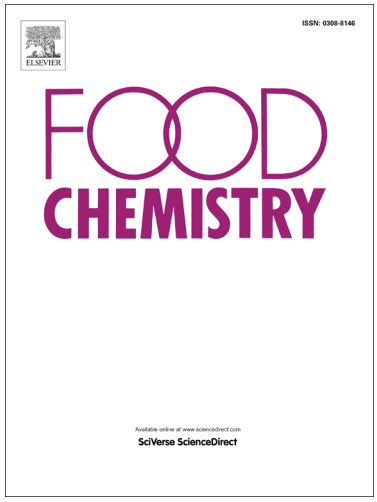

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Effect of temperature, oxygen and light on the degradation of β-carotene, lutein and α-tocopherol in spray-dried spinach juice powder during storage

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Running title: Nutrient retention in spray-dried spinach juice

Abstract

The aim of this study was to evaluate the interaction between packaging parameters (transmission of light and oxygen) and storage temperatures (4, 20, 40°C) on nutrient retention of Spinach (*Spinacia oleracea*) juice, spray-dried in the absence of an added encapsulant. β-carotene was more susceptible to degradation compared with lutein and α-tocopherol. Under our experimental conditions, it was observed that excluding low fluorescent light intensity and air by vacuum packaging at 20°C did not seem to improve nutrient retention loss over time (p>0.05). The rate of β-carotene, lutein and α-tocopherol loss displayed first order reaction kinetic with low activation energy of 0.665, 2.650 and 13.893 kJ/mol for vacuum, and 1.089, 4.923 and 14.142 kJ/mol for non-vacuum, respectively. The reaction kinetics and half-life for β-carotene, lutein and α-tocopherol at 4°C and non-vacuumed were 2.2 $x10^{-2}$, 1.2 $x10^{-2}$, and 0.8 $x10^{-2}$ day^{-1}, and 32.08, 58.25 and 85.37 day, respectively.

Keywords: spinach juice, spray-drying, carotenoids, tocopherol, degradation kinetics

1. Introduction

Green leaf material is rich in essential nutrients and can be consumed fresh, pureed or processed. In general, bioactive materials like β-carotene, lutein and α-tocopherol within green-leaf material are concentrated in the chloroplasts (Gedi et al., 2017; Soll, Kemmerling, & Schultz, 1980; Torcello-Gómez et al., 2019). In the diet, β-carotene is a pro-vitamin A, has antioxidant potential by quenching oxygen or free-radicals, and reduce the risk of chronic diseases (diabetic, cancer, cardiovascular diseases). Lutein were reported to improve eye health, while vitamin E (α-tocopherol) is a lipid-soluble antioxidant required for the preservation of cell membranes. With the growing public awareness of healthy eating, there are increasing opportunities for the commercial development of spray-dried, nutrient-rich juice obtained from green biomass. Examples are increasing vitamin A intake for who suffer from anaemia or, supplementation of α-tocopherol for individuals suffering with fat malabsorption like abetaliproteinemia to maintain neurological function. Spinach (*spinach oleracea*) is a member of the Amaranthaceae family which is rich in β-carotene, lutein and α-tocopherol and can be cultivated throughout the year, so is ideal for experiments into green leaf material as well as for potential commercial production of these nutrients.

One of the main obstacles for maintaining nutrients in spray drying is the application of relatively high processing temperatures and the stickiness of the green material. Our preliminary study on spray-dried spinach juice encapsulated with 10% skimmed-milk showed that there was no significant differences in β-carotene, lutein and α-tocopherol's retention when compared to the non-encapsulated version within a week of storage (data not published). The choice of skimmed-milk as the encapsulant was due to the need of malnourished patient that need more protein in

their diet instead of carbohydrate such as maltodextrin and gum Arabic. There are a limited number of studies that report the successful stabilisation of spray-dried vegetable or fruit juices in the absence of a carrier. Among the reported studies were: tomato pulp with total solid recovery lower than 45.8% and moisture content up to 11.3% (Goula & Adamopoulos, 2005); and spinach juice with rapid chlorophyll loss and 0.31 of water activity (Çalışkan Koç & Nur Dirim, 2017). Regardless, to date, there is no published data on the β-carotene, lutein, and α-tocopherol stability of non-encapsulated spray-dried plant juices upon storage. Quality deterioration of powders during storage occurs due to common factors such as exposure to temperature, oxygen, light, and water activity. Hence, it is beneficial to determine the stability of the non-encapsulated spinach juice as it is a cheaper alternative to reduce vitamin A deficiency and increase food availability especially in the developing country and in rural populations.

