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ORIGINAL PAPER

Polyphenolic profiles in lettuce (*Lactuca sativa* L.) after $CaCl_2$ treatment and cold storage

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Abstract

Lettuce (*Lactuca sativa* L.) is a popular vegetable with the health-enhancing properties determined by high levels of antioxidant polyphenols as chlorogenic acids and other derivatives of caffeic acid or flavonoids. In this study, changes in the phenolic compound profiles in the lettuce leaves induced by application of $CaCl_2$ before harvest and cold storage were studied. For the first time quantitative analysis of individual phenolic compounds on the basis of standards isolated from lettuce leaves was performed. Compounds were identified using HPLC, LC-MS, ^{1}H and ^{13}CNMR techniques. The dominant compounds were 2,3-dicaffeoyltartaric (2,3-diCTA), 5-*O*-caffeoylquinic (5-OCQA) and caffeoyltartaric (CTA) acids, with content of 5.7, 2.5 and 0.981 mg/g DM, respectively. The levels of individual phenolic compounds, total phenolics and antioxidant activity (DPPH assay) in plants treated by $CaCl_2$ were determined throughout the storage period (7 and 14 days) at 4 °C. To ascertain the relationship between the content of individual compounds, total phenols, antioxidant activities and storage time, Pearson's correlation analysis was used. The best correlation between the storage length and compound concentration was observed for 2,3-diCTA ($R^2 = 0.866$) and caffeoylmalic acid (CMA) ($R^2 = 0.750$). Application of $CaCl_2$ (0.05M) on lettuce resulted in an increase in the levels of CTA, 2.3-diCTA and 5-OCQA about 120, 65 and 57%, respectively, compared to the control stored for 7 days in the same conditions and had a favourable effect on the antioxidant activity ($R^2 = 0.985$). The present paper shows that $CaCl_2$ may be used as an agent that influences the stability of health-promoting compounds of cold-stored lettuce.

Keywords Lettuce · Storage · $CaCl_2$ · NMR analysis · Antioxidant activity

Introduction

Lettuce is a representative of the family Asteraceae and is a popular vegetable used in salads, which are being increasingly consumed as 'healthier' food. The health-enhancing properties of these products are determined by their high levels of antioxidant compounds, primarily vitamin C and polyphenols, as well as fibre [1]. Recent studies in rats and humans have demonstrated the health-enhancing effects of lettuce in the prevention of cardiovascular diseases [2]. The primary reason that lettuce is thought to have strong antioxidant activity is its large amount of phenolic compounds. They are synthesised during normal plant growth or are produced in response to environmental stress and other stresses such as UV, radiation, injuries and processing techniques, including postharvest treatment [3]. In addition, phenolic compounds play an important role in plant–plant and plant–pathogen signalling [4]. They are components of

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many plant-derived drugs and currently attract considerable attention due to their applications in the prevention of cancer, cardiovascular and neurodegenerative diseases as a result of their antioxidant activity [5].

Investigations of the composition of polyphenols in lettuce have demonstrated the presence of caffeic acid derivatives and flavonoids [6, 7]. High contents of sugar-conjugated quercetin in the leaves of lettuce comparable with those in tomatoes, lemons and celery have been detected [8]. Noteworthy is the presence of quercetin-3-*O*-D-glucuronide (Q3GA), which exhibits antioxidant, anti-atherosclerotic and antiviral activities in both in vivo and in vitro models. It can also promote the anti-inflammatory properties of RAW264.7 macrophages and modulate immune response effects with pro-inflammatory stimuli [9]. The major phenolic compounds in lettuce include 2,3-dicaffeoyltartaric acid (2,3-diCTA) and 5-caffeoylquinic acid (5-OCQA) [10]. Caffeic and chlorogenic acids have health-enhancing potential effects (antioxidant, antitumor and anti-mutagenic). The biological activity of caffeic acid derivatives is associated with the inhibition of LDL oxidation or the reduction of plasma cholesterol. Many studies have demonstrated that caffeic acid is an inducer of tumor cell apoptosis and is able to inhibit cancer cell growth [11]. Antitumor activity has also been determined for chlorogenic acid. It was found to inhibit the growth of a variety of large intestine, liver and tongue cancers in experimental animals [11]. Recent research results have demonstrated that another caffeic acid derivative, 3,5-dicaffeoylquinic acid (3,5-diCQA), exhibits α-glucosidase inhibitory activity. Its presence in extracts of *Cichorium glandulosum* seeds contributed to the attenuation of type I diabetes [12].

Lettuce is susceptible to mechanical damage, and it has a short shelf life. To more effectively preserve fresh vegetables, researchers focused on the use of preservatives such as anti-browning and/or firming agents [3, 13, 14]. The main goal of this study was to evaluate the effect of $CaCl_2$ applied before harvest on phenolics as quality parameters and the possible changes through the shelf life of lettuce. Calcium plays an extremely important role in vegetables and fruits because of its role in cell wall structures. The US Food and Drug Administration approved this compound for use as a postharvest agent [15]. It has been found that the application of $CaCl_2$ after harvest at the recommended doses does not produce any harmful effects that could lower consumer acceptance of the treated fruit [16] but reduces damage and preserves the quality of stored fruit and vegetables. Postharvest treatments of apples with $CaCl_2$ reduced fresh mass loss [15]. Dips of kiwifruit slices in $CaCl_2$ delayed their softening and browning [17]. Suppavanish et al. [18] reported that dips of fresh-cut sweet leaf bush in hot $CaCl_2$ significantly maintained the overall quality enhanced total antioxidants and total phenolic contents during storage. In addition, dipping fruit in iced water containing $CaCl_2$ increases the storability and upgrades the nutritional quality by increasing the levels of nutrients in sweet cherries [19]. Currently, the literature does not provide data on the effect of the pre-harvest application of $CaCl_2$ by spraying of plants on the quality of lettuce and other leafy vegetables reflected in the content of health-enhancing polyphenols during storage. Therefore it was hypothesised that the pre-harvest treatment of lettuce with $CaCl_2$ solution stabilizes its quality during storage. The aim of this study was to determine the effect of $CaCl_2$ treatment on lettuce before harvest on phenolic profiles in leaves during storage for 14 days at 4 °C. The analyses consisted of the determination of structures using HPLC-DAD, LC-MS, ^1H, ^{13}C NMR spectroscopy and changes in the levels of the individual phenolic compounds that accumulated in lettuce leaves after pre-harvest treatment with $CaCl_2$ and during cold storage. In addition, possible correlations between the content of the identified compounds and the storage length, $CaCl_2$ application, antioxidant activity (DPPH assay) and PAL activity are within the scope of this study.

