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Formation of a bioactive cyclopentenone and its adducts with amino acids in sterilized-fruits and - vegetables baby foods

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ABSTRACT

The formation of the molecule 4,5-dihydroxy-2-cyclopenten-1-one (DHCP) from the thermal treatment of pectin-containing foods was investigated in small-scale laboratory preparation of sterilized vegetable puree (carrot, zucchini and tomato) and fruit puree (peach and mixture of pear and apple) and in commercial baby foods. DHCP attracts attention due to its cytotoxicity as well as potential antiviral and anti-inflammatory effects. However, its effects and the difficulty of its identification in food are mediated in part by the formation of Michael adducts of DHCP with amino acids. The results revealed that DHCP reacted efficiently with cysteine and glutathione, and to a lesser extent with histidine. Mass spectrometry analysis confirmed the formation of adducts of DHCP with amino acids in a model system, being in a real food system difficult to investigate. However, these formed adducts are of potential interest, although it is not known whether they are safe, bioactive or reversible.

1. Introduction

The direct consequences derived from the application of thermal treatment on foods, in addition to ensuring the safety and extend shelf-life, are those caused by the physical or chemical changes that make the products more pleasant. However, the reactions produced by high temperatures can generate compounds that not only influence the aroma, flavor and color, but can promote certain beneficial or toxic biological activities (Koszucka & Nowak, 2019; Palermo, Pellegrini, & Fogliano, 2014). Thermal processing at high temperature, including the cooking and sterilization of fruits and vegetables, plays an important role in dictating the magnitude of the health effect. Heating induces reactions that lead to the appearance of browning products by Maillard reactions, including products like hydroxymethylfurfural, melanoidins, or acrylamide, among others, which have both positive (antioxidant, bactericidal, antiallergic) and negative (pro-oxidant, carcinogens) impacts on health (Tamanna & Mahmood, 2015). Also, the pectins are depolymerized by chain slitting *via* β-elimination and acid hydrolysis, which leads to pectin fragmentation and solubilization and then destroys galacturonan chains by degradation (Diaz, Anthon, & Barret, 2007). D-Galacturonic acid, the main monomer of the pectin molecule, is a highly reactive compound in nonenzymatic browning. The decarboxylation of D-galacturonic acid leads to the formation of 4,5-unsaturated 4-deoxy-L-arabinose followed by dehydration and cyclization to yield 4,5-dihydroxy-2-cyclopenten-1-one (DHCP), among others, which can form a colored polymer resin (Bornik & Kroh, 2013).

DHCP was identified as a molecule present in the active fraction of citrus pectin modified by heat treatment, showing a high cytotoxicity activity on several cancer cell lines (Leclere et al., 2016; Koyama et al., 2000). This molecule would partly explain the antiproliferative effect of heat-treated citrus pectin (HTCP) on human cancer cells such as breast, cervix, colorectal and liver, which decreases the growth of tumors in rat models (Hao, Yuan, Cheng, Xue, Zhang, Zhou, & Tai, 2013), and induces apoptosis in prostate cancer cells (Jackson, et al., 2007) and in colon cancer cells HL-60 (Koyama et al., 2000). Also, it has been proven that ginseng pectin, modified by high temperatures, exhibits remarkable antiproliferative effects on HT-29 cells of human colon cancer, compared to non-modified pectin (Cheng, et al., 2011). In addition, Guan, Zhang, Yu, Yan, Zhou, Cheng, and Tai (2018) demonstrated that the inhibitory effect on CT-26 colon cancer cells implanted in mice was due to the DHCP molecule obtained from heat-treated *Helianthus annus L.* pectin. More recently citrus-derived DHCP has been described to induce cell death in colon cancer cells *via* the induction of mitochondrial ROS (Chen, Hao, Yan, Sun, Tai, Cheng, & Zhou, 2021).

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DHCP is a cyclopentenone ring with an α,β-unsaturated carbonyl group in its structure, being therefore a potent electrophile, which allows it to potentially react with nucleophilic residues –SH or $–NH_2$ of the different cellular proteins by Michael addition to form covalent adducts (Koyama et al., 2000). In fact, the thiolate groups present in cysteine and glutathione have high reactivity, while histidine and lysine present less nucleophilic capacity (Vasiĺev, Tzeng, Huang, & Maier, 2014). It should be noted that the mechanism of action of DHCP should be very similar to that of endogenous prostaglandin (15-$dPGJ_2$ and PGA_1), with a cyclopentenone ring in its structure, which exerts its effect *in vivo*. These cyclopentenone prostaglandin (cyPGs), with an α,β-unsaturated carbonyl group in their molecule, exhibit anti-inflammatory, antitumor and antiviral properties by activating the peroxisome proliferator-activated gamma receptors (PPARγ) (Oeste & Perez-Sala, 2014) or through covalent adducts with cellular thiol groups of protein and nucleic acids which alter their functions (Burstein, 2020; Grau, Iñiguez, & Fresno, 2004).

Pectin is present in all fruits and vegetables. Most plant foods are consumed only after mechanical and/or thermal treatment. In this work, the presence of DHCP in baby food samples was evaluated as a model system of heat-treated processed food. In industrial samples the treatment is slightly different with blending for fruits, and cooking followed by blending for vegetables; a subsequent autoclaving step on the puree in both cases may influence the food through a pronounced degradation of the pectin polysaccharide and consequently the different DHCP contents in these food samples. The mechanical disruption of parenchyma-rich plant tissues results in a combination of liquid-phase-containing pectic material and a dispersed phase formed of all the plant insoluble solids, such as cell wall, skin and seeds (Lopez-Sanchez, Nijsse, Blonk, Bialek, Schumm, & Langton, 2011). Moreover, the cooking and autoclaving of fruit and vegetable puree leads to an important pectin solubilization from the middle lamella of the cell wall to the liquid phase, which is attributed to the β-elimination depolymerization of pectin at the high temperatures reached during these processes (Christiaens, Mbong, Van Buggenhout, David, Hofkens, Van Loey, Hendrickx, 2012).

