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Application of pulsed light to patulin reduction in McIlvaine buffer and apple products

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A B S T R A C T

Numerous studies around the world have reported the occurrence of patulin in commercialized apple products. The persistence of this mycotoxin in apple products indicates that current methods used to reduce it during the manufacturing process are not entirely successful and reflects the need to evaluate new detoxification methods. The purpose of the present study was to investigate the effect of pulsed light (PL) dose on patulin degradation in McIlvaine buffer, apple juice and apple purée. The exposure of all samples to PL doses between 2.4 and 35.8 J/cm^2 resulted in a significant decrease in patulin levels. Patulin reduction in McIlvaine buffer did not depend markedly on the initial concentration of the mycotoxin. At the maximum dose tested, the remaining average patulin level dissolved in McIlvaine buffer was approximately 5–15%, while in apple juice the values declined up to 22%. In apple purée naturally contaminated with 29 μg/kg of patulin, exposure to a PL dose of 12 J/cm^2 provoked a 51% reduction in patulin concentration, while no residual contamination was detected for higher irradiation times.

These results suggested that PL treatment would be a potential alternative method to reduce patulin contamination in apple products. However, further investigations need to be conducted to evaluate toxicological safety of patulin degradation product(s).

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1. Introduction

Patulin (PAT), an unsaturated heterocyclic lactone (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one), is a toxic secondary metabolite produced by a wide range of fungal species belonging to *Penicillium*, *Byssoclamy* (anamorph *Paesilomyces*) and *Aspergillus* genera (Boonzaaijer, Bobeldijk, & Van Osenbruggen, 2005). *Penicillium expansum* is probably the most common pre-harvest and post-harvest contaminant specie in apples, while *Byssoclamy nivea* is the most heat resistant between the known producing species, being a potential productor of patulin in pasteurized fruit juices.

Mutagenic, teratogenic, neurotoxic, inmunotoxic and gastrointestinal effects in rats and rodents have been attributed to patulin (Hopkins, 1993). The International Agency for Research on Cancer (IARC) has classified PAT as category 3. Therefore, juice, jam and purée elaborated with apples contaminated with PAT pose a serious health risk, particularly to children who consume high levels of these products. Due to its toxicity, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA, 1995) have established a provisional maximum tolerable daily intake (PMTDI) for PAT of 0.4 μg/kg body weight/day. Based on this PMTDI, CODEX (2003) has set a maximum PAT concentration of 50 μg/L in single-strength and reconstituted apple juices, while the European Commission (2003) has established a maximum concentration of 50 μg/kg in fruit juices and nectar, reconstituted fruit juices, spirit drinks, cider and other fermented drinks derived from apples or containing apple juice, and a level of 25 μg/kg for solid apple products. In addition, to protect infants and young children, a maximum amount of 10 μg/kg is allowed for PAT in apple-based products intended for these consumers (European Commission, 2006).

Numerous studies around the world have revealed the occurrence of patulin in commercialized apple products, and occasionally, in other fruits such as pears, apricots, peaches and grapes (Baert, Meulenaer, Kamala, Kasase, & Devlieghere, 2006; Barreira,

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Alvito, & Almeida, 2010; Bonerba, Ceci, Conte, & Tantillo, 2010; Boonzaaijer et al., 2005; Marín et al., 2011; Ritieni, 2003). Although in general a low or moderate incidence of patulin at concentrations below the recommended limits was found (De Souza Sant'Ana, Rosenthal, & Rodriguez de Massaguer, 2008), some studies reported higher levels of patulin. Wheeler, Harrison, and Koehler (1987) found concentrations from 244 to 3993 μg PAT/L cider produced in Georgia. Funes and Resnik (2009) reported that 21.6% of 51 solid and semisolid apple and pear products marketed in Argentina were contaminated (range = 12–221 μg/kg, average of positive samples = 61.7 μg/kg), but the highest levels were found in apple purée with 50% contaminated samples (average of positive samples = 123 μg/kg).