Therefore, this study explores the nutrient stability of non-encapsulated spray-dried spinach juice powder and the interaction between the nutrients and external environment factors during 56 days of storage at different temperatures (4, 20, 40°C) and different packaging conditions (vacuum-sealed foil bag, non-vacuum sealed foil bag, and vacuum-sealed transparent multi-layered polyamide/ polyethylene bag). Foil bags were used to provide light-excluded packaging, while transparent bags allowed transmission of light. The target nutrients for this study were β-carotene, lutein and α-tocopherol.

2. Materials and methods

2.1 Spray-dried sample preparation

Baby spinach leaves were purchased from a local supermarket (Morrisons, UK). The spinach leaves were juiced using a domestic cold press juicer (ANNDY All-in-One Slow Juicer TD-1002, UK). The juice (6.4°Bx) was filtered through a 75 µm mesh filter to remove particulates and avoid blockage during spray drying. Spray drying experiments were conducted on an Armfield FT80 Tall Form Spray Dryer (Ringwood, UK), with a 1 mm two-fluid atomizer nozzle, inlet temperature of 132 ± 2°C, outlet temperature of 65 ± 5°C, constant feed rate of 420 ml/h, and compressed air pressure of 1.5 bar.

2.2 Packaging and storage conditions

Approximately 5 g of powders were sealed with or without vacuum using a commercial kitchen food chamber seal vacuum (VacMaster VP215, Kansas, US) and stored independently at 4 ± 1°C, 20 ± 1°C and 40 ± 1°C for 14, 28, 42 and 56 days, in triplicate. Samples were analysed every 14 days from day 0 to day 56.

The packaging materials used were foil or transparent multi-layered polyamide/ polyethylene vacuum bags. The vacuum packaging (10 x 15 cm, 0.13 mm thickness, 130 µm mesh, Fresherpack, UK) is claimed to protect from the ultra-violet of sunlight and from oxygen penetration. Samples were always kept in the dark and vacuum-sealed unless stated. An oxygen indicator (Ageless Eye, Mitsubishi Gas Chemical, Japan) was used as an in-package colorimetric monitor where a pink colour indicated 0.1% or less oxygen, and a blue colour indicated 0.5% or more oxygen is present. For samples incubated with exposure to light, the samples were exposed to light (fluorescent lamp (FL15D), 15 W, 30-40cm distance) at 600-800 lux

(VWR Traceable Light Meter, USA) for 24 h and rotated daily to minimize temperature differences.

A triplicate of vacuum sample stored at 4°C in dark, and non-vacuum sample stored at 20°C in dark were also stored for 365 days and analysed as additional data.

2.3 Chemical and standards

Acetone, acetonitrile, chloroform, hexane and methanol used for lipid extraction and analysis of β-carotene, lutein, and α-tocopherol, were HPLC grade, purchased from Merck (Darmstadt, Germany) and VWR International (Lutterworth, England). Butylated hydroxytoluene (BHT) and standards of β-carotene, and α-tocopherol were purchased from Merck (Darmstadt, Germany) and lutein from Pan Reac AppliChem (Barcelona).

2.4 Microscopy and Scanning electron microscopy

A light microscope was used with 100x magnification to observe the morphology of fresh spinach juice. Scanning electron microscopy was used to observe physical changes in the spinach juice samples after the spray drying. Platinum coating was carried out and samples were subsequently viewed with a JEOL 6060LV Variable Pressure Scanning Electron Microscope (Jeol. (UK) Ltd, Hertfordshire, UK) at an accelerating voltage of 10 kV and under a pressure of 40 Pa. SEM was operated at magnification of 1000x.

2.4 Total colour difference (Hunter Lab)

Colour of the samples was measured in terms of the CIE L^*, a^*, b^* values using a Hunter Lab Colorimeter. L^* represents lightness, a^*=red (+) to green (−) axis, b^*=yellow (+) to blue (−) axis.

$$Chroma = (a^{*2} + b^{*2})^{1/2}$$

$$Hue\ (H^{\circ}) = tan^{-1}(b^* / a^*)$$

$$Total\ Colour\ Difference\ (\Delta E^*) = [\![(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2]\!]^{1/2}$$

2.5 Lipid extraction

Lipids were extracted from the spray-dried spinach juice powders using a modified Folch et al. (1957) technique. Chloroform: methanol in a 2:1 ratio (1.2 ml) was added to 0.1 g of powder and vortexed for 1 min. A 0.9% sodium chloride solution (0.5 ml) was added and the mixture was vortexed again for 1 min before centrifugation at 17507 RCF (3000 rpm) (Rotina 380R, Hettich Zentrifugen, Germany) for 10 min at 4°C to separate into 3 phases. The lower phase, containing the lipids and chloroform was transferred to a clean centrifuge tube. The residue was extracted twice with 1.2 ml portions of chloroform: methanol (2:1), vortexed and centrifuged again. The procedure was repeated three times to ensure complete lipid extraction (the lipid phase to be clear). The lipid phases were removed and pooled with the original lipid layer. The pooled lipids were centrifuged again to separate the lipids from any residual contents, and then dried under a flow of nitrogen. The dried lipid extracts were weighed to calculate the total lipid content and subsequently used for the analysis of β-carotene, lutein and α-tocopherol. All extracts were prepared in triplicate.