The objective of the present investigation was therefore to identify the presence of the DHCP molecule in in small-scale laboratory preparations of sterilized vegetable and fruit purees. Due to its high reactivity, it is possible that a Michael covalent adduct with free amino acids or protein nucleophiles is formed from the food itself during the technological process. To find the presence of these adducts, which are not known to be reversible under certain conditions, or even whether or not the DHCP molecule or its adduct could be possess a beneficial effect or be a potential health risk, was another challenge. To study the reactivity of DHCP with three amino acids (i.e., cysteine, histidine and lysine) and glutathione, a tripeptide containing a –SH residue, under three temperatures and two pH values, and to characterize the formed adduct by mass spectroscopy, were additional objectives. Finally, the presence of DHCP in commercial sterilized baby food samples was evaluated. At present, there is no study on the occurrence of DHCP in small-scale laboratory preparations of autoclaved vegetable and fruit puree (baby food). A total of 20 commercially baby food samples were analyzed, including fruits and vegetables in jars.

2. Materials and methods

2.1. Chemical

Galacturonic acid, protease, cysteine, histidine, lysine and glutathione amino acid were purchased from Sigma-Aldrich (St Louis, MO).

2.2. Fruit and vegetable collection

Carrot, zucchini, tomato, pear, apple and peach were purchased from a local market in Seville, Spain. Fruits were stored at 4 °C before use. All fruits and vegetables were homogenous in size, color, and appearance, without signs of mechanical damage or fungal infection.

2.3. Small-scale laboratory preparation of sterilized vegetable and fruit puree

Fruit (peach and a mixture of 1:1 pear:apple) and vegetable (carrot, zucchini and tomato) were prepared in the form of puree to simulate the industrial preparation of baby food. Briefly, all fruits were peeled (except for tomato) and cut into 2–3 pieces. The pieces were directly ground in a domestic blender. However, for the vegetables (except for tomato), they were peeled and cut and then boiled in water until fully cooked, and then directly ground in a domestic blender. After that, about 100 g of fruit or vegetables were added in triplicate to Pyrex® bottles. All samples were heat-treated for 20, 30 and 60 min at 121 °C in an autoclave in the laboratory. Aliquots of 2 mL were centrifuged at 13,000 *g* for 5 min (centrifuge Eppendorf™ Minispin™, Germany). The supernatant was collected and filtered through a 0.45 μm membrane and analyzed by HPLC (conditions in *Section 2.7*)

2.4. Detection of DHCP in sterilized baby food

All samples were treated as described in the previous section. However, prior to heat treatment, the samples (100 g of fruit or vegetable puree) were washed with abundant distilled water. They were deposited in a nylon cloth and rinsed with about 3 L of distilled water. The resulting solid was rehydrated with water until reaching its initial weight (100 g). Other batches of sample were not washed. In another experiment, a known amount of DCHP was added to the resulting solid and liquid, washed and unwashed samples. In addition, samples (100 g of fruit or vegetable puree) were treated with and without protease. To carry out these experiments, the pH was changed to 7.5 in all samples to which protease was added (5 mg protease / 100 mg fruit or vegetable). The samples were shaken in a thermostated bath at 50 °C for 30 min. Then all the samples with and without protease were washed with abundant distilled water. The resulting solid was rehydrated with water until reaching its initial weight (100 g). After that, all samples were heat-treated at 121 °C for 20, 30 and 60 min. The samples were centrifuged at 13,000 *g* for 5 min (centrifuge Eppendorf™ Minispin™, Germany). The supernatant was collected and filtered through a 0.45 μm membrane and analyzed by HPLC (*Section 2.7*). The process is summarized in Fig. 2

2.5. Identification in commercial baby food

A total of 20 commercial baby food samples were collected randomly from local supermarkets in Seville, Spain. These puree samples were fruit, vegetable, or mixtures of fruits or mixtures of vegetables. Aliquots of 1.0–1.5 g of sample were centrifuged at 13,000 *g* for 10 min (centrifuge Eppendorf™ Minispin™, Germany). The supernatant was collected and filtered through a 0.45 μm membrane and analyzed by HPLC (*Section 2.7*).

2.6. Obtaining purified DHCP

DHCP was produced from heat-treated galacturonic acid and further purified for obtaining a standard. Briefly, 2.5 g of galacturonic acid in 200 mL of H_2O were heat-treated at 121° C for 4 h in an autoclave. The solution was concentrated to dryness in a rotary evaporator; 50 mL of chloroform were added to the dry extract, and it was kept for 20 min in an ultrasonic bath. Then, the chloroform extract was dried under vacuum and re-dissolved in 5 mL of water. The extract was then passed through a HiTrap® Q HP, prepacked 5-mL resin, ready-to-use Q Sepharose high-performance strong anion exchange column purchased from Sigma-Aldrich (St Louis, MO); the column was equilibrated with 25 mL of H_2O at a flow rate of 1 mL/min. The sample (5 mL) was injected and eluted with another 25 mL of water. Fractions were collected every 5 mL and then the column was washed with 25 mL of 0.1 M acetic acid