The presence of patulin in commercial apple products indicates that to a certain extent this mycotoxin is stable to the steps of the manufacturing process. This fact is not unexpected in the case of thermally preserved products since patulin is highly heat resistant at low pH and the standard pasteurization process for apple juice and cider (71.1 °C for 6 s) fails to eliminate an appreciable amount of this mycotoxin (Dong et al., 2010; Lovett & Peeler, 1973). During apple processing, methods and techniques that can contribute to reduce PAT levels include removal of the decayed portions from the raw fruit, washing, pulping, filtration through activated carbon, depectinization, apple juice fermentation, addition of sulphur dioxide, fortification with ascorbic acid and ascorbate, thermal treatment and UV-C radiation (De Souza Sant'Ana et al., 2008; Dong et al., 2010; Janotová, Cízková, Pivonka, & Voldrich, 2011; Stinson, Osman, Huhtanen, & Bills, 1978). However, these methods cannot completely degrade or remove the mycotoxin and can alter the quality attributes of the final product. Therefore, there is a need to develop new detoxification processes to reduce PAT levels in apple products.

Pulsed light (PL) is a "non-thermal" preservation technique that is being studied as a feasible alternative to conventional processes. It involves the use of intense and short-duration (1 μs–0.1 s) pulses of broad spectrum light of wavelength ranging from UV to near-infrared (200–1100 nm) (Gómez-López, Ragaert, Debevere, & Devlieghere, 2007). The high-energy pulses would cause changes in DNA, proteins, membranes and other cellular components of microorganisms. Its use has been approved by the FDA for the decontamination of food and food surfaces (FDA, 1996). Various studies have demonstrated the positive effect of pulsed light on the reduction of microorganisms in many foods (Gómez-López, Devlieghere, Bonduelle, & Debevere, 2005; Jun, Irudayaraj, Demirci, & Gêiser, 2003; Lagunas-Solar, Piña, McDonald, & Bolkan, 2006; Sauer & Moraru, 2009). Recently, Moreau et al. (2011) studied the potential application of pulsed light for the degradation of mycotoxins, such as zearalenone, deoxynivalenol, aflatoxin B1 and ochratoxin in solution. They found that the application of few flashes allowed a decrease in the toxicity of evaluated mycotoxins and that PL treatment of aflatoxin B1 can completely eliminate the mutagenic potential of this mycotoxin. Besides this study, there are not other reports in the scientific literature that evaluate the use of this technology for mycotoxin degradation.

The aim of the present study was to investigate the effect of different PL doses on patulin degradation in artificially contaminated McIlvaine buffer, apple juice and purée and in naturally contaminated apple purée.

2. Materials and methods

2.1. Reagents and chemicals

Acetonitrile (ACN) (HPLC grade), ethanol anhydrous (HPLC grade) (Carlo Erba, Rodano, Italy), glacial acetic acid (A.C.S. grade) and perchloric acid for trace metal analysis were ordered from J.T. Baker (NJ, USA); methanol (MeOH) (HPLC grade) and ethyl acetate (A.C.S. grade) were from Tedia (OH, USA), and nitrogen was purchased from Oxygeno Central SA (C.A.B.A., Argentina). Water (HPLC grade) was obtained from NANOpure Diamond water purification systems (model D11911, Barnstead International, Iowa, USA).

Monohydrate citric acid (analysis grade) was from Merck KgaA (Darmstadt, Germany), anhydrous di-sodium hydrogen phosphate (A.C.S. grade) was from Carlo Erba (Rodano, Italy), and sodium hydroxide (A.C.S. grade) from J.T. Baker (NJ, USA). Pectinase from *Aspergillus niger* (1.47 U/mg) was purchased from Fluka BioChemika (Denmark) and 5-hydroxymethylfurfural (HMF) and PAT standard were ordered from Sigma–Aldrich (St. Louis, MO, USA). PAT standard stock solution was prepared with ethanol anhydrous (1 mg/ml) and stored in a freezer (−18 °C) until be used (less than one week). To calculate PAT concentration, appropriate dilutions with absolute ethanol were done before measuring ultraviolet (UV) absorption (AOAC, 2000). PAT standard working solutions and PAT fortification solutions were prepared by evaporating appropriate portions of the stock solution and diluting with 0.1% (v/v) acetic acid.