Materials and methods

Chemicals

Acetonitrile (ACN) and methanol (MeOH), were obtained from Merck (Darmstadt, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH), chlorogenic acid, formic acid (HCOOH), CD_3OD (99.8%), and D_2O 99.9% were purchased from Sigma-Aldrich. Folin and Ciocalteu's phenol reagent, $CaCl_2$, ethanol 96%, toluene, ethyl acetate, HCl, and Na_2CO_3 were obtained from POCh, Poland. All reagents were of ACS grade or better.

Caffeoyltartaric acid (CTA), 5-*O*-caffeoylquinic acid (5-OCQA), caffeoylmalic acid (CMA), 2,3-dicaffeoyltartaric acid (2,3-diCTA), quercetin-3′-*O*-glucuronide (Q3GA), quercetin-3-*O*-(6″-*O*-malonyl)-glucoside (Q3MG), and 3,5-dicaffeoylquinic acid (3,5-diCQA) were isolated using medium-pressure liquid chromatography and identified with ^1H and ^{13}C NMR.

General experimental procedure

The mass spectra were recorded on a Thermo Finningan LCQ Advantage Max ion-trap spectrometer using an electrospray ion source (Thermo Electron Corp., Bellefonte, PA). The spray voltage was 4.2 kV at the capillary inlet and 60 V at the outlet of the capillary. The temperature of the capillary was 493 K and the samples were injected in methanol. The full scan mass covered the range from *m*/*z* 200 to 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision

gas. MS data were acquired in the negative ionisation mode and in the positive mode.

^{1}H NMR and ^{13}C NMR spectra were recorded at a temperature of 300 K in a CD_3OD or D_2O solution on a Bruker DRX-600 spectrometer working at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Chemical shifts in methanol extracts were calibrated on solvent signals at 3.30 ppm (^{1}H) and 49.15 (^{13}C). The signal assignments were based on analysis of ^{1}H and ^{13}C 1D NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and HMBC spectra.

High-performance liquid chromatography (HPLC) was performed using an Empower-Pro chromatograph (Waters, Milford, MA, USA), which consists of a quaternary pump (M2998 Waters) with a degasser and a UV–Vis diode array detection (DAD) system. Separation was performed on a column filled with a modified silica gel RP-18 (Atlantis T3—Waters, 3 µm, 4.6 mm × 150 mm). The gradient elution consisted of mobile phases A (0.1% formic acid in water) and B (100% acetonitrile). The solvents were used in the following scheme: 0–20 min, 87%, (v/v) A; 20–40 min, 80% (v/v) A; 40–45 min, 60% (v/v) A; 45–50 min, 20% (v/v) A. The flow speed was 1.0 mL/min, the dose volume was 20 µL, and the detector was set at wavelength of 330 nm.

Spectrophotometric analyses were performed using a Cary 50 (Varian, Palo Alto, CA, USA) spectrophotometer.

Plant material

The study material was butter-head lettuce cv. Omega. The plants were grown in the greenhouse of the Department of Horticulture, University of Life Sciences in Lublin in standard cultivation and fertilisation conditions. 2 weeks before harvest (40 days after sowing), the plants were divided into four groups and sprayed with a $CaCl_2$ solution at concentrations of 0.05, 0.1 and 0.2 M, control samples were sprayed with water. After harvesting, the whole heads of lettuce were divided into three groups that each included five plants from each treatment. Fresh plants from the first group were utilised in laboratory analyses. The second and third groups of lettuce were placed in dark polyethylene bags and stored at 4 °C for 7 and 14 days.

Prior to the chemical analyses, fresh and cold-stored plants were weighed, shredded and averaged. Part of the material was used immediately for the analyses, while the other part was lyophilised in an LABCONCO device (temp. -43 ± 2 °C; $p = 0.03$ mBar). The lyophilisates were stored in tight containers in dark at a room temperature until further analysis.

Preparative chromatography

Lyophilised material (115 g) obtained from fresh plants was used to isolate pure phenolic compounds. The lyophilisate was homogenised with aqueous ethanol (85%) for 30 min with the use of a Diax 900 homogenizer. The extract was filtered and concentrated at a temperature of 45 °C until an oily residue was obtained, which was dissolved in approximately 10 mL of water. Next, the initial purification was carried out by extraction in the solid phase on a 40–63 µm RP-18 LichroPrep silica gel (Merck) funnel. The aqueous extract was washed with water and then with a 60% aqueous methanol solution to isolate the phenolic compound fractions. The aqueous and water–methanol fractions were pooled and concentrated at 45 °C before being dissolved in water. The phenolic fractions dissolved in water were applied to a preparative column (3 × 50 cm) filled with the Lichroprep RP-18 phase with a particle diameter of 25–45 µm (Merck), which had previously been rinsed with methanol and water. Gradient elution (water–methanol) with increasing methanol concentrations up to 100% was used to separate the phenolic compounds.

Individual fractions were collected with a fraction collector and subjected to preliminary thin-layer chromatography analysis with a solvent system containing formic acid, toluene, ethyl acetate and water (3.45:1:8:0.05 v/v) using Silica Gel F_{254} (Merck) as a stationary phase. The spots on the TLC plates were observed on UV light and fractions containing identical bands were pooled, and their purity was checked by HPLC as described in the 'General' section. Pure fractions were concentrated, dried and analysed using MS. Spectrometric analysis was performed at the Department of Biochemistry and Crop Quality, IUNG, Puławy. Contaminated fractions were purified further.