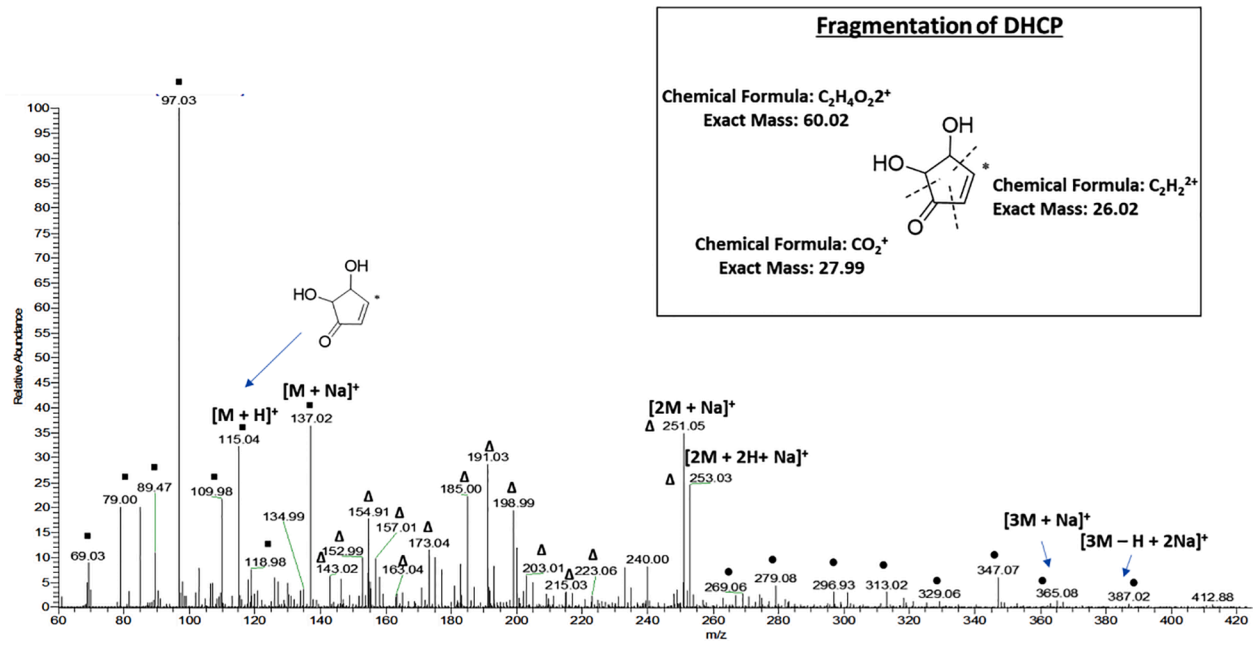

Fig. 1. Mass spectrum of isolated and purified 4,5-dihydroxy-2-cyclopenten-1-one (DHCP) from heat-treated galacturonic acid, identified as $[M + H]^+$ and $[M + Na]^+$ ions and fragment ions formed (■), $[2\ M + Na]^+$ ion and fragment ions (Δ), and $[3\ M + Na]^+$ ion and fragment ions (•). Fragmentation pattern proposed for DHCP.

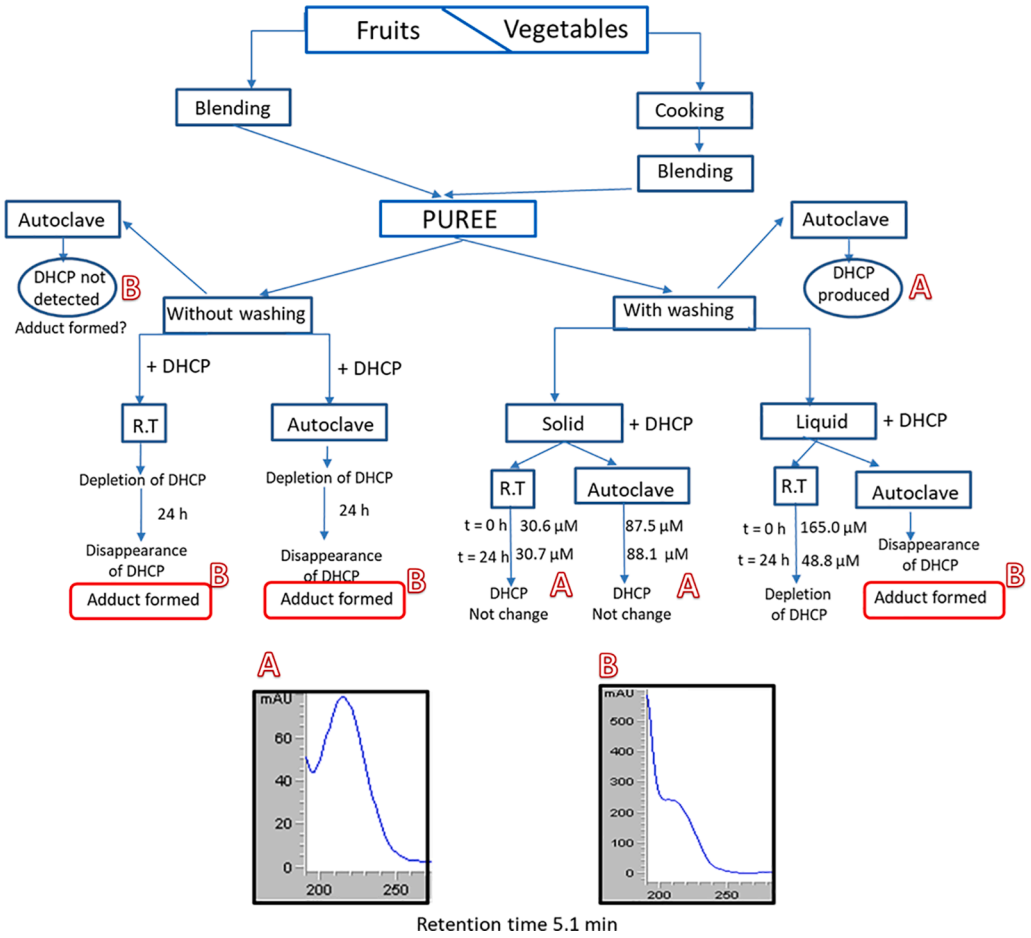

Fig. 2. Summary diagram of the process followed in the investigation of the presence of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP) in sterilized baby food. RT: room temperature.

and finally with another 25 mL of water. This process was repeated three times. The entire purification process of DHCP was monitored by the HPLC method. The detection of DHCP, unreacted galacturonic acid and other reaction products from each fraction were conducted at wavelengths of 210, 254, 280, and 340 nm. The DHCP was contained in Fraction 1 and the purity of DHCP was confirmed by mass spectrometric analysis (Fig. 1)

2.7. Quantification of DHCP by HPLC

DHCP was quantified using a Hewlett-Packard 1100 liquid chromatography system with a C18 column (Tracer Extrasil ODS-2; 250 mm × 4.6 mm i.d., 5 μm; Teknokroma Analítica S.A., Barcelona, Spain) and equipped with a diode array detector (DAD). The mobile phase was 0.01% trifluoroacetic acid in water isocratic at a flow rate of 0.6 mL/min with a total run time of 30 min. Fraction 1 from HiTrapQ column collected from the three-time purification process was used for preparing aqueous solutions of the calibration curve. Previously, the fractions were concentrated to dryness under vacuum and weighed, then dissolved in water to make a concentration of 0.4 mg/mL. The quantification of DHCP was carried out through the integration of peaks at 210 nm. The linearity of the standard curve was expressed in terms of the determination coefficient plots of the integrated peak area against the concentration of the same standard, with the first concentration of the standard curve of 0.08 mg/mL. These equations were obtained over a wide concentration range in accordance with the levels of these compounds in the samples. Samples containing higher concentration than the highest concentration in the calibration curve were diluted to ensure that the analysis was carried out over the calibrated concentration range. The system was linear in all cases ($r > 0.99$). Three replicates on the same day were carried out.