2.6 Moisture content and water activity (a_w)

Triplicate samples of spray dried spinach juice (50 mg) were weighed and dried in oven at 105°C. The samples were cooled in a desiccator for 4 h before weighing. The processes were repeated until constant weights were obtained. Measurement of water activity was carried out using Aqualab Dew Point Water Activity Meter 4TE (Decagon Devices Inc., Pullman, WA, USA). Before opening the samples' packaging, they were left for 30 min to 1 h at room temperature to avoid further moisture loss or intake by the samples. Triplicate samples were analysed and the mean values were recorded.

2.7 β-carotene and lutein analysis

The β-carotene and lutein content of the spray-dried spinach juice powders was analysed using HPLC (Agilent 1100, Germany) with Photo Diode Array (PDA) detection. The dried lipid extract obtained in Section 2.5 was dissolved in 10 ml of acetone containing 0.1% butylated hydroxytoluene (BHT) and syringe filtered (0.45 µm) into a 2 ml of amber HPLC vial. The mobile phase consisted of acetonitrile, methanol and ethyl acetate at a flow rate of 0.5 ml/min. At the start of the run the proportions of the solvents were 95:5:0 which changed to 60:20:20 after 20 min and these latter proportions were maintained until the end of the run. Re-equilibration took 15 min. Samples (10 µl) were injected through a security guard-column (Phenomenex C18, 4 µm x 3.0 mm), and separated using a Waters Spherisorb S3ODS (3 µm x 15 cm x 4.6 mm) column, with the temperature set at 22°C; carotenoids were detected at 454 nm. The concentration of β-carotene and lutein was determined using a linear equation created using a calibration curve produced

from a range of external (β-carotene/ lutein) standards (2.5-100 µg/ml) in acetone containing 0.1% BHT.

2.8 α-tocopherol analysis

The α-tocopherol content of the spray-dried spinach juice powders was determined by HPLC (Agilent 1100, Germany) with fluorescence detector using a modification of the method of Rogers et al. (1993). The dried lipid extracts obtained in Section 2.5 were further dissolved in 1 ml of the methanol containing 0.1% BHT and syringe filtered (0.45 µm) into a 2 ml of amber HPLC vials. Samples (10 µl) were injected through a security guard column (Phenomenex C18, 4 µm x 3.0 mm) and separated on a Zorbax RX-C8 5 µm (250 x 4.6 mm) column with the oven set at 20°C. A gradient system of two mobile phases was employed; the first solvent (A) contained 45:45:5:5 acetonitrile: methanol: isopropanol: 1% acetic acid solution and the second solvent (B) contained 25:70:5 acetonitrile: methanol: isopropanol. The flow rate of the mobile phase was set at 0.8 ml/min starting with 100% solvent A. After 6 min of solvent A running isocratically, the mobile phase changed linearly to 100% solvent B over 10 min. This was held for 12 min before being returned to the initial conditions. Detection of α-tocopherol was achieved at excitation and emission wavelengths of 298 and 328 nm and quantitation achieved using the linear formula produced from a calibration curve of external standards (4-100 µg/ml) in methanol containing 0.1% BHT.

2.9 Degradation kinetic during storage

The kinetics of experimental data were evaluated using first order kinetic (Eq.1):

$$\ln C_t - \ln C_0 = -kt \quad \text{(Eq.1)}$$

Where *k* is first order rate constant, t is storage time, C_0 is the nutrient's initial concentration while C_t is their concentration at time *t*. The reaction rate constant was determined by calculating the slope non-linear fit on linear regression plots (GraphPad 7.0) of ln (C_0/C_t) vs *t*.

The half-life ($t_{1/2}$) for the nutrient's concentration in the spray-dried spinach juice powder to degrade to 50% of their initial concentrations was calculated by (Eq.2):

$$t_{1/2} = \frac{0.693}{k} \quad \text{(Eq.2)}$$

The relationship between the reaction storage and temperature was quantified by the Arrhenius equation (Eq.3).

$$k = k_0 e^{-(Ea/RT)} \quad \text{(Eq.3)}$$

Where *Ea* is the activation energy of the reaction (kJ/mol), and k_0 is the pre-exponential constant; R is the gas constant (8.314 J mol^{-1} K^{-1}); T is the absolute temperature of the storage temperature (K).