The structure of the compounds isolated was determined using NMR at the Institute of Organic Chemistry of the Faculty of Chemistry, Technical University of Łódź, and at the Department of Pharmaceutical Sciences of the University of Salerno, Italy. The structure of the compounds isolated was elucidated based on the analysis of the ^{1}H and ^{13}C NMR spectra and the use of homonuclear correlation spectroscopy ^{1}H-^{1}H Cosy and the heteronuclear correlation techniques ^{1}H-^{13}C HSQC and HMBC. The phenolic compounds that were isolated and identified served as standards to quantify the phenolic fraction profiles obtained from Omega lettuce leaves.

Extraction of phenolic compounds

Fractions containing phenolic compounds isolated from powdered lyophilised samples of whole lettuce leaves cv Omega were used in the study as previously described [20] with some modifications. A lyophilised lettuce sample (250 mg) was homogenised in a Diax 900 homogeniser with a 70% aqueous methanol solution (50 mL) for 30 min. The extract was filtered and evaporated to dryness at a temperature of 47 ± 1 °C. The dry residue was dissolved in water and

purified on Sep-Pak C18 columns (waters) pre-conditioned with methanol and then with HCl (0.03 M). Prior to their application on the column, HCl (0.1 M) was added to the sample to achieve acidification and reverse the dissociation of the phenolic acids. Next, the column was washed with HCl (0.03 M), and the phenolic compound fraction was eluted with an aqueous methanol solution (60%).

The fractions obtained were used to quantify individual phenolic compounds, total phenolic content and antioxidant activity.

Quantification of individual phenolic compounds

The quantification of the phenolic compounds was carried out using the HPLC-DAD method described in the 'General' section. Analyses were conducted with the method described previously by Llorach et al. [21] with some modifications. The individual phenolic compounds were identified by comparison of the retention times and spectra with those of pure standards obtained by the preparative method and identified by HPLC-DAD and ^{1}H and ^{13}C NMR. Standard curves obtained from pure compounds were used for the quantitative analysis of the phenolic compounds in the fractions prepared from leaves of lettuce.

PAL activity

The activity of phenylalanine ammonia-lyase (PAL) was assayed using a method described by Ke and Salveit [22] with some modifications. PAL activity was determined in acetone powder prepared from the leaves of lettuce. The acetone powder (0.2 g) was mixed with 8 mL of 0.1 M borate buffer at pH 8.5. The resulting mixture was cooled in a refrigerator at 4 °C for 15 min and then centrifuged at −4 °C (10,000×*g*) for 20 min. For the PAL assay, 0.5 mL of the extract was incubated with 0.5 mL of 15 mM L-phenylalanine and 0.5 mL of 0.1 M borate buffer (pH 8.5) at 37 °C. The absorbance of the reaction mixture was measured at 290 nm every 20 min for 2 h. The quantity of *trans*-cinnamic acid released was measured from the respective curve. The enzyme activity was expressed as ΔA_{290} mM *trans*-cinnamic acid/h × g FM.

Total phenolic compounds

The total content of phenolic compounds was determined using Folin–Ciocalteu's phenol reagent as described previously [20, 23, 24]. Four millilitres of Folin–Ciocalteu's reagent (diluted with distilled water at a ratio 1:10) and a Na_2CO_3 solution (3.2 mL at a concentration of 75 g/L) were added to the phenolic compound fraction (0.8 mL). The reaction mixture was stored for 1 h at room temperature and absorbance was measured at $\lambda = 760$ nm. The content of total phenolic compounds was expressed as chlorogenic acid equivalent based on the calibration curve prepared previously. The result are the means of three replications in mg of chlorogenic acid/g DM.

Antiradical activity in the DPPH system

Antioxidant activity was determined in accordance with Llorach et al. [21] and own modifications. A methanol DPPH solution at a concentration of 0.0394 mg/mL was prepared. Test tubes with phenolic compound extracts (0.5 mL) were supplemented with DPPH solution (4 mL) and stored at room temperature for 30 min. Changes in the absorbance of the extract and DPPH mixture against methanol as a reference were measured at $\lambda = 517$ nm. Absorbance of the blank sample (DPPH solution in 60% methanol) was determined in the same conditions. The antioxidant activity was expressed as % inhibition calculated according to the equation below [21, 23].

$$\mathrm{AA} = \left[\left(A_0 - A_\mathrm{p}\right)/A_0\right] \times 100\%,$$

where A_0—absorbance of the blank sample, A_P—absorbance of the sample.

Statistical analysis

The statistical analysis compared the results obtained in three replications, and the data were expressed as mean values ± standard deviation (SD). The analysis of variance (ANOVA) was used to determine the effects of $CaCl_2$ treatments and storage period on the content of identified compounds, PAL activity, total phenolic content and antioxidant activity. The significance of differences between the mean values was determined with LSD multiple range test with 5% error probability. To ascertain the relationship between the antioxidant activities, storage length, $CaCl_2$ application and content of the compounds investigated, Pearson correlation analysis was used. Statistical analysis was conducted using Statgraphic Centurion software, version XVI (Statgraphics Technologies Inc. 2013).

Results and discussion

Isolation and identification of phenolic compounds

Mass spectrometry and nuclear magnetic resonance spectroscopy analyses identified the structure of seven compounds contained in the phenolic compound fraction isolated from lettuce leaves using preparative liquid chromatography. The compounds are indicated by the large letters in the chromatogram (Fig. 1).