2.2. Samples

PAT degradation by PL was analyzed in different substrates: a) McIlvaine buffer (70.2 ml 0.1 M citric acid and 29.8 ml 0.2 M Na_2HPO_4; pH 3.5) artificially contaminated to contain 100 or 500 μg/L of PAT standard; b) store-bought heat-pasteurized apple juice (pH 3.5) artificially contaminated with 129 μg/L of PAT standard; c) apple purée artificially contaminated with 90 μg/kg of PAT standard; and d) store-bought canned apple purée naturally contaminated with PAT (29 μg/kg).

Commercial apple products were purchased locally.

2.3. Pulsed light equipment and dosimetry

PL treatments were performed with an RS-3000B Steripulse-XL system (Xenon Corporation, Woburn, MA, U.S.A.), which produced polychromatic radiation in the 200–1100 nm wavelength range. The system consisted of an RC-747 power/control module, a treatment chamber that houses a xenon flash lamp (non-toxic, mercury free) and an air cooling system attached to the lamp housing to avoid lamp overheating during operation. The system generated high intensity PL at a pulse rate of 3 pulses per second and a pulse width of 360 μs. According to the specifications supplied by the manufacturer, each pulse delivered 1.27 J/cm^2 for an input of 3800 V at 1.9 cm from the quartz window surface of the lamp. The fluence (light dose) was modified by altering the number of applied pulses.

Fluence measurements were taken by a pyroelectric head model ED500 (Gentec Electro-Optics, Québec, Canada) connected to an oscilloscope model TDS 2014 (Tektronix, Beaverton, USA), with an aperture cover of 20.3 cm^2. Measurements were performed in triplicate.

2.4. Pulsed light treatments

Samples to be irradiated were placed on a stainless steel shelf located at 10 cm of vertical distance from the quartz window of the lamp. Variations in radiation dose absorption were minimized by placing the samples within a uniform area of the radiation field (beneath the lamp and around the central point). For irradiation of McIlvaine buffer solution and apple juice, 11 mL of the samples were put in a Petri dish (9 cm in diameter) and irradiated with PL

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during periods of time between 2 and 30 s, being the corresponding doses between 2.4 and 35.8 J/cm^2. On the other hand, samples of apple purée (≅15–16 g) were placed in a Petri dish and irradiated during 10, 20, 60, and 100 s, being the corresponding doses between 11.9 and 119.0 J/cm^2. Treatments were performed in duplicate.

2.5. Temperature measurement

Temperature evolution of samples during irradiation was monitored using a T-type thermocouple connected to a data logger Digi-Sense model 69202-30 (Barnant Company Division, Barrington, USA). Measurements were made in triplicate.

2.6. Patulin extraction and clean-up

Liquid samples (10 ml) were extracted three times with 10 ml of ethyl acetate. Extracts were gathered and evaporated to dryness under N_2. The resulting residue was reconstituted with 300 µl acetic acid (0.1% v/v) and stirred in a minishaker (MS1, IKA, Wilmington, USA) at 2500 rpm until total dissolution.

The extraction of PAT from apple purée samples was performed using the methodology described by Funes and Resnik (2009). A pectinase step before the extraction was used. Briefly, enzyme solution at 5 mg/mL was made with citrate buffer (pH 4.0). The buffer citrate was prepared by adding 55.1 mL of sodium citrate 0.1 M (21.014 g of monohydrated citric acid + 200 ml of NaOH 1 M and the volume was completed to a liter with water) + 44.9 mL of HCl 0.1 M. Eight milliliters of water and 150 µl of the enzyme solution were added to 10 g of apple purée. This volume allowed total dissolution of the apple purée after the enzyme was left to act overnight at room temperature (24–26 °C).