2.10 Statistical analysis

Data were subjected to PCA (Principal Component Analysis) and ANOVA (Analysis of Variance) with 95% confidence interval. Data were analysed using the software Microsoft® Excel 15/XLSTAT©-Pro (2018.6, Addinsoft SARL, 40 rue Damrémont, 75018 Paris, France).

3. Results and discussion

3.1 Physical and chemical properties of spray-dried spinach powder

All spray-dried spinach juice powders were analysed every 14 days over 56 days. The initial β-carotene, lutein and α-tocopherol contents were 2.57 ± 0.03, 2.26 ± 0.02, and 1.01 ± 0.02 mg/g dry mass (DM). Despite of being exposed to a high temperature, the β-carotene and α-tocopherol contents in the spray-dried spinach juice were not significantly different from the contents found in a freeze-dried spinach juice (2.64 ± 0.05 and 1.02 ± 0.05 mg/g DM, respectively) (p>0.05). This may due to a short period of juice exposed to a high temperature in the spray drying chamber, and faster water removal compared to longer drying time applied in freeze drying (V. Lavelli & Corti, 2011). A comparable or superior polyphenol content of spray-dried apple pomace compared with the freeze-dried materials was also reported by Ramírez, Giraldo and Orrego (2015).

In this present study, the initial moisture content and water activity of spray-dried spinach juice powder was 6.06 ± 0.06% and 0.23 ± 0.00 a_w. The moisture content of the powder was lower than the limit for dried powder (10%) and is consistent with the moisture content in the spray-dried spinach obtained by Çalışkan Koç and Dirim (2017), but with slightly lower water activity (a_w). Throughout storage in dark, the moisture content and water activity of all samples fluctuated between 4.5 and 7.1% and 0.190 and 0.277a_w, respectively (data not shown). At the end of storage time (Day 56), there was no significant difference in moisture content between samples stored under different conditions, but in terms of water activity, the majority of samples had an a_w value of 0.200-0.220a_w, except the sample stored at 40°C and with no vacuum had an a_w of 0.260a_w (p<0.05).

On the other hand, throughout storage, the moisture content and water activity of samples stored under vacuum and at 20°C, and either in dark or under the light, fluctuated between 4.8 to 7.3% and 0.210 to 0.360a_w, respectively. The highest moisture content recorded (6.7-7.3%) and water activity recorded (0.290-0.360a_w) were the vacuum samples stored for 42 days and 56 days under light and are significantly higher than the rest of the samples (p<0.05). The variation of water activity of 0.20 to 0.35 in stored spinach powder were also reported by Çalışkan Koç and Dirim (2017). This indicated that the presence of light could affect the moisture content and water activity of the sample. However, the fluctuation of the moisture content and water activity in all storage conditions in our study may mainly due to the humidity of the storage conditions that is not controlled (Bechoff et al., 2010; Goldman, Horev, & Saguy, 1983). Regardless, the moisture content and water activity of all samples were still in the acceptable limits for safety storage.

Colour plays an influential role in the acceptance of food materials and changes in colour during storage of dried powder may affect not only the visual quality but could reflect the nutritional quality too. After 56 days of storage, regardless of the presence of air, the samples stored at 4°C retained the best colour appearance, while the samples stored at 40°C had obvious colour changes from green to olive-brown (Fig.1). Low temperature of storage were reported to has no significant effect on the chlorophyll *a* between dried spinach and frozen storage of blanched spinach stored for 6 months in both under air and under nitrogen-flushed packaging (Kidmose, Edelenbos, Christensen, & Hegelund, 2005). Meanwhile, there was no obvious change of colour observed between samples stored in the dark or light at 20°C, suggesting no or very little photochemical changes occurred to the powder matrix.

The colour changes between the samples at Day 0 and the samples stored at Day 56 were measured and determined as a total colour difference (*ΔE*). A total colour difference of more than *ΔE* 5 indicates that the colour difference can be distinguished easily by the naked eye (Obón, Castellar, Alacid, & Fernández-López, 2009). Importantly, a lower *ΔE* may indicate preservation of sensitive components like β-carotene and lutein. The lowest colour difference were samples store at 4°C non-vacuum (*ΔE* 5.04 ± 1.88) and 4°C vacuum (*ΔE* 6.17 ± 1.42), followed by 20°C vacuum (*ΔE* 7.60 ± 0.66), 20°C non-vacuum (*ΔE* 9.08 ± 2.63), 40°C vacuum (*ΔE* 15.34 ± 0.97) and 40°C non-vacuum (*ΔE* 21.43 ± 1.17). There is no significance in between vacuum and non-vacuum samples within the same storage temperature ($p>0.05$) but there is significant difference between samples stored at different temperatures ($p< 0.05$). Samples stored at 20°C, vacuum and exposed to light have scored *ΔE* 7.01 ± 1.80 and is not significant with the other samples stored at the same temperature.