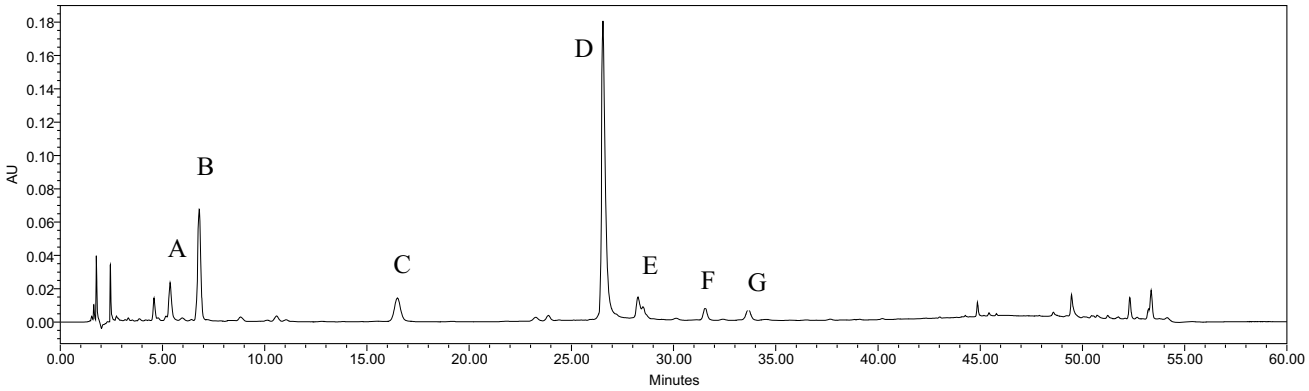

Fig. 1 Chromatogram of fractions of lettuce leaves with identified compounds at $\lambda = 330$ nm. **A** Caffeoyltartaric acid (CTA); **B** 5-*O*-Caffeoylquinic acid (5-OCQA); **C** Caffeoylmalic acid (CMA); **D** 2,3-diCaffeoyltartaric acid (2,3-diCTA); **E** Quercetin-3-*O*-glucuronide (Q3GA); **F** Quercetin 3-*O*-(6″-*O*-malonyl)-glucoside (3-O-MGQ); **G** 3,5-diCaffeoylquinic acid (3,5-diCQA)

Peak A (1.8 mg) corresponds to a compound with the shortest retention time of 5.4 min (Fig. 1). Its spectrum exhibits three absorption maxima at 219.5, 243.1, and 328.7 nm (Fig. 2A). The analysis of the mass spectra indicated the presence of a deprotonated molecule with *m*/*z* 311 $[M–H]^-$ and fragmentation ions with *m*/*z* (%): 179 (35), 149 (100), and 135 (50). These values confirm the data reported by other authors for caffeoyltartaric acid (CTA) detected in lettuce leaves [3]. The data provided by the analysis of ^1H and ^{13}C NMR spectra confirm the presence of CTA (Supplementary material).

Compound B (21.9 mg) was eluted over the course of 6.6 min (Fig. 1). Three maxima were observed in the absorption spectrum of this compound: at 219.5, 241.9, and 326.3 nm (Fig. 2B). The mass spectrum revealed the presence of a deprotonated molecule with *m*/*z* 353 $[M–H]^-$ and fragmentation ions at *m*/*z* (%): 191 (100) and 179 (7). The results of the MS analysis indicate that compound B is 5-*O*-caffeoylquinic acid (5-OCQA). Its presence has been confirmed in lettuce leaves [3], *Anastatica hiererochuntica* seeds [25], apple [26], black and red currant [27], quince [28], coffee [29–31] and tomatoes [32]. The identification of the 5-OCQA was confirmed from the analysis of the ^1H and ^{13}C NMR spectra (Supplementary material), which are comparable with data reported by other authors for 5-OCQA in coffee [29].

Compound C (4.8 mg) is visible in the chromatogram with a retention time of 16.7 min (Fig. 1). The UV–Vis spectrum of this compound shows three absorption maxima: 219.5, 243.1, and 328.7 nm (Fig. 2C). The mass spectra of the analysed compound exhibited the presence of a deprotonated molecule with *m*/*z* 295 $[M–H]^-$ and fragmentation ions with *m*/*z* (%): 179 (100), 133 (60), and 135 (5). The analysis of the ^1H and ^{13}C NMR spectra confirmed that compound C is caffeoylmalic acid (CMA). These data are comparable to the results previously obtained in *Brassica rapa* leaves [33].

Compound D, isolated in 17.7 mg, was eluted at a retention time of 26.9 min. (Fig. 1). Its UV–Vis spectrum is characterised by three absorption maxima: 219.5, 245.4, and 329.9 nm (Fig. 2D). The mass spectrum showed a deprotonated molecule with *m*/*z* 473 $[M–H]^-$ and fragmentation ions with *m*/*z* (%): 311 (100) and 293 (85). The analysis of the ^1H and ^{13}C NMR spectra confirmed that compound D was 2,3-dicaffeoyltartaric (chicoric) acid (2,3-diCTA). The results are in agreement with the values presented for 2,3-diCTA in a study conducted by Sobolev et al. [34].

Compound E with a retention time of 29 min (Fig. 1) was isolated by preparative chromatography in an amount of 1.9 mg. Two absorption maxima at 256.1 and 353.8 nm are visible in the UV–Vis spectrum of this compound (Fig. 2E). The mass spectrum showed a deprotonated molecule with *m*/*z* 477 and a fragmentation ion at *m*/*z* 301. This compound was identified as quercetin-3′-*O*-glucuronide (Q3GA). The data were confirmed by results reported by other authors [2] and by the ^1H and ^{13}C NMR analyses (Supplementary material).

Compound F (2 mg) was eluted with a retention time of 32.3 min (Fig. 1). The absorption maxima at 256.1 and 355 nm are shown in the UV–Vis spectrum of this compound (Fig. 2F). The mass spectrum revealed the presence of a deprotonated molecule with *m*/*z* 549, which was reported by other authors [3, 21], and fragmentation ions at *m*/*z* (%): 505 (100), 463 (35), and 301 (100). The ^1H and ^{13}C NMR analyses confirmed that compound F was quercetin-3-*O*-(6″-*O*-malonyl)-glucoside (Q3MG). The values are consistent with those obtained by other researchers who used other conditions for H^1 NMR (300 MHz, DMSO-d_6) and C^{13} NMR (60 MHz, DMSO-d_6) [35].