After the pectinase step, 32 ml of acetonitrile were added and the sample was stirred for 2 min with a magnetic stirrer (Precytec modelo AE28). Then 30 mL of the sample were filtered and passed through a Multisep® 228 AflaPat Multifunctional Columns (Romer Labs®, US). Twenty milliliters of the purified extract were reduced to approximately 2 mL in vacuum conditions at 40 °C in a rotary evaporator (Model RE200, Yamato Scientific CO., Japan). This extract was transferred to a test tube with 3 portions of 3 mL of methanol; the solution was completely evaporated to dryness in a nitrogen stream at room temperature and immediately added with 300 µL acetic acid (0.1% v/v). The mixture was shaken in the minishaker until it completely dissolved. This solution was then transferred into an HPLC vial. All samples were prepared by triplicate for HPLC analysis.

2.7. HPLC patulin analysis

A Waters 2695 separation module equipped with a quaternary pump and photodiode array detector (Waters Corporation, Milford, USA) was used. The analytical column was from Thermo Scientific, BDS Hypersil C18 250 × 4.6 mm, 5 µm (UK), with a guard column BDS Hypersil BDS C18 10 × 4.6 mm, 5 µm (UK). The solvents used were 0.01% (v/v) perchloric acid (solvent A) and acetonitrile (solvent B). The gradient started with a concentration of 95–5% A–B until 4 min, from 4.5 min to 18 min linear gradient until 98-2 A/B, from 18 min back to initial concentration. To adjust the gradient, PAT standard was injected with HMF (2 mg/L, working standard made with water HPLC grade). The UV detection was performed at 275 nm. All injections were made by triplicate.

A calibration curve using external PAT standards was performed to determine the amounts of PAT in the samples. Measurements were made in triplicate using independent solutions. Eleven calibration standards were used in the range of 0.12–112.73 ng ($R^2 = 0.9993$).

Recoveries studies were determined at three contamination levels (10, 50 and 140 µg/kg of PAT), each level by triplicate. For liquid samples the recoveries were higher than 95% at different speaking levels (Relative standard deviation %, RSD % <7). The recovery values for apple purée at 10 and 50 µg/kg levels were 74% (RSD % <8.4), and at 140 µg/kg was 105 (RSD % <5) .The LOD and LOQ values were calculated considering a signal-to-noise ratio of 3:1 and 5:1, respectively. LOD was 3 µg/kg for liquid samples and 3.8 µg/kg for apple purée, while LOQ was 5 µg/kg for liquid samples and 6.3 µg/kg for apple purée.

PAT in positives samples was confirmed through acquiring the spectra of the eluting compounds (200–400 nm). The UV spectrum was compared to that of the external PAT standard solution analyzed under the same conditions.

A chromatogram of apple purée treated with PL during 10 s is illustrated in Fig. 1; it clearly shows the separation of pure HMF and PAT.

2.8. Patulin dose–response curves

PAT degradation data were fitted with an empirical dose–response type model (De Lean, Munson, & Rodbard, 1978) expressed as:

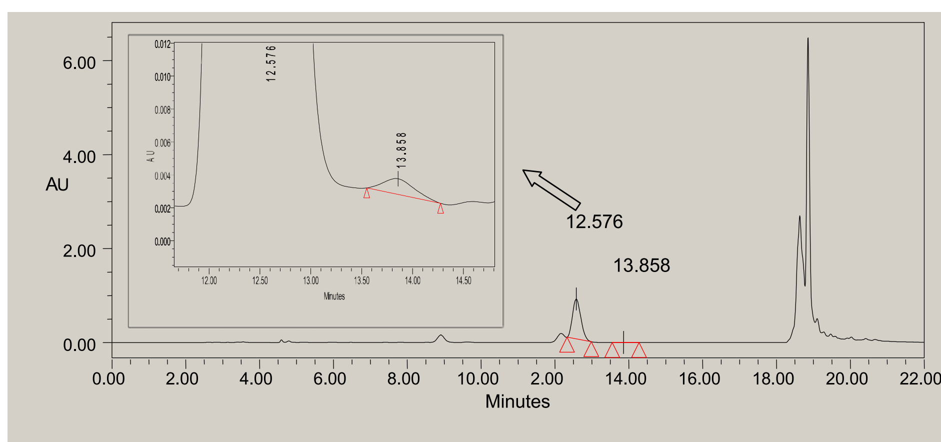

Fig. 1. Chromatogram for apple purée exposed 10 s to pulsed light. Retention time: 2.576 min for HMF and 13.858 min for PAT.