This change is likely due to the degradation of chlorophyll to pheophytin where the magnesium ion (Mg^{2+}) of the chlorophyll is replaced by two hydrogen atoms, due to heat treatment and increased acidity during storage. These chemical changes affect the molecular chromophores and lead to the brown colour (Canjura, Schwartz, & Nunes, 1991; White, College, & Jones, 1963).

3.2 Chloroplast particle morphology

The green colour of chlorophyll is located in chloroplasts, which were first described by a German botanist, Hugo von Mohl, in 1837. Chloroplast sizes vary between 5 to 10 µm in long-axis diameter (Coombs & Greenwood, 1976). Microscopy of a fresh spinach juice extract showed liberated green particles around

5-10 µm in diameter (Figure 2a), without presence of plant cell wall. The images of the chloroplast showing the compact granum of chloroplast is shown in Supplementary Fig.1.

Irrespective of the presence of air and the storage temperature, there were no pronounced changes in the microstructure of the particles at the end of storage time (Fig.2 b-c, d-e, f-g). None of the stored samples showed bridging between particles indicating that there was no caking, a phenomenon that is usually associated with moisture migration within the sample (Zafar, Vivacqua, Calvert, Ghadiri, & Cleaver, 2017).

The irregularity and indentations in the spray dried particles (Fig.2b-h) are thought to be due to water evaporation during spray drying and similar changes were reported in spray-dried bioactive compound from cactus pear (Saénz, Tapia, Chávez, & Robert, 2009) and spray-dried acai encapsulated with maltodextrin (Tonon, Brabet, & Hubinger, 2008). However, the indentations are not as severe as the samples obtained by Çalışkan Koç and Dirim (2018). These authors used an inlet temperature of 160°C for spray-drying spinach juice without an encapsulant, compared to the lower inlet temperature used in this study (132°C) and this suggests that the level of heat is responsible for the degree of indentation. In spray drying, the water content of plant material like spinach is reduced significantly (from about 95 to 5%). The loss of water causes the cell structures to collapse during evaporation as can be seen in Fig 2.b-h which shows many hollow, cracked particles.

3.3 Kinetic degradation of bioactive compounds during storage

Knowledge of degradation kinetics is useful to predict the quality changes that occur during storage and determine the shelf life of a product. In general,

degradation of carotenoids in dried food systems, under the influence of one or more factors has been demonstrated to be a first order kinetic reaction (Haralampu & Karel, 1983). In this work, β-carotene was the most sensitive nutrient during storage with a rapid decrease, followed by lutein and α-tocopherol which showed more gradual decreases of content. For the β-carotene, a final loss of 68.2%, 65.3% and 70.2% when stored at 4°C, 20°C and 40°C in vacuum. Meanwhile, storage in non-vacuum at 4°C, 20°C and 40°C has the final loss of 68.3%, 71.5% and 66.2% respectively. For the lutein, a final loss of 48.5%, 46.9% and 55.2% was recorded when stored at 4°C, 20°C and 40°C in vacuum. Meanwhile, storage in non-vacuum at 4°C, 20°C and 40°C has the final loss of 47.9%, 49.6% and 57.7% respectively. The α-tocopherol had final loss of 36.6%, 39.2% and 60.2% was recorded when stored at 4°C, 20°C and 40°C in vacuum. Meanwhile, storage in non-vacuum at 4°C, 20°C and 40°C has the final loss of 37.3%, 41.1% and 59.1%, respectively.

Fitting of the data to a first order reaction equation was good with R^2 values around 0.9 (Table 1) and is in line with β-carotene degradation in dehydrated products like sweet potato (Bechoff et al., 2010; Haralampu & Karel, 1983), and carrots (Vera Lavelli, Zanoni, & Zaniboni, 2007). However, the rate of β-carotene, lutein and α-tocopherol loss/degradation are extremely high compared with other published works. High nutrient degradation rate at low water activity suggests that the reaction was autoxidative in nature, and it could be argued that the similar nutrient degradation rates in powder stored under a vacuum and in the presence of air may be due to the presence of residual oxygen in the packaging. It is known that dissolved residual oxygen is responsible for β-carotene deterioration during long-term storage, even if the headspace of the packaging is purged with N_2 (Vásquez-Caicedo, Schilling, Carle, & Neidhart, 2007). To estimate the amount of oxygen in

the vacuum-sealed samples, an oxygen indicator was included as a control. Upon opening, the indicator showed a pink colour which is equivalent to 0.1% oxygen or 0.74 Torr demonstrating the vacuum samples contained significantly less oxygen than the non-vacuum samples (20.95% oxygen or 155.50 Torr).