2.8. Effect of three amino acids and a tripeptide on DHCP decrease in a model reaction system

A model reaction system was prepared in order to determine the changes in the concentration of DHCP with three amino acids (cysteine, histidine, and lysine) and a tripeptide (glutathione). The changes in the concentration of DHCP were followed after 20 min of reaction at 40, 80 °C in a thermostated bath and 120 °C on a heating plate and two-pH values, 4.0 and 7.0 for a molar ratio of nucleophile agent (amino acids or glutathione): DHCP (5:1) (12.5 μM/2.5 μM). Briefly, DHCP was obtained after heat treatment of galacturonic acid at 120 °C for 3 h. Cysteine, histidine, lysine and glutathione were added to DHCP solutions at pH 4 and 7 at the ratios indicated above. For solutions at pH 4, water was used and the pH was adjusted with 0.5 M HCl. For solutions at pH 7, the solution was made with 0.01 M phosphate buffer. These changes were compared with the change of DHCP alone, at these temperatures and pH conditions, named as control. Other DHCP samples at the two pHs without heating were carried out as an initial control. The solutions were filtered through a 0.45 μm membrane and DHCP was quantified by HPLC.

2.9. Determination of adducts formed between DHCP and amino acids (cysteine, histidine, lysine) and the tripeptide (glutathione) by mass spectrometry (MS)

To investigate the effect of three amino acids and a tripeptide on DHCP elimination and the adduct formation, a range of pH values was applied to test their reactivity. Specifically, the reaction mixture in a test tube contained 400 μL of cysteine, methionine, lysine and glutathione and 100 μL of DHCP to a final concentration of 12.5 μM of amino acids and 2.5 μM of DHCP. The pHs were adjusted to 4.0, 5.0, 6.0 and 7.0 using 0.1% HCl (*v/v*) and 0.4% NaOH (*w/v*). The reaction was performed at 80 °C for 20 min. The reaction products were analyzed by mass spectrometry and diluted and filtered through 0.45 μm membrane to determine the amount of DHCP using HPLC. The mass spectra of the precursor (DHCP) and product ion of DHCP-Cysteine, DHCP-Histidine, DHCP-Lysine, and DHCP-glutathione adducts were obtained using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The samples were injected by direct infusion by syringe pump into the mass spectrometer and the mass spectra were acquired in positive ion mode over a mass to-charge ratio (*m/z*) range of *m/z* 0–900. The instrument was operated at 22 V.

2.10. Statistical analysis

Results were expressed as mean values ± standard deviation. STATGRAPHICS® plus software was used for statistical analysis. Comparisons among samples were made using one-way analysis of variance (ANOVA) and the LSD method. A p-value < 0.05 was considered significant

3. Results and discussion

3.1. Isolation and identification of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP) from autoclaved galacturonic acid

In order to obtain a pure DHCP standard from galacturonic acid the procedure used was adapted from that of Koyama et al. (2000). DHCP was isolated and identified as described in *Materials and Methods*. The DHCP produced from heat-treated galacturonic acid was extracted with chloroform and subjected to a purification step with a HiTrapQ column. The eluting fractions were analyzed by HPLC–DAD at different wavelengths, the strong anion exchange column showing a reasonable chromatographic separation. The chromatographic profiles of the different fractions at 210, 254 and 280 nm differed mostly in the relative abundance of a variety of compounds from galacturonic acid degradation. The presence of DHCP was detected as an intense peak at 210 nm in Fraction 1 only. A peak eluting at 5.00 min with an absorption maximum at 214–215 nm was detected, which coincided with the molecule DHCP described by other authors (Leclere et al., 2016; Koyama et al., 2000; Guan et al., 2018).

Fraction 1 was analyzed by mass spectrometry to confirm the identification of DHCP and to determine the purity. DHCP was identified in the mass spectrum (Fig. 1) as protonated $[M + H]^+$ and sodiated $[M + Na]^+$ species, with molecular ions at *m/z* 115 and 137, respectively. Fragments that were in accordance with the fragmentation of DHCP were also identified (marked in the spectrum by ■). These results corroborated in part with the fragmentation profile of DHCP described by Leclere et al. (2016). The main fragment ion represented in the spectrum was obtained by the loss of 18 Da (ion at *m/z* 97) from the precursor ion $[M + H]^+$, suggesting that it arose from the loss of water from the –OH group. This loss of water, giving a peak at *m/z* 119, was also observed in the sodium adduct. These loss of water from the hydroxyl group was previously observed in the mass spectrum of oligosaccharides that occurred at the reducing end, abstracting a hydroxyl proton rather than a carbon proton (Hofmeister, Zhow, & Leary, 1991). Although the presence of the other fragment, attributed to the loss of a second water molecule by the loss of 36 Da (molecular ion at *m/z* 79) might explain that a complex reengagement from a fragmentation of cyclic ketones with two –OH groups helped stabilize the product. Also, the formation of the sodium-bound dimer (non-covalent) $[2\ M + Na]^+$ and sodium-bound trimer (non-covalent) $[3\ M + Na]^+$ were observed in mass spectrum, where two or three DHCP molecules were solvated by Na^+ cation, which corresponded to ions at *m/z* 251 and 365, respectively. The fragmentation patterns were similar between them, and similar to that previously explained for $[M + H]^+$ and $[M + Na]^+$, where a series of ions were identified in the spectrum by (Δ), which corresponded to fragments from $[2\ M + Na]^+$ and the one marked with (•), which corresponded to fragments from $[3\ M + Na]^+$ species. The fragmentation ions at *m/z* 347, 329 and 215 represented losses of one and

Table 1
DHCP concentration of the different baby food samples, fruits and vegetables autoclaved (121 °C) at different times (20, 30 and 60 min). Roman numerals represent independent assays. Each value is the average of three replicates ± SD.

