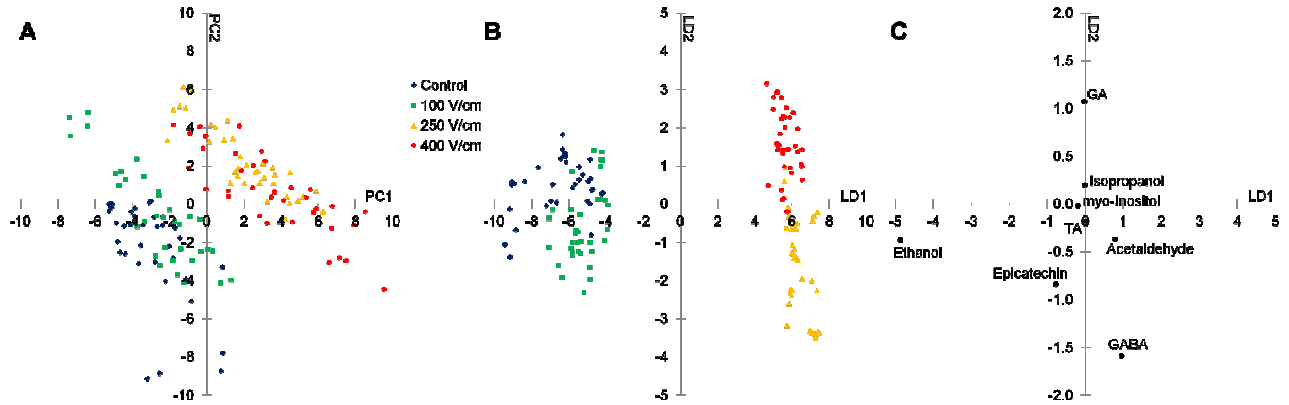

Fig. 3. PCA scores of the first two components (A), sPLSDA-LDA scores (B) and loadings (C) of the first two latent variables; GA = Glutamic acid, GABA = γ-Aminobutyric acid .Further details are reported in Material and methods section.

The two steps data process was tailored to both enhance the separation of the different treatments and, simultaneously, reduce the complexity of the model by selecting and sorting the metabolites by importance. Firstly, the iterative sPLSDA step analysis resulted in parsimonious selection of 8 important metabolites which showed average V.I.P. values higher than 1 in more than 70 % out of one thousand repetitions. Secondly, a LDA model was built, based on the selected metabolites, so that an improved discrimination of the four classes was achieved. **Fig. 3b-c** illustrate score and loading plots while **Table 3** shows the confusion matrix arisen from the chemometric analysis. LD1 accounted for the 93.37 % of the variance and led to clearly discriminate samples permanently electroporated from the others which, in agreement with PCA results, gave rise to highest differences in the metabolic profiles. LD2 and LD3 aided the fine tuning of the modifications of the profiles between 250-400 V/cm and Control-100 V/cm, respectively. In addition, the confusion matrix (Table 3) describes the correct assignment of the 95 % of the cases. Interestingly, the remaining 5 % of the samples were incorrectly assigned by the model only between Control-100 V/cm and 250-400 V/cm.

Table 3. Confusion matrix of 4-class LDA

	Control	100 V/cm	250 V/cm	400 V/cm
Control	0.231	0.006	0.000	0.000
100 V/cm	0.019	0.244	0.000	0.000
250 V/cm	0.000	0.000	0.231	0.006
400 V/cm	0.000	0.000	0.019	0.244

Being intrinsically quantitative and high reproducible HR-NMR analysis allowed the accurate estimation of the concentrations of the 8 metabolites which are illustrated in **Fig. 4**. Interestingly,

the concentrations of ethanol, acetaldehyde and isopropanol were significantly affected by the application of the different electric fields confirming that anaerobic fermentative metabolisms took place (Cortellino et al., 2015).