The calculated half-life ($t_{1/2}$) of β-carotene in samples stored in the dark, regardless of the temperature and air exposure, was around 30 to 33 days. Conversely, 50% of lutein and α-tocopherol was lost at: 56.96 to 58.25 days and 84.43 to 85.37 days respectively when stored at 4°C; 56.08 days and 70.94 to 73.44 days respectively when stored at 20°C; and 45.63 to 49.98 days and 42.34 to 42.45 days respectively when stored at 40°C (Table 1). At lower temperature, α-tocopherol had a higher half-life compared to β-carotene and lutein. The lower half-life of β-carotene and lutein could be due to the opening of the carotenoid's β-ionone ring and the chromophore extension into apocarotenols which is more amenable to reaction with peroxyl radicals ($ROO^{\bullet}$) compared to α-tocopherol (Supplementary Fig.1) (Mordi, 1993; Pénicaud, Achir, Dhuique-Mayer, Dornier, & Bohuon, 2011; Rodrigues, Mariutti, Chisté, & Mercadante, 2012). In addition to that, thermal stability of α-tocopherol is due to the increase of steric hindrance by the extra methyl group(s), thus reducing the rate of addition and polymerization reactions (Supplementary Fig.2) (Al-Khusaibi, Gordon, Lovegrove, & Niranjan, 2012; Sabliov et al., 2009).

The rate constant for lutein loss was slightly lower than β-carotene in all conditions, suggesting that lutein has relatively greater heat resistance, similar to the stability of carotenoids reported in dehydrated pumpkin (Song et al., 2018), and in a light-induced model system (Xiao et al., 2018). In addition to that, the low R^2 for the

Ea of β-carotene in this sample indicated that Arrhenius equation could not be applied as it is temperature independent and is very labile even at 4 ˚C.

The degradation rate of exposed β-carotene at the surface is higher compared with internal β-carotene due to exposure to the air (Stefanovich & Karel, 1982). The activation energy (*Ea*) for the degradation reaction of total carotenoids (10.2736 kJ/mol) in maltodextrin-encapsulated spray-dried spinach juice (Çalışkan Koç & Nur Dirim, 2017) is higher than the *Ea* of β-carotene in a non-encapsulated spray-dried spinach juice in this study. This suggests that even though the spray drying of spinach juice without any encapsulant yielded a high total solid or product recovery, an addition of encapsulant can provide a more stable products that is protected from the environment, and especially against oxygen diffusion (Mahfoudhi & Hamdi, 2015). Regardless, the *Ea* of total carotenoids reported for encapsulated spray-dried spinach juice (Çalışkan Koç & Nur Dirim, 2017) is still low compared with the *Ea* of β-carotene in the other dehydrated products like sweet potato chips (64.2 kJ/mol) (Bechoff et al., 2010), and sliced pumpkins (13.82 kJ/mol) (Song et al., 2018).

The high reaction rate, low half-life and low activation energy of β-carotene, lutein and α-tocopherol degradation in the spray-dried spinach juice is probably due to oxidation. At this stage we cannot rule out the possible role of catalysts such as enzymes (though their activity at low water actives seems unlikely); nor can we discount the action of other reactive oxygen species such as hydrogen peroxide (H_2O_2). Due to the nature of the juice that was used, fresh and not heat-treated, the presence of active enzyme is possible. Enzyme not only reduce the energy barrier, but also induced completely different reaction pathways such as carotenoid hydroxylation by β-carotene hydroxylase (Supplementary Fig.3) (Davison, Hunter, & Horton, 2002). Hydrogen peroxide (H_2O_2) is widely used as packaging sterilant and

may cause autoxidation. Hydrogen peroxide can decompose and form highly reactive products like hydroxyl radical ($^{\bullet}OH$). The presence of H_2O_2 residues as low as 0.5ppm, which is the maximum allowed level allowed by the FDA in the food packages can caused rapid degradation of anthocyanins in fruit juices (Özkan, Yemenicioğlu, & Cemeroğlu, 2005). Therefore, an action must be made to ensure a complete inactivation of endogenous enzyme, and protection from peroxide residues to obtain good quality of spray-dried spinach juice.

The spray-dried spinach juice powder used in this experiment contained around 9-13% of lipid, of which 60% and 10% of it is α-linolenic acid (triunsaturated) and linoleic acid (diunsaturated), respectively (data not shown). The higher the degree of fatty acid unsaturation, the higher the rate of autoxidation; the initial (intermediate) product of fatty acid autoxidation is a lipid hydroperoxide, this can act as a pro-oxidant that oxidised other organic molecules such as carotenes (Budowski & Bondi, 1960; Holman, 1954). The oxidation of carotene is autocatalytic and begins after an induction period in which radicals are built up and antioxidants are depleted (Stefanovich & Karel, 1982). It is also possible for lipid hydroperoxides to be generated via the action of lipoxygenase enzymes; in our system these enzymes could be active during spinach leaf grinding, generating oxidants that take time to react in a dry system.