		DHCP (μM)		
	assay No.	20 min	30min	60 min
Carrot (cooked + blended)	I	45.99 ± 0.92	67.40 ± 0.49	89.53 ± 5.34
	II	-[a]	-	42.98 ± 1.10
	III	-	-	91.90 ± 0.80
	IV	28.35 ± 1.10	44.40 ± 2.30	57.08 ± 1.70
	V	77.72 ± 0.20	-	-
Zucchini (cooked + blended)	I	44.25 ± 3.41	61.18 ± 8.12	66.90 ± 0.77
	II	19.74 ± 1.40	25.58 ± 3.20	42.10 ± 2.60
Tomato (blended)	I	45.62 ± 0.86	53.38 ± 1.49	110.78 ± 6.61
Pear + Apple (blended)	I	10.35 ± 0.29	12.70 ± 0.33	21.53 ± 1.57
	II	ND	-	11.85 ± 0.16
	III	-	-	15.94 ± 0.54
	IV	ND	-	15.35 ± 0.23
	V	ND	21.77 ± 1.3	29.76 ± 0.74
Peach (blended)	I	ND	9.03 ± 0.01	13.39 ± 0.70

-[a]: assay not realized; ND: not detected.

two water molecules, while the rest of fragment ions with mass difference with respect to their former –26 Da, –28 Da, –34 Da, –60 Da and –86 Da, could be attributed to cross-ring cleavage according to the fragmentation proposed (Fig. 1).

In this manner, since almost all ions were identified, it can be concluded that the isolated compound from heat-treated galacturonic acid was a pure molecule of DHCP.

3.2. Presence of DHCP in sterilized baby foods

In order to investigate the presence of DHCP in a real system of heat-treated food, fruit (peach and a mixture of pear-apple) and vegetables (carrot, zucchini and tomato) were prepared in the form of puree to simulate the industrial preparation of baby foods, which a finally autoclaved. The results of this study are summarized in Fig. 2. When foods rich in pectin are heat-treated, two mechanisms of pectin degradation might occur, β-elimination and acid hydrolysis, until reaching its main monomer, the galacturonic acid, and the subsequent formation of carbocyclic compounds such as DHCP as degradation products (Bornik & Kroh, 2013). This was demonstrated by Leclere et al. (2016) and Koyama et al. (2000), when the pectin or galacturonic acid monomers were heat-treated to produce the cytotoxic molecule, DHCP. In our case, however, when the samples of fruit puree and tomato or cooked-vegetable puree were autoclaved at 121 °C for 60 min and analyzed by HPLC, although a peak appeared at the same retention time as DHCP, the absorption spectrum did not match. It might be expected that changes in the cell wall polymers by mechanical disruption and cooking would facilitate accessibility to pectic substances. However, the formation of DHCP in the sterilization step apparently did not occur. Since DHCP possesses a very reactive α,β-unsaturated carbonyl group in its structure, covalent adducts with residues of –SH and $–NH_2$ of protein may form through a Michael addition, which is probably the reason DHCP was not detected. To confirm this hypothesis, the samples were treated with protease followed by washing with water to remove the decomposed protein, and when the washed solid was autoclaved the DHCP was produced. This is in agreement with the report on cabbage by Koyama et al. (2000). Also, when the samples were washed with water only, without protease treatment, and the washed solid was autoclaved, DHCP was also produced. This observation seemed to indicate that the presence of free amino acids, which were removed by washing, was sufficient for adduct formation. The next experiments with the puree both washed with water and without washing, to which a known amount of DHCP, obtained from a solution of galacturonic acid heat-treated at 121 °C/3 h, was added, resulted in the depletion of DHCP until complete disappearance in 24 h in the case of unwashed, while the concentration of DHCP remained constant in the case of the washed solid. In addition, the washing liquid, containing the free amino acids from the fruit and vegetables (data not shown), to which the DHCP was added, also caused a significant decrease in the molecule, which disappeared when the liquid was autoclaved, giving an idea of the formation of the adduct with amino acids.

3.3. Effect of different times of autoclaving on the formation of DHCP from different fruit and vegetable purees

The above results show that DHCP formation occurred in baby foods during sterilization, although due to their high reactivity and the adduct formation, this makes the identification of DHCP difficult. The different processing conditions applied to the washed solid of the different fruits and vegetables, autoclaved at 121 °C for 20, 30 and 60 min (Table 1), showed that DHCP was formed, with increasing concentrations at longer periods of time, forming even after 20 min for cooked-vegetables.

Carrot, zucchini and tomato showed similar behavior with significantly higher DHCP levels than those found in fruits. In both cooked vegetable samples (carrot and zucchini) the molecule showed an important variation in a range from 28.3 to 91.9 μM in the case of carrot and 19.7 to 66.9 μM for zucchini. In tomato, not cooked, DHCP was also found at 20 min of sterilization with a value of 45.6 μM, similar to cooked vegetables. These variations would depend possibly on the moisture content, origin, variety, seasonal change and/or agronomic conditions of each sample analyzed. In addition, the structure, composition and degree of esterification of pectin of each type of fruit or vegetable can influence the level of DHCP produced. In fact, since the carboxylic group of uronic acid is responsible for the generation of DHCP, a greater degree of esterification correlated with less DHCP generation (Aoyagi et al., 2008). In the case of fruit-based puree (peach and mixture of pear and apple), DHCP was only detected in one of the samples, at 20 min of autoclaving, although it does appear after 30 min, with the samples of washed solid treated at 121 °C for 60 min reaching a maximum of 30.0 μM. During the wet-heat processing of vegetables, such as boiling, the solubility of pectin has been reported to increase through β-elimination-cleavage of glycosidic bonds in the polysaccharide (De Roeck, et al., 2009), which can lead, as in our case, to significant losses in this soluble pectin during the water washing of the puree, which would lead to a minor DHCP production. At the same time, the cooking also induced pectin depolymerization and/or demethoxylation (Christiaens et al., 2012), which also promoted greater degradation of the small galacturonan chains during sterilization, accelerating the breakdown and subsequent formation of DHCP. Since the washing step, necessary for the detection of the molecule by eliminating important nucleophilic components such as free amino acids that would form adducts, can lead to significant losses in soluble dietary fiber such as pectin, the actual value for the DHCP obtained during the autoclaving of the puree might be much higher than the values found. Therefore, our study has shown that DHCP was produced upon sterilizing vegetable- and fruit-based lab-scale model systems and might be formed in industrial preparations.