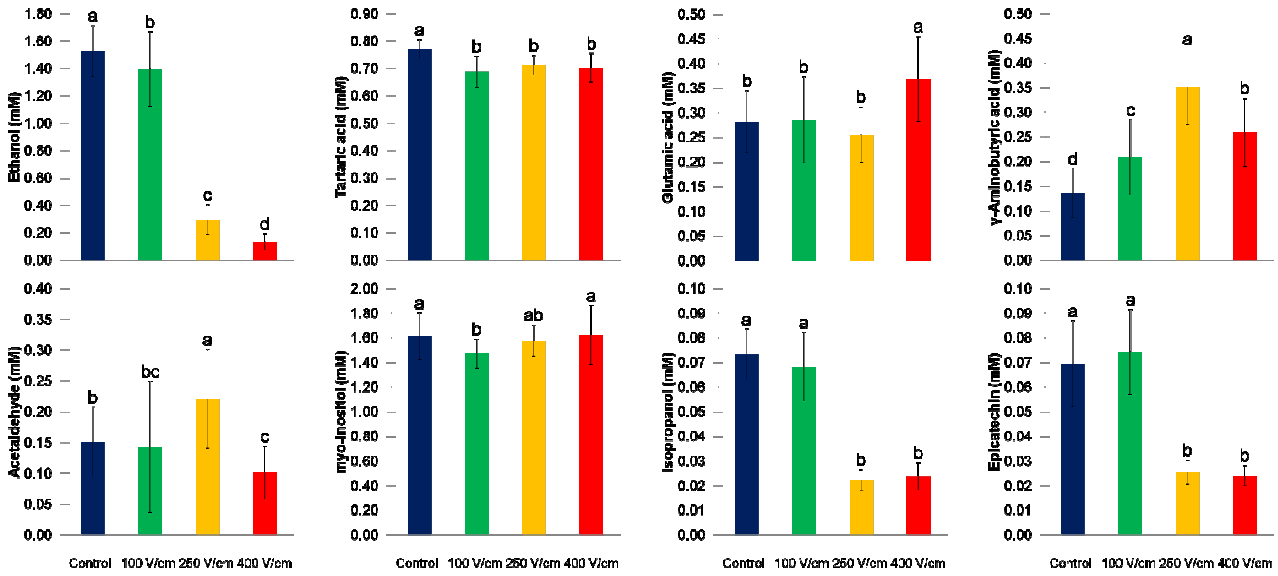

Fig. 4. Concentrations of the important metabolites as arisen from metabolomic analysis. Values are means ± standard deviations (n=36) and differences between means with the same letter are not significant at $p<0.05$.

Both ethanol and isopropanol contents were lowered by the PEF treatments especially when the threshold of the irreversible electroporation was exceeded. The high alcohol levels detected could be a consequence of microbial metabolism (Barth et al., 2009), but the reduction observed at the highest PEF treatment adopted confirm their endogenous generation, as a consequence of apple tissue metabolism. Actually, according to Heinz et al (2001), threshold value for the onset of microbial inactivation is about 5 kV/cm, extremely higher than the one applied in this study. In this direction, according to the calorimetric and respiration results, the loss of the cell viability was the most probable cause.

Moreover, the different concentrations of acetaldehyde was able to discriminate samples treated at 250 V/cm from those subjected to 400 V/cm. however, it is worth noting that acetaldehyde has been reported to be produced in small amount, in particular, during the first day of storage after fresh tissue cutting and remains constant afterwards (Soliva-Fortuny, Ricart-Coll, & Martín-Belloso, 2005). Due to the high volatility of this metabolite it was usually found in the package headspace (Cortellino et al., 2015) and this can explained the high variability of the data collected, in the present work, by the analysis of the metabolome.

Beside anaerobic respiration, epicatechin showed a similar trend to the one evidenced for the two alcohols. In fact, PEF treatment above the irreversibility threshold lowered the amount of

epicatechin without a linear correlation with the applied electric field. In addition, tartaric acid was diminished by the application of PEF at every field strength. Both the metabolites could be affected by the oxidative stress induced immediately after the formation of pores (Teissie et al., 1999). Indeed, epicatechin, as well as other phenolic compounds of plant tissues, is a source of active antioxidants which are easily oxidized by the technological processes (Berregi, Santos, del Campo, & Miranda, 2003). On the other hand, the pathway that leads to the biosynthesis of tartaric acid involves the degradation of ascorbic acid to threonic acid and, subsequently, to tartaric acid (Saito, Morita, Kasai , 1984). Similarly to the present work, this pathway was also subjected to PEF-specific response in potato tissue as observed by Galindo et al. (2009).

Another metabolic pathway previously described in potato tissue is the one which involves the alteration of the Krebs cycle. Galindo et al (2009) stated that glutamic acid was affected by the application of PEF, within few hours after treatment, in a similar way to the wounding response. In the present work, two metabolites, i.e. glutamic acid and γ-Aminobutyric acid (GABA), showed an accordant behaviour. Shelp et al (2012) demonstrated that the production of γ-Aminobutyric acid in plant tissue, including apples, was the results of the abiotic stress. In addition, the alteration of the Krebs cycle might also accounted for the lower heat and CO_2 productions.

To the best of our knowledge, hitherto the PEF-induced stress of vegetable tissues has been mainly described as short-term response. Indeed, previous works demonstrated that the generation of reactive oxygen species (ROS) occurs within seconds after the application of electric fields (Teissie et al., 1999; Ye, Huang, Chen, & Zhong, 2004). However, the effect of electroporation on plant tissue can last for hours or days due to the recovery processes, for instance the resealing of pores (Teissié, Escoffre, Rols, & Golzio, 2008; Vorobiev & Lebovka, 2009). In the present work, both short and long term effects were observable, nevertheless, in agreement with Galindo et al. (2009), the latter predominantly affected the metabolic profiles of fresh-cut vegetable products. The combination of data acquired by a multi-analytical approach allowed to clarify the PEF-induced stress responses and hypothesize which metabolic pathways were triggered in accordance with the applied electric field.

4. Conclusions

The metabolic stress response induced by pulsed electric fields was strongly related to the electric field strength. Therefore, the accurate control of the process parameters is fundamental for the feasible application of PEF in fresh-cut products since irreversible damages of the membranes led to a severe loss of the cell viability with likely undesirable effects on the shelf life. Conversely, by applying electric field strength below the threshold of irreversibility, merely slight effects on

metabolic profiles of fresh-cut apple tissue were noticed, promoting the employment of PEF at those conditions.

The multianalytical approach based on calorimetry, gas analysis and NMR-based metabolomics led to clarify important metabolic aspects of apples. Indeed, different PEF-induced metabolic pathways were revealed by analysing tens of metabolites simultaneously through a non-targeted approach. The measurement of heat production adequately described both reversible and irreversible treatments, encouraging the use of this method for the assessment of PEF treatments in the fresh-cut sector.

Nonetheless, only the combination of complementary techniques based on different physical principles resulted in a clear and comprehensive picture of the effects of pulsed electric fields on the metabolic response.

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