3.4 Effect of storage temperature

Storage temperature significantly impacted the nutrient stability. Samples kept at 4°C showed the highest retention of nutrients at each time interval (p<0.05) (Figure 3). The PCA plots that are presented in Supplementary Fig.4a-b show that samples stored at 40°C did not cluster with the samples stored at lower temperature

within the same days except for day 28 at 20°C in vacuum due to insignificant differences in α-tocopherol content (Supplementary Table 1); indicating that the losses of β-carotene and lutein are more rapid at high storage temperature. After 56 days of storage, regardless of the presence or absence of air, β-carotene experienced extreme losses in samples stored at 4°C (68.2-68.3%), 20°C (65.3.-71.5%) and 40°C (66.2-70.2%). Under the same conditions, lutein had lower losses at 4°C (47.9-48.5%), followed by 20°C (46.9-49.6%) and 40°C (55.2-57.7%). Meanwhile, α-tocopherol loss was 36.6-37.3% at 4°C, 39.2-41.1% at 20°C and 59.1-60.2% at 40°C. A three-way ANOVA confirmed that throughout 56 days of storage, regardless of the presence of air, there was a decrease but not significant in nutrient contents during storage at 4 and 20°C (p>0.05), whereas samples stored at 40°C experienced higher losses (p<0.0001) from those at 4°C (Supplementary Table 1). This probably due to the free radical activity that is more active at high temperature. These findings support evidence from previous observation that higher storage temperature has a great influence on the stability of carotenoid pigments of dried sweet potatoes (Bechoff et al. 2010).

3.5 Effect of oxygen exposure

The effects of storage time over a period of 56 days and storage temperatures 4°C, 20°C and 40°C in vacuum and non-vacuum samples on nutrient stability of spray-dried spinach juice were studied (Figure 4). Contrary to expectations, this study did not find a significant effect of air on the nutrient stability of spray-dried spinach juice. In general, both vacuum and non-vacuum samples stored at 4°C retained nutrients better than the rest of the storage conditions (p<0.0001) (Supplementary Table 1). The PCA results (Supplementary Fig.5a-c) showed that

most of the vacuum (V) and non-vacuum (NV) samples for the same days clustered together, indicating negligible difference of nutrient retention between the samples. An exception was observed for Day 28 samples stored at 20°C (Supplementary Fig.5b) where non-vacuum samples retained significantly better lutein and α-tocopherol concentration. This result contradicts a previous study on the effect of oxygen exposure on the degradation of carotenoids in sweet potato flakes stored for more than 140 days in a (Bechoff et al., 2010; Goldman et al., 1983; Walter & Purcell, 1974). This is possibly due to the short observation period applied in work presented here which is only 56 days, and without pre-established water activity by adjusting the relative humidity of the sample using salt as employed by Bechoff et. al (2010) and Goldman et. al (1983).

3.6 Effect of light exposure

Interestingly, the effect of light exposure at 20°C did not have a significant impact on the nutrient loss (p>0.05) (Table 3). Since unsaturated fatty acids are present in the extracts, it might be expected that some photo-oxidation might occur in sample stored in the light. However, in the PCA plots (Fig 3.d), it can be seen that the major factor driving degradation is storage time. The samples stored in the light and in the dark appear in the same cluster (D0-D14; D42-D56), and two-way ANOVA confirmed there were no significant differences in nutrient contents of these samples (p>0.05). The only exception is for the samples stored for 28 days where the samples stored in the dark had higher lutein and α-tocopherol retentions (p<0.05, p<0.0001) but this result seems more like an outlier than a significant trend. Results may also be due to the low light intensity of the fluorescent lamp (600-800 lux) that was used in this study, compared to the significant impact of light on the colour and

odour of Harvati cheeses (1300 lux) (Mortensen, Sørensen, & Stapelfeldt, 2002), carotenoids in carrot juice (1500 lux) (Chen, Peng, & Chen, 1996), and raw spinach in cold storage (2000 lux) (Kopas-Lane & Warthesen, 1995).

To observe the correlation of the β-carotene, lutein and α-tocopherol concentration in all storage conditions tested in this study, a principal component analysis was carried out (Supplementary Fig.5). Due to many overlapping data points, which made the score plots difficult to interpret, four separate plots were prepared; three plots for storage in the dark at 4°C, 20°C and 40°C for vacuum and non-vacuum samples (Fig.5a-c), and one plot for storage in vacuum at 20°C in dark and exposed to light (Fig.5d). In all plots the amount of variation explained by the F2 axis was very low (<5%) indicating that the factors behind the F1 axis were driving the degradation of the carotenoids.