The mechanism by which DHCP exerts its effect most likely coincides with the mechanism of a family of biologically active molecules present

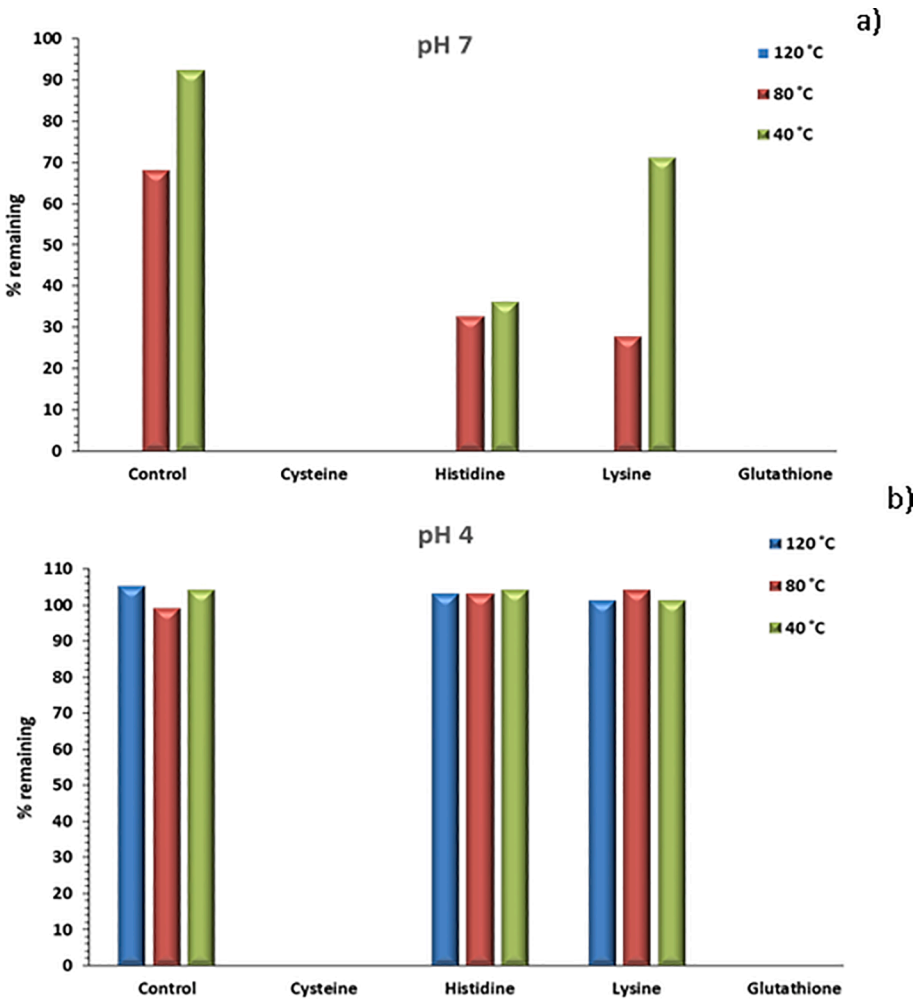

Fig. 3. % remaining DHCP in the presence of three amino acids (cysteine, histidine and lysine) and a tripeptide (glutathione). The changes in the % of retention of DHCP were followed after 20 min of reaction at 40, 80 and 120 °C and two pH values, 4.0 (a) and 7.0 (b) for a molar ratio of the nucleophile agent: DHCP (5:1).

in the human organism, the prostaglandins with a cyclopentenone ring in their structure (Leclere et al., 2016). The presence of an α,β-unsaturated carbonyl group is key for their antiproliferative, anti-inflammatory, antidiabetic, and antiviral effects (Guan et al., 2018; Bie, Dong, Jin, Zhang, & Zhang, 2018; Grau, Iñiguez, & Fresno, 2004). DHCP and cyclopentenone prostaglandins are potent electrophiles that can react with different cellular proteins containing nucleophile groups such as cysteine, histidine or lysine, *via* Michael addition, giving rise to important biological activities (Oeste & Perez-Sala, 2014). Therefore, the direct consequence derived from the application of thermal treatment to pectin-containing foods, all fruits and vegetables and most plant-derived foods, is the generation of compounds, such as DHCP, which promote certain biological activities that may play a role in maintaining human health.

It is likely that humans consume some DHCP on a daily basis, after thermal preparation of food by cooking and especially by high temperatures in a pressure cooker, without any apparent harmful effect on health. It has been reported that DHCP has a favorable toxicity profile, and is shown to be a promising preventive agent for combating human colon cancer (Chen et al., 2021; Koyoyama et al., 2000). However, it is important to identify the presence of adducts in sterilized baby foods. Due to the high reactivity of DHCP, it is possible that it forms covalent adducts with proteins or free amino acids from the food itself. It is not known whether the possible adducts are bioactive, or whether they are reversible under certain conditions, or even, more importantly, whether they can be a potential health risk. In this sense, in some studies it was found that the stability of the thio-adducts formed for example between cyclopentenones and selected cysteine derivatives was inversely proportional to the pH, becoming reversible at physiological pH, with some of the adducts showing potent antiviral and anti-inflammatory activities (Bickley et al, 2004). On the other hand, a similar adduct, formed between 5-hydroxymethylfurfural (HMF) (with an α,β-unsaturated carbonyl group) and cysteine seems to act as an oxidative stress-inducing agent when fed to rats (Zhao et al, 2018), although these same authors also indicated that it was less absorbed and less toxic against Caco-2 cells than HMF (Zhao et al, 2017).