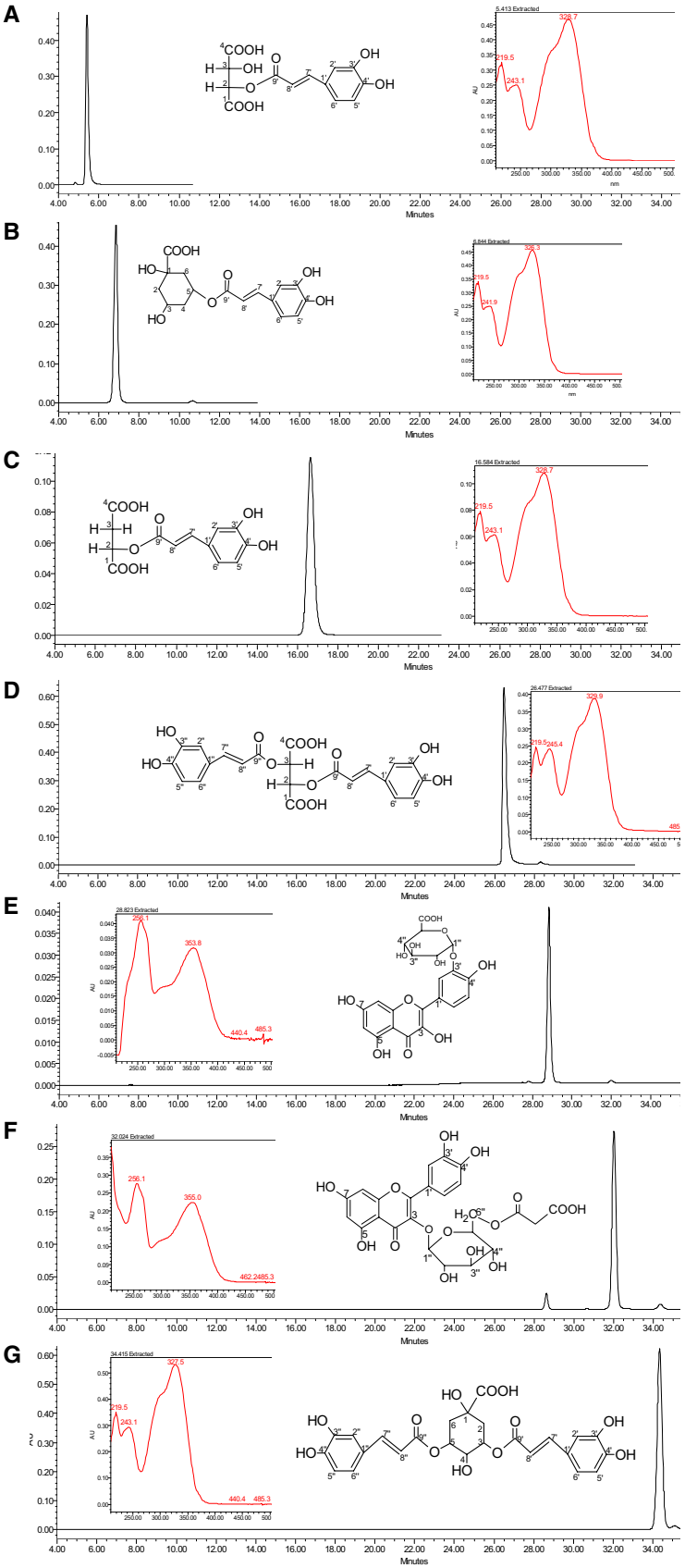

Fig. 2 Chromatograms, UV-spectra, and structures of the isolated compounds from leaves of lettuce

Compound G (4 mg) had a retention time of 34 min (Fig. 1). The absorption spectra 219.5, 243.1, and 327.5 nm are visible in the UV–Vis spectrum of this compound (Fig. 2G). The mass spectrum shows a deprotonated molecule with *m/z* 515 and fragmentation ions at *m/z* (%): 353 (100), 179 (3), 191 (100), and 179 (72). The *m/z* values for the MS^1 MS^2 base peak shown in this study agree with data in the literature describing this compound in coffee as dicaffeoylquinic acid [29]. Compound G was identified as 3,5-dicaffeoylquinic acid (3,5-diCQA) based on the results of ^1H and ^{13}C NMR analyses. The spectroscopic data are consistent with the results of previous investigations of *Cichorium glandulosum* seeds [12].

Individual phenolic compounds content during storage and after $CaCl_2$ treatment

The compounds that were isolated and identified by MS and H^1 and C^{13} NMR were used as standards to quantify individual polyphenols in extracts from Omega lettuce leaves. Standard solutions of each compound were diluted to six different concentrations to construct calibration curves by plotting the peak areas against the corresponding concentrations in the range of 5–500 µg/mL. The calibration curves enabled the determination of a regression equation, and the linear range of seven compounds was determined (Table 1). Calibration curves were examined based on high correlation coefficient values ($R^2 > 0.9989$). The limit of detection (LOD) and limit of quantitation (LOQ) for each analyte were defined by the signal-to-noise values of 3 and 10, respectively. As shown in Table 1, the LODs and LOQs of the seven compounds ranged from 0.957 to 6.171 µg/mL and from 3.189 to 20.570 µg/mL, respectively, indicating that the analytical method was effective and sensitive.

This study showed that 2,3-dicaffeoyltartaric acid (2,3-diCTA) was found at much higher levels than other phenolic compounds in the leaves of lettuce cv. Omega (Table 2). The concentration of 5-OCQA was twofold while those of CTA and Q3GA were fivefold lower than 2,3-diCTA, which was also the dominant acid in the green leaves of cv. Simpsum Elite lettuce [3]. According to Nicole et al. [23] the levels of 2,3-diCTA in green lettuce of different cultivars were from 5.58 to 6.1 mg/g DM, which agrees with our results (Table 2). Another study of three green and two red varieties of lettuce indicated that chicoric acid (2,3-diCTA) was present at the highest concentrations (0.84–1.95 mg/g FM) and accounted for more than 55% of the total caffeic acid derivatives [21]. The investigations of the outer leaves of lettuce cv. Audran demonstrated that the sum of the 3,5-diCQA and 2,3-diCTA derivatives represented more than 93% of the total phenolic compounds [10]. In this study, 2,3-diCTA and 5-OCQA accounted for 74.55% of the total phenolic compounds (Table 2).