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$$y = (A_1 - A_2)/\left[1 + (x/x_0)^P\right] + A_2 \quad (1)$$

where y is the response (C/C_0%, where C is the PAT concentration at time t and C_0 the initial PAT concentration); x, the PL exposure time; A_1, the response when $x = 0$; A_2, the response for "infinite" dose; x_0, the dose resulting in a response halfway between A_1 and A_2; and P is a "slope factor" that determines the steepness of the curve. To analyze all the degradation curves simultaneously, and obtain reasonable estimates of the parameters, a constrained curve fitting was used by setting the parameter A_1 as 100.

A general nonlinear, least-squares curve-fitting routine using Marquardt algorithm was employed. Nonlinear regression analysis and statistical analyses were carried out using STATGRAPHICS PLUS for Windows 3.0®Package (Statistical Graphics, Washington, USA). Significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Patulin degradation in McIlvaine buffer and in apple juice

The effect of PL dose on the reduction of PAT in McIlvaine buffer and in apple juice, both artificially contaminated, is shown in Fig. 2. The initial PAT levels (100 or 500 μg/L) in the buffer solution declined with increasing irradiation time. As can be observed, the percentage of PAT degradation at different PL doses did not appear to depend markedly on the initial mycotoxin concentration. The remaining PAT level after 30 s of irradiation (fluence: 35.8 J/cm^2) was about 5–15%. PAT level in apple juice added with 129 μg/L of the mycotoxin also decreased with increasing PL dose, but the reduction pattern was different from that observed in McIlvaine buffer. In the low dose-region, the degradation in apple juice was more pronounced than in buffer solution. For instance, after 11 s irradiation (13.1 J/cm^2), the remaining PAT in apple juice was approximately 46%, while for the same fluence the retention of the mycotoxin dissolved in McIlvaine buffer was about 72–76%. On contrast, for long treatment times, the reduction achieved in the juice was slightly lower than in buffer solution. At the highest dose tested (35.8 J/cm^2), residual PAT level in apple juice was slightly greater than in buffer ($\approx$22% vs 5–15%).

The kinetics of PAT degradation in McIlvaine buffer and in apple juice versus PL exposure was nonlinear. Fig. 2 shows the fitting of experimental degradation data using the dose–response type model described by Eq. (1). Since confidence intervals for A_2 covered 0 in all curves, this parameter was eliminated without affecting substantially the adjustment. Table 1 displays the estimated parameters and the corresponding specific statistics. The two-parameter phenomenological equation seemed to appropriately fit the dose–response curves. The confidence limits of parameter estimates in nonlinear regression indicated the curves significantly differed between media, buffer and juice, with PAT showing more degradation at lower doses in apple juice than in the buffer. Then, after a period of important decrease in the concentration, PAT level diminished and clearly tended to a plateau. In buffer solution, the reverse was observed: the degradation was low at low doses, raised as doses increased and then slightly declined and would approach to a plateau when doses became higher. On contrary, comparison of dose–response curves in McIlvaine buffer indicated the same response for both PAT levels assayed.