3.4. Effect of amino acids on the decrease in the concentration of DHCP in a model system. Study of DHCP in commercial sterilized baby foods

The reaction of DHCP with three selected amino acids (cysteine, histidine and lysine) and a tripeptide (glutathione) was investigated in a model system. The changes in the concentration of DHCP were monitored after 20 min of reaction at 40, 80 and 120 °C and two pH values, 4.0 and 7.0 (Bickley et al., 2004; Zhao et al., 2017) for a molar ratio of nucleophile agent: DHCP (5:1) (Zhao et al., 2017; Hamzalıoğlu & Gökmen, 2018) (Fig. 3). These changes were compared to the change in DHCP alone, at these temperature and pH conditions, named as control. The pH showed a greater influence on the self-degradation of DHCP, with complete elimination at pH 7.0 and 120 °C and a loss of almost 30% at 80 °C and 10% at 40 °C; while at pH 4.0 the molecule was much more stable for the three temperatures assayed. However, the elimination of

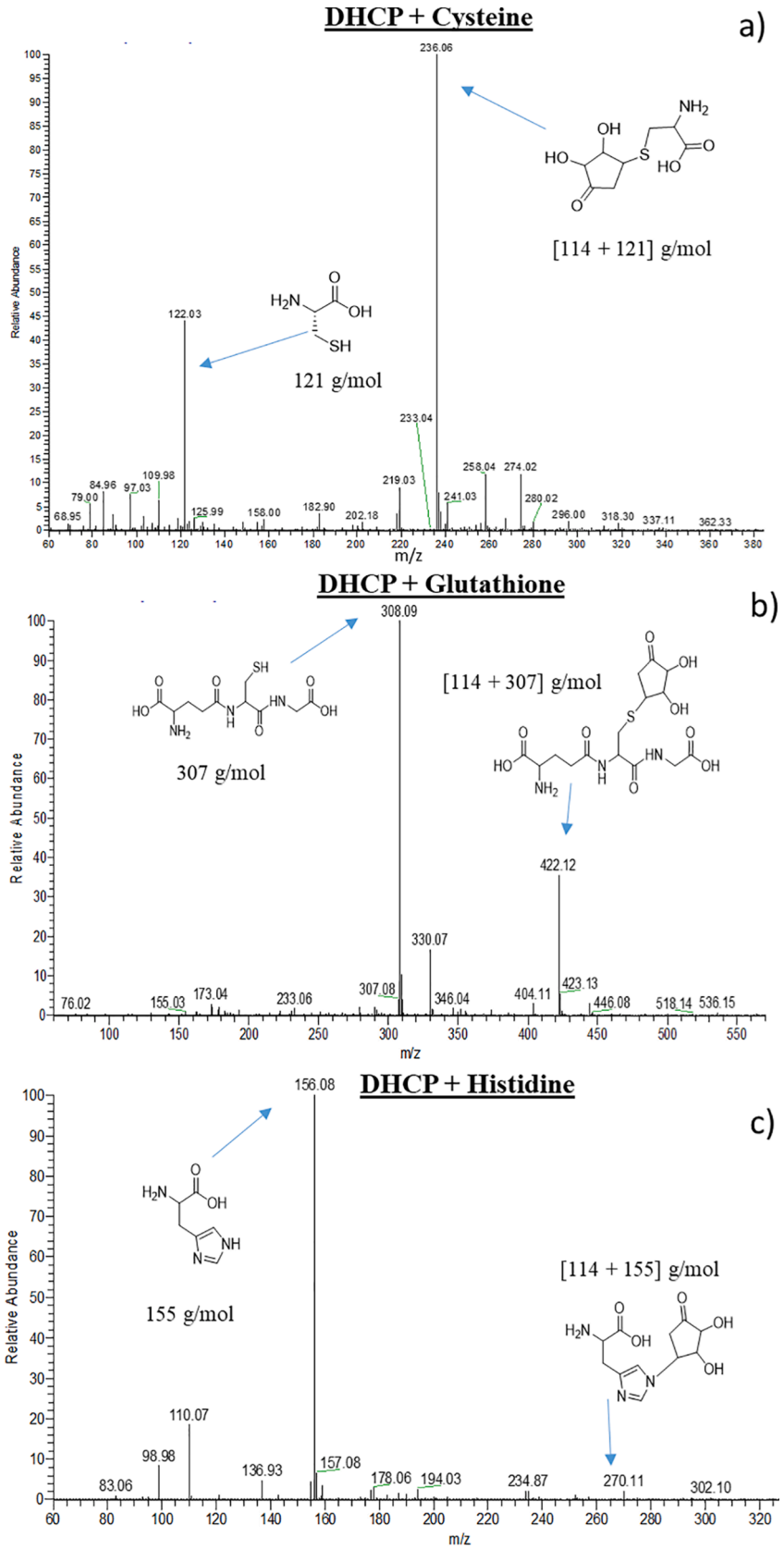

Fig. 4. Mass spectrometry of the adducts formed from the reaction model of DHCP–cysteine (a), DHCP–glutathione (b) and DHCP–histidine (c).

DHCP increased in the presence of the amino acid cysteine, and the tripeptide, glutathione, which contain one –SH group, showing a greater reactivity, with complete elimination at all conditions studied. High pH favored DHCP elimination by histidine and lysine even at low temperatures, with 64 and 30% elimination at 40 °C, respectively, and only a moderate increase in elimination due to increasing the temperature in the case of the lysine, with 68 and 71% loss, at 80 °C; whereas at pH 4.0 no effect on the elimination of DHCP for these amino acids was detected.