As far as we know, there are no reports comparing the phenolic profiles of leaves of lettuce during cold storage control and pre-harvest $CaCl_2$ treatment. Therefore, we evaluated the effect of storage time on individual phenolic compounds in non-treated lettuce of *Lactuca sativa* L. cv. Omega and treated with $CaCl_2$ at different concentrations. We found that when non-treated plants were kept at 4 °C storage for 7 days, the levels of all of the compounds measured decreased compared with the control (Table 2). The most stable compound was Q3MG, and its content was lower by approximately 12%, while the greatest losses were observed in the levels of 5-OCQA, Q3GA and CTA, which were reduced by more than 61, 56 and 51%, respectively, compared with the amounts detected in the lettuce leaves at the beginning of the experiment. The storage of lettuce up to 14 days contributed to an increase in the content of the analysed compounds to a similar degree (2,3-diCTA), lower (5-OCQA, CTA, Q3GA) or higher (CMA, 3,5-diCQA) than that of the control (Table 2). The increase in the levels of 3,5-diCQA of approximately twofold and the decrease in 5-OCQA (20%) after 14 days of storage at 4 °C can be the result of the conjugation of 5-OCQA to 3,5-diCQA. This study revealed significant correlations at $p < 0.05$ between the length of the lettuce storage and the content of CTA, CMA, and 2,3-diCTA confirmed by high correlation coefficients (from 0.750 to 0.866) (Table 3). The results of the influence of storage time on the phenolic contents in non-treated plants are in agreement with the observation reported by Ferreres et al. [35] who noticed a decrease (57.8%) in

Table 1 Linear regression, LOD and LOQ values for compounds isolated and identified in lettuce leaves

Compound	t_r (min)	Regression equation	R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
CTA	5.40	$y = 19315x - 966.90$	0.9991	0.2–100	3.236	10.787
5-OCQA	6.90	$y = 22107x - 5174.4$	0.9997	0.4–50	1.724	5.747
CMA	16.7	$y = 23945x - 8717.0$	0.9996	0.4–50	0.957	3.189
2,3-diCTA	26.9	$y = 27351x + 24{,}930$	0.9992	0.8–150	4.204	14.014
Q3GA	29.0	$y = 13065x + 56{,}273$	0.9994	3–150	4.313	14.377
Q3MG	32.3	$y = 14488x + 18{,}540$	0.9995	0.4–150	3.643	12.145
3,5-diCQA	34.0	$y = 28884x - 396.60$	0.9989	0.2–150	6.171	20.570

Table 2 Content of individual caffeic acid and flavonoid derivatives (mg/g DM) in lettuce leaves after $CaCl_2$ treatments and cold storage

Compound	Time of storage (days)	Control	Treatment $CaCl_2$		
			0.05 M	0.1 M	0.2 M
CTA	0	$0.981^{a} \pm 0.040$*	$0.859^{b} \pm 0.025$	$0.565^{c} \pm 0.017$	$0.944^{a} \pm 0.023$
	7	$0.476^{c,d} \pm 0.009$	$1.057^{a} \pm 0.029$	$0.796^{b} \pm 0.018$	$0.883^{a,b} \pm 0.015$
	14	$0.865^{a,b} \pm 0.019$	$1.037^{a} \pm 0.024$	$0.708^{b} \pm 0.001$	$0.650^{c} \pm 0.011$
5-OCQA	0	$2.540^{a} \pm 0.060$	$1.734^{c} \pm 0.030$	$2.354^{a} \pm 0.070$	$2.019^{b} \pm 0.050$
	7	$0.983^{f} \pm 0.010$	$1.546^{d} \pm 0.010$	$1.442^{d} \pm 0.030$	$1.293^{d,e} \pm 0.020$
	14	$2.035^{b} \pm 0.020$	$1.525^{d} \pm 0.060$	$1.031^{f} \pm 0.020$	$1.304^{d,e} \pm 0.010$
CMA	0	$0.458^{d,e} \pm 0.021$	$0.393^{e} \pm 0.013$	$0.457^{d,e} \pm 0.008$	$0.406^{e} \pm 0.011$
	7	$0.316^{f} \pm 0.006$	$0.564^{d} \pm 0.019$	$0.408^{e} \pm 0.006$	$0.486^{d} \pm 0.013$
	14	$0.843^{a} \pm 0.014$	$0.785^{b} \pm 0.022$	$0.622^{c} \pm 0.008$	$0.615^{c} \pm 0.010$
2,3-diCTA	0	$5.730^{a} \pm 0.200$	$4.493^{b} \pm 0.108$	$4.000^{b,c} \pm 0.165$	$4.723^{b} \pm 0.151$
	7	$2.536^{e} \pm 0.100$	$4.176^{b,c} \pm 0.124$	$3.597^{c} \pm 0.142$	$3.433^{c,d} \pm 0.089$
	14	$5.802^{a} \pm 0.060$	$4.810^{b} \pm 0.057$	$3.781^{c} \pm 0.053$	$3.627^{c} \pm 0.109$
Q3GA	0	$1.045^{a} \pm 0.040$	$0.444^{d} \pm 0.023$	$0.647^{b} \pm 0.023$	$1.025^{a} \pm 0.044$
	7	$0.218^{f} \pm 0.005$	$0.440^{d} \pm 0.009$	$0.370^{e} \pm 0.021$	$0.351^{e} \pm 0.021$
	14	$0.641^{b} \pm 0.009$	$0.543^{c} \pm 0.009$	$0.383^{e} \pm 0.010$	$0.360^{e} \pm 0.004$
Q3MG	0	$0.162^{c} \pm 0.005$	$0.148^{c,d} \pm 0.005$	$0.169^{c} \pm 0.004$	$0.159^{c} \pm 0.002$
	7	$0.143^{c,d} \pm 0.002$	$0.169^{c} \pm 0.002$	$0.141^{c,d} \pm 0.003$	$0.152^{c,d} \pm 0.004$
	14	$0.249^{a} \pm 0.002$	$0.250^{a} \pm 0.002$	$0.143^{c,d} \pm 0.002$	$0.160^{c} \pm 0.003$
3,5-diCQA	0	$0.177^{c} \pm 0.005$	$0.117^{d} \pm 0.004$	$0.170^{c} \pm 0.006$	$0.159^{c} \pm 0.004$
	7	$0.091^{e} \pm 0.002$	$0.219^{b} \pm 0.007$	$0.188^{b,c} \pm 0.007$	$0.089^{e} \pm 0.002$
	14	$0.366^{a} \pm 0.003$	$0.235^{b} \pm 0.002$	$0.243^{b} \pm 0.001$	$0.192^{b,c} \pm 0.002$