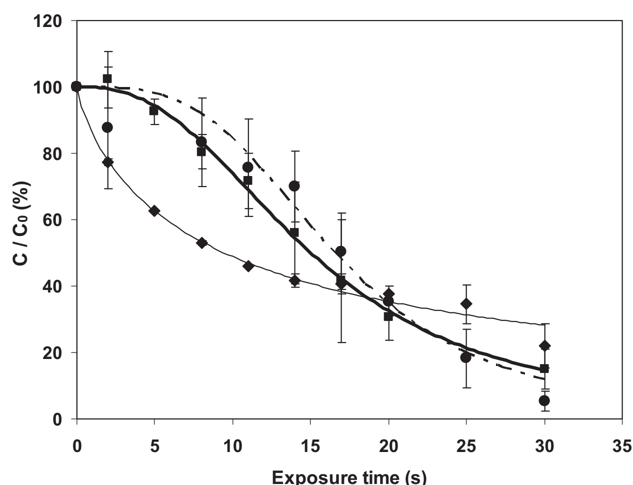

Fig. 2. Dose–response curves of PAT exposed to pulsed light, fitted with Eq. (1) as a model. McIlvaine buffer, 100 μg/L PAT (●: experimental; –––: predicted) and 500 μg/L PAT (■: experimental; ▬: predicted). Apple juice, 129 μg/L PAT (◆: experimental; –: predicted).

3.2. Patulin degradation in apple purées

An apple purée naturally contaminated with 29 μg/kg of PAT and an apple purée artificially contaminated with 90 μg/kg of PAT standard were selected for this study. Exposure of apple purée with 29 μg/kg of PAT to PL during 10 s (11.9 J/cm^2) provoked a 51% reduction in PAT levels (14 μg/kg). This decrease was very similar to that occurred in apple juice after the same PL dose (Fig. 2). For tested irradiation times greater than 10 s, no residual PAT contamination was detected. A similar response was found in the apple purée artificially contaminated: after 20 s of PL treatment, the levels of PAT were below the detection limit of the method.

3.3. Discussion

Photochemical damage to a compound structure can be induced by irradiation with photons having energy levels corresponding to the bond energies of biomolecular chemical bonds. Many aromatic and heterocyclic carcinogenic chemicals, including mycotoxins, have been reported to be UV radiation sensitive. Dong et al. (2010) evaluated continuous UV-C radiation (254 nm) as a possible alternative for PAT reduction in fresh apple cider. They reported a linear decrease (by 9.4–43.4%) in PAT levels when applying doses from 14.2 to 99.4 mJ/cm^2 in a CiderSure 3500 system. Yousef and Marth (1985) found that low energy UV treatment caused aflatoxin M_1 to degrade 42, 65 and 100% after 10, 30 and 60 min of exposure respectively in artificially contaminated milk.

It was reported that temperature does increase measurably during PL treatments as a consequence of the absorption of light by the samples (Gómez-López, Ragaert, Debevere, & Devlieghere, 2007; Gómez, Salvatori, García Loredo, & Alzamora, 2012; Jun et al., 2003; Ozer & Demirci, 2006) and so it would be reasonable

Table 1
Dose–response model (Eq. (1)) parameters for pulsed light degradation of patulin in McIlvaine buffer and in apple juice.

Sample	Estimated parameters[a]		R^2_{adj}	F-value	p-value
	x_0 (s)	P			
Patulin dissolved in buffer (100 μg/L)	16.5 ± 1.7	3.3 ± 1.2	0.9560	410.1	1.8E^{-7}
Patulin dissolved in buffer (500 μg/L)	15.1 ± 0.6	2.6 ± 0.3	0.9946	4853.7	3.3E^{-11}
Patulin dissolved in apple juice (129 μg/L)	9.5 ± 1.2	0.8 ± 0.1	0.9854	2038.5	6.4E^{-11}

[a] Mean values ± confidence interval.

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to suppose that heat may play a role in toxin degradation. Temperature evolution in apple juice and purée during irradiation is shown in Fig. 3. In PL treated purée samples, the temperature reached 30 and 40 °C after being exposed to irradiation during 11 s and 30 s, respectively. In apple juice, the heat build-up was lower and for the same treatment times mentioned above the temperature only increased to 26 and 30 °C. Accordingly, it is unlikely that toxin reduction were caused by a thermal mechanism. In addition, PAT is heat resistant in acidic conditions. Lovett and Peeler (1973) demonstrated PAT to be resistant to thermal destruction at a pH range of 3.5–5.5 when heated up to 125 °C. For instance, they reported a decimal reduction time value equal to 29 h when PAT was heated at 105 °C in an aqueous solution with pH 4.5.