The results obtained in the model system showed that although DHCP degrades to some extent, depending on pH, DHCP reactions in the presence of amino acids can easily take place and explain the depletion of the molecule during the autoclaving process of food. These observations coincide with those reported by Vasíĺev, Tzeng, Huang, and Maier, (2014), who predicted that cyclopentenones have high reactivity towards the thiolate state of single cysteine and tripeptide, and predicted histidine and lysine to be less effective nucleophiles in the reaction with cyclopentenones. Glutathione, a tripeptide present at high concentrations in all human cells, showed high reactivity with DHCP. This can have importance since glutathione has a maintenance function in cell homeostasis as an antioxidant (Hatai et al., 2019).

Nucleophilic groups (–SH and $–NH_2$) of amino acids are easily added to the β-carbon of DHCP through Michael-type addition forming adducts. The high contents of free amino acids are widely distributed in food (Ou, Zheng, Huang, Ho, & Ou, 2020), which together with proteins, can lead to large amount of adducts with DHCP during the thermal processing of food. These interactions are much more complex in real foods than in a model reaction system which contains fewer reactant species. The presence of DHCP was evaluated in commercial sterilized baby food puree in jars on the market. In no case was DHCP identified. However, in many samples analyzed by HPLC, a peak was detected at the same time of retention of DHCP but with an UV absorption spectrum similar to adducts. Taking into account the complexity of the samples, it was sometimes not possible to distinguish the peak; although the adduct could be formed but its quantification could be complicated due to the matrix of the food itself. Thus, all these observations seem to indicate that DHCP–amino acids and/or DHCP–protein adduct are inevitably produced during the sterilization process of baby foods. However, the safety, activity and metabolism remain unknown.

3.5. Identification of DHCP–amino acid adducts in the model system

With the objective of confirming the formation of DHCP-amino acid adducts in the model reaction at different pH conditions, the reaction products were characterized by mass spectrometry (MS). At pH 4.0, DHCP can only react with cysteine and glutathione. Fig. 4a shows the presence of $[M + H]^+$ ion *m/z* of 236, and $[M + Na]^+$ ion *m/z* of 258 as the result of the addition of one molecule of DHCP (MW = 114 g/mol) (Fig. 4a) to cysteine (MW = 121 g/mol) in the reaction mixture, confirming the formation of Michael adduct (MW = 235 g/mol) at 80 °C. Cysteine contains $–NH_2$ and –SH groups, which might result in the formation of two different Michael adduct structures with the same ion *m/z*. However, the possibility of adding a –SH group to the double bond of DHCP is higher than that of adding an amine group, because, as mentioned above, the cyclopentenone has more reactivity toward the thiolate state of cysteine. Similarly, DHCP showed high reactivity with glutathione (MW = 307 g/mol) at pH 4.0 to give $[M + H]^+$ ion *m/z* of 422 (Fig. 4b), which confirmed the formation of Michael adduct as a result of the addition of one molecule of DHCP to glutathione in the reaction mixture. The presence of an $[M + H]^+$ ion *m/z* of 536 , which is an adduct formed of two moles of DHCP with glutathione, was also confirmed (Fig. 4b). However, at pH 7.0 the presence of –amino acids or DHCP–glutathione adducts was not confirmed. After incubating the histidine (MW = 155 g/mol) with DHCP no adducts were observed until the pH decreased to 6. In this case, the compound formed an adduct by a nucleophilic addition with the ring NH group of histidine, as was confirmed by the presence of $[M + H]^+$ ion with *m/z* of 270 (Fig. 4c). Lysine has an α-NH_2 and a more reactive ε-NH_2. However, no significant depletion of DHCP was observed at 80 °C for pH 4.0, 5.0 or 6.0, and no adduct was confirmed.

4. Conclusions

The results presented in this work show the presence of a small active molecule, DHCP, formed from pectins during the thermal processing of food. To the best of our knowledge, this is the first time that its formation in sterilized vegetable- and fruit-based baby foods has been demonstrated. The α ,β-unsaturated carbonyl group in its structure can easily react with the amino and thiol groups in amino acids and proteins, *via* Michael addition, leading to important biological activities in a similar way as the cyclopentenone prostaglandins. The results of this work demonstrated the high reactivity of DHCP, which forms covalent adducts with selected amino acids (cysteine and histidine) and a tripeptide (glutathione), which were tentatively identified through a mass spectrometry approach. Therefore, the formation of adducts with free amino acids and protein from the food itself during thermal processing is the reason for the difficulty in identifying DHCP in heat-treated food and in the commercial sterilized baby food tested. However, once the free amino acids were removed from fruit- and vegetable-based puree by washing with water, and the rest of the washed solid material was autoclaved at 121 °C for 20 min, the molecule DHCP was detected, and its amount increased with the increase in time of autoclaving. The formation of DHCP was higher in vegetables, either previously cooked (i.e. carrot and zucchini) or uncooked (i.e. tomato), than fruit (i.e. peaches and mixture of pear and apple). In both cases, they were prepared in the form of puree by blending. In addition, in the case of cooked vegetables it could be assumed that true values were much higher than the values found, which can be due to the β-elimination and thermo-solubilization of the pectin which occurs during prior cooking, and can lead to significant losses in soluble pectin during the washing necessary for their detection. Therefore, the formation of DHCP or its adducts, especially in sterilized baby food, which is also sometimes consumed by elderly and/or sick people, and in our daily lives by the use of pressure cookers with plant foods, has great potential interest, since DHCP together with heat-modified pectin, may play an important role in the treatment and prevention of certain diseases. In the case of adducts, their formation cannot be measured quantitatively in food. This needs to be addressed, since it is not known whether the DHCP–amino acid adducts are bioactive, reversible, or more importantly, whether they are safe.

CRediT authorship contribution statement

Alejandra Bermúdez-Oria: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Data curation. **Guillermo Rodríguez-Gutiérrez:** Writing – review & editing, Supervision, Funding acquisition. **Africa Fernández-Prior:** Formal analysis, Writing – review & editing. **Rlisa Rodríguez-Juan:** Formal analysis, Writing – review & editing. **Juan Fernández-Bolaños:** Conceptualization, Methodology, Writing – original draft, Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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