*Data are mean values ± SD, ($n = 4$); for each compound value not sharing the same letter were significantly different at $p < 0.05$

Table 3 Pearson's correlation coefficients between the length of storage and application of $CaCl_2$, DPPH scavenging activity, total phenolic compounds, and the content of compounds isolated from lettuce leaves

Factors	AA	TP	CTA	5-OCQA	CMA	2,3-diCTA	3,5-diCQA
Time storage	0.660	n.s	0.750**	n.s	0.757**	0.866**	0.600
$CaCl_2$	0.985*	n.s	0.736**	n.s	n.s	n.s	n.s
AA	–	0.679	0.453	0.753**	n.s	0.729**	n.s

AA Antioxidant activities (DPPH assay), *TP* total phenolic compounds

*Correlation is significant at $p < 0.01$

**Correlation is significant at $p < 0.05$

CQA content from its initial values during 7 and 14 days of storage at 5 °C in green lettuce cv. Lollo Rosso and by Degl'Innocenti et al. [6] who also indicated lower values of CTA and 2,3-diCTA levels in lettuce after 72 h of storage at 4 °C.

The pre-harvest treatment of the plants with $CaCl_2$ caused different concentration-dependent effects on the level of phenolic acids and quercetin derivatives. The leaves of lettuce treated with $CaCl_2$ before harvest were characterised by a reduced concentration of the dominating compounds compared with control at the beginning of the experiment except for CMA and Q3MG. However, the pre-harvest treatment of lettuce with $CaCl_2$ had a beneficial effect on the quality expressed as the content of caffeic acid and quercetin derivatives after 7 days of cold storage. The highest increase in the contents of 3,5-diCQA (2.5-fold) and CTA (twofold) was observed in the lettuce treated with 0.05 M $CaCl_2$ compared with the non-treated plants stored in the same conditions. The longer cold storage (14 days) of plants treated with 0.05 M $CaCl_2$ resulted in increasing concentrations of the analysed phenolic compounds to the same values (CTA and Q3MG) or lower ones compared to the control (Table 2). The results of the correlation analysis revealed significant correlation coefficient at $p < 0.05$ between the $CaCl_2$ concentration and the CTA content in the lettuce samples, while the correlations for

the content of the other caffeic acid derivatives were not statistically significant (Table 3).

The increase in the caffeic acid derivative contents is involved in the de novo synthesis of phenolic compounds in the phenylpropanoid pathway in which the first step is the conversion of the L-phenylalanine to *trans*–cinnamic acid catalysed by the enzyme phenylalanine ammonia-lyase (PAL) [11]. Both wounding and exposure to plant hormones such as ethylene [31, 32] or ABA (abscisic acid) or environmental and salt stresses [3] stimulate the phenylpropanoid pathway and induce new enzymatic activity leading to increased production of the major phenolic compounds and synthesis of new compounds such as 5-OCQA, 3,5-diCQA, CTA and 2,3-diCTA [9, 14, 33], which have been related to browning in cut lettuce [35–37].

Effects of $CaCl_2$ and cold storage on PAL activity, total phenolic content and antioxidant activity (DPPH assay) in the leaves of lettuce

Phenylalanine ammonia-lyase (PAL) is one of the main enzymes from the first step of phenolic synthesis. Therefore, the changes in PAL activity, total phenolic contents and antioxidant activity during cold storage were measured in control and lettuce treated with $CaCl_2$ before harvest. As shown in Fig. 3A, PAL activity in the non-treated plants increased after 7 days of storage at 4 °C and then declined after 14 days, but not significantly. These results agree with studies of different types of lettuce stored for 0, 7 and 14 days at 5 °C, when the initial rate of induction PAL activity in the heads of lettuce increased with increasing storage time. However, maximal levels of PAL activity decreased [38]. Other researchers noticed a gradual increase in the PAL activity in whole leaves during storage [3, 36].

In our research, PAL activity varied not only in terms of storage period but also $CaCl_2$ concentrations that were applied to plants before harvest (Fig. 3A). Treatment of plants with 0.05 M $CaCl_2$ led to a doubling of PAL activity in lettuce at the beginning of the experiment, and the levels remained the same for up to 7 days of cold storage. The longer time of storage (14 days) resulted in a decrease in PAL activity in plants treated with 0.05 M $CaCl_2$ to the levels shown in non-treated plants. It is interesting that the use of the higher $CaCl_2$ concentrations (0.1 and 0.2 M) on lettuce slightly increased PAL activity compared to controls at the beginning of the experiment and then stabilized it for up to 7 days of storage. After 14 days of storage, the PAL activity increased compared to untreated lettuce and lettuce treated with 0.05 M $CaCl_2$.

The total phenolic content of lettuce cv. Omega was 10.75 mg/g DM with chlorogenic acid as its equivalent (Fig. 3B). These data are consistent with the results obtained with other green lettuce cultivars [23, 24, 39].