In all PCA analysis plots for the samples stored under vacuum and non-vacuum (Fig5.a-c), the major factor is storage time (D0-D56) and this is related to the F1 axis. An ANOVA analysis confirmed that the storage time was the major factor in nutrient degradation compared to the effects of temperature and the presence of air (p<0.05). This study supports results from previous observations. For example, storage of dehydrated carrots at 40°C for a month (Vera Lavelli et al., 2007) and storage in different packaging materials at room temperature (Singh, Kulshrestha, & Kumar, 2013) also showed that time was the major factor in nutrient degradation.

3.8 Additional data

After 365 days of storage, additional measurements were carried out on the vacuum sample stored at 4°C in dark, and non-vacuum sample stored at 20°C in

dark. The conditions of vacuum sample at 4°C in dark was chosen as it is the best storage condition to keep the powder, whilst the non-vacuum sample at 20°C in dark is the ambient storage commonly applied in a dry food warehouse or on the market-shelf.

Surprisingly, the sample stored in vacuum at 4°C dark still retained 26.24% of β-carotene and 35.40% of lutein (0.69 ± 0.05 and 0.80 ± 0.03 mg/g dm, respectively), meanwhile the sample stored in non-vacuum at 20°C dark still retained 20.15% of β-carotene and 14.60% of lutein (0.53 ± 0.03 and 0.33 ± 0.03 mg/g dm, respectively). This suggests that there is a further loss of nutrients during storage after 56 days to 365 days (about 23.5 to 35.4%), but the rate of loss is slower than during the first 56 days. Therefore, it could be possible for spray-dried spinach juice to retain enough nutrients to be viable for health benefit. With this limited investigation, intervention of technology to avoid further loss of nutrients in the spray-dried spinach juice powder is needed.

4. Conclusions

This study showed that direct spray drying of spinach juice (i.e. without carriers) could be a method to produce a potential functional food as it largely preserved the key nutrients measured. Although moisture content and water activity of the powders fluctuated to some extent (4.5-7.3%; 0.19-0.36 a_w) this did not appear to affect the concentration of target nutrients during a period of storage. However, temperature and length of storage had a significant impact on the stability of β-carotene, lutein and α-tocopherol. Reducing oxygen levels by vacuum packaging and excluding light intensity lower than 800 lux did not improve the stability of micronutrients. It is possible that residual oxygen in the powder is sufficient to cause

the degradation of the nutrients under study, or that other molecular oxidants (endogenous or exogenous) are present. These results will guide future work to design experimental approaches to reduce nutrient degradation, for example by different juice preparation methods and the potential use of encapsulants that could protect the micronutrients from oxidation.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure 1

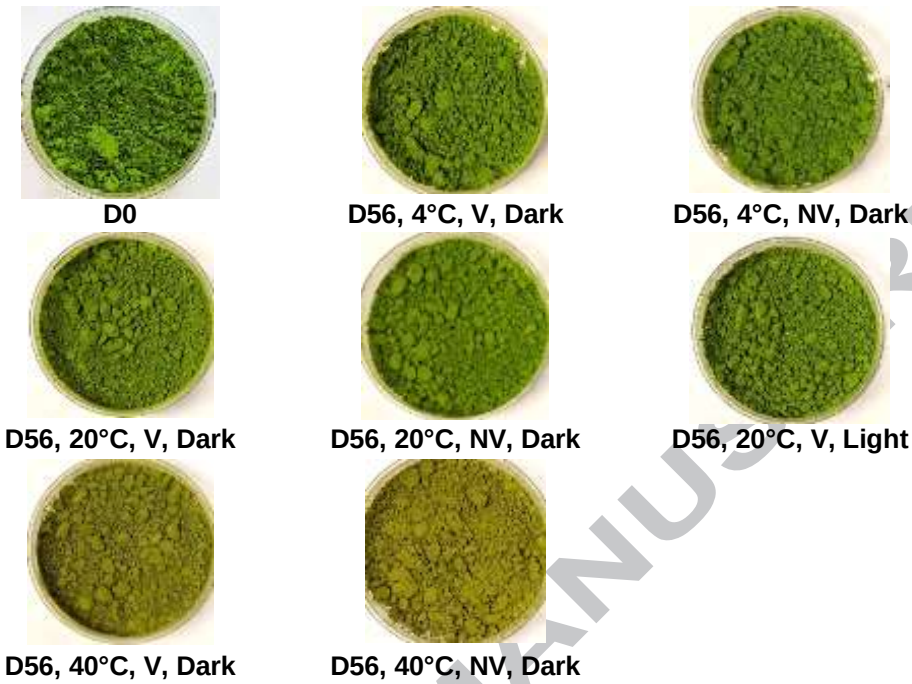

Figure 1: Images of spray-dried spinach juice powder at D0 and D56.
(D: Day, V: Vacuum, NV: Non-Vacuum)

Figure 2