Present results suggested a significant role of the matrix in the sensitivity of PAT to PL treatment. Further studies are needed to clarify these trends. However, it has been reported that the presence of certain components in foods limit the amount of PAT. Brackett and Marth (1979) found that vitamin C could caused disappearance of PAT from a buffer solution and apple juice. When apple juice containing 300 μg PAT/L was fortified with 5.0% ascorbic acid and then held at 4 °C, less than 40% of the toxin was recovered after 8 days of storage. In a more recent investigation, Drusch, Kopka, and Kaeding (2007) verified that, at acidic pH, the presence of ascorbic acid reduced the stability of PAT in an aqueous juice-like model system. They reported a 30% reduction of PAT in the presence of ascorbic acid compared to 68% in samples without ascorbic acid after 34 days. Their data indicated that PAT was decomposed by free radicals generated either from metal ion catalysed oxidation of ascorbic acid to dehydroascorbic in the presence of oxygen, or from metal chelate-catalysed oxidation of ascorbic acid (reaction not dependent on the presence of oxygen). After complete oxidation of ascorbic acid, PAT degradation levelled off; thus initial concentration of ascorbic acid and its rate of degradation would be important factors to be considered in the decontamination process. So the greater effectiveness of PL for PAT decontamination in apple products could be due, at least, to the coadjutant effect of ascorbic acid on PAT decomposition by PL radiation.

The findings of this study demonstrate that PL would be an alternative strategy for PAT detoxification in apple products. However, further investigations need to be conducted to assess the safety of degradation product(s) of PAT and the effect of matrix composition on degradation rate. Moreover, the effect of PL treatment on quality attributes of apple juice and purée should be also evaluated. In previous studies, Palgan et al. (2011) examined the effect of PL on chemical, physical and sensory aspects of quality in apple juice. They found no significant changes in pH, °Brix, color, total phenol content, total antioxidant capacity, sweetness, acidity and odour in apple juice treated up to a PL dose of 14 J/cm^2. However, for higher PL doses a negative impact on flavour and antioxidant capacity was detected. Therefore, the selection of an optimal PL dose taking into account degradation and quality aspects would be important to achieve adequate reductions of PAT without compromising the quality parameters of apple products.

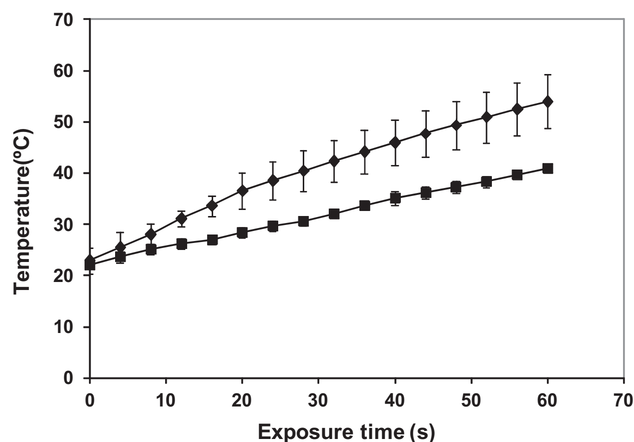

Fig. 3. Temperature evolution in apple juice (■) and apple purée (◆) during PL exposure.

4. Conclusions

PAT levels in McIlvaine buffer, apple juice and apple purée were reduced by PL exposure, and the magnitude of the degradation depended on the PL dose and the matrix evaluated. These experiments, intended to provide a first insight into the effect of PL irradiation on PAT stability, indicated that this treatment would be promising and could be implemented at industrial scale to help in reducing PAT contamination in apple products. However, disappearance of PAT does not necessarily eliminate the potential health hazard posed by its initial presence and further investigations need to be conducted to evaluate mechanisms of degradation, toxicity of degradation product(s), and effects of the degradation reactions on nutritional value of apple products.

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