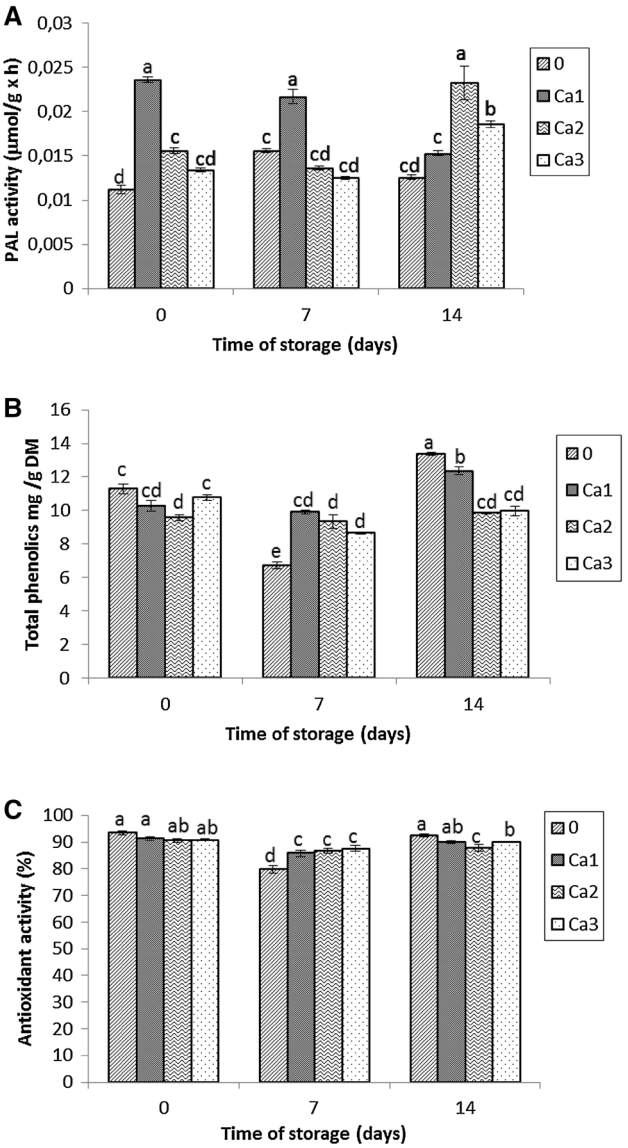

Fig. 3 Changes in **A** PAL activity, **B** total phenolic compounds and **C** antioxidant activity of the extracts from lettuce leaves after $CaCl_2$ treatments and cold storage. 0: control, Ca_1: 0.05 M, Ca_2: 0.1 M, and Ca_3: 0.2 M $CaCl_2$. Error bars represent the SD of the mean values ($n=3$). Different letters indicate statistically significant differences ($p<0.05$)

Changes in the level of these compounds during the cold storage of lettuce were in contrast to PAL activity (Fig. 3B). During the storage for 7 days of untreated plants, a reduction of the total phenolic compounds by 55% was observed, while after 14 days, the levels of those compounds were significantly higher compared to control. These values are similar to results reported by Ferreres et al. [35] for green lettuce cv. Lollo Rosso stored for 7 days at 5 °C where losses of the total phenolic content were not statistically significant and subsequently increased to the values of the control plants after 14 days.

Spraying of the lettuce with $CaCl_2$ before harvest influenced the stability of total phenolic compounds (Fig. 3B) and no significant differences between their content in plants treated with $CaCl_2$ at the beginning of the experiment and stored at 4 °C for 7 and 14 days were noticed, except for lettuce sprayed with 0.05 M $CaCl_2$. In this case the level of total phenolic compounds increased significantly compared to non-treated plants after 7 days but was lower as in the control stored for 14 days (Fig. 3B). The changes in the total phenolic compounds were a result of PAL activity. The increase in total phenolics after 0.05 M $CaCl_2$ treatment was preceded by the higher activity of this enzyme in the lettuce stored for 7 days at 4 °C (Fig. 3A). However, no linear relationship was found between PAL activity, total phenolic compounds and $CaCl_2$ treatment before harvest and at the time of storage.

This study is the first to examine bioactive phenolic levels in lettuce after pre-harvest $CaCl_2$ application and during cold storage, so the comparison of results with other scientific literature is difficult. Alternatively, when postharvest wild cherry fruits were submerged in $CaCl_2$ at a concentration in the range of 0.2–0.5%, an increase in total phenolic compounds after 4 days of storage of the fruit at a temperature of 0 °C was found [19]. The treatment of kiwi fruits with a 2% $CaCl_2$ solution stored for 8 days at a temperature of 2 °C also exerted a positive effect on their phenolic compound content and increased their antioxidant activity (DPPH assay) [17].

The changes in the antioxidant activity of the extracts obtained from lettuce reflected in the DPPH radical scavenging ability were similar to the changes in the level of total phenolic compounds (Fig. 3C). Plants stored in the cold for 7 days were characterised by lower antioxidant activity (DPPH assay) than those at the beginning of the experiment. However, in the extracts from the $CaCl_2$-treated plants, the reduction of the activity was lower than in the control stored in the same conditions and was not independent of $CaCl_2$ concentration. After the subsequent cold storage (14 days) of the control plants and treatment with 0.05 M and 0.2 M $CaCl_2$, there was an increase in the antioxidant activity to values similar to the beginning of the experiment. Results reported by other authors indicated that the storage process had no negative impact on the antioxidant activity of fruit and vegetables, and they observed a linear correlation between total phenolic compounds and antioxidant activity based on measurements in the DPPH radical system [39].

The results of the relationship between the length of the storage period, application of $CaCl_2$, and antioxidant activity showed a correlation coefficient significant at $p < 0.01$ (0.985) between the application of the different concentrations of $CaCl_2$ and the value of antioxidant activity (Table 3).

Conclusion

In this study, the use of HPLC-DAD, LC-MS, ^{1}H-NMR and ^{13}C-NMR techniques resulted in the identification of five caffeic acid and two flavonoid derivatives in the leaves of lettuce of cv. Omega. In addition, the quantification of these compounds during cold storage of lettuce treated with $CaCl_2$ before harvest was evaluated for the first time on the basis of isolated standards. Presented study shows a positive effect of $CaCl_2$ at a concentration of 0.05 M on nutritional characteristic of lettuce after a 7-day cold storage at 4 °C. It increased the levels of CTA, CMA, and 3,5-diCQA and inhibited the reduction of 5-O-CQA, 2,3-diCTA, and Q3MG, total phenolic compounds and antioxidant activity determined with the DPPH method in comparison with the control stored in the same conditions. The relationship between antioxidant activity and the total phenolics was confirmed by the significant correlations for 5-OCQA (0.753) and 2,3-diCTA (0.729). The results of this paper indicated that application of $CaCl_2$ on lettuce before harvest influenced the stability of health-promoting phenolic compounds in lettuce stored at 4 °C for 7 days.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest statement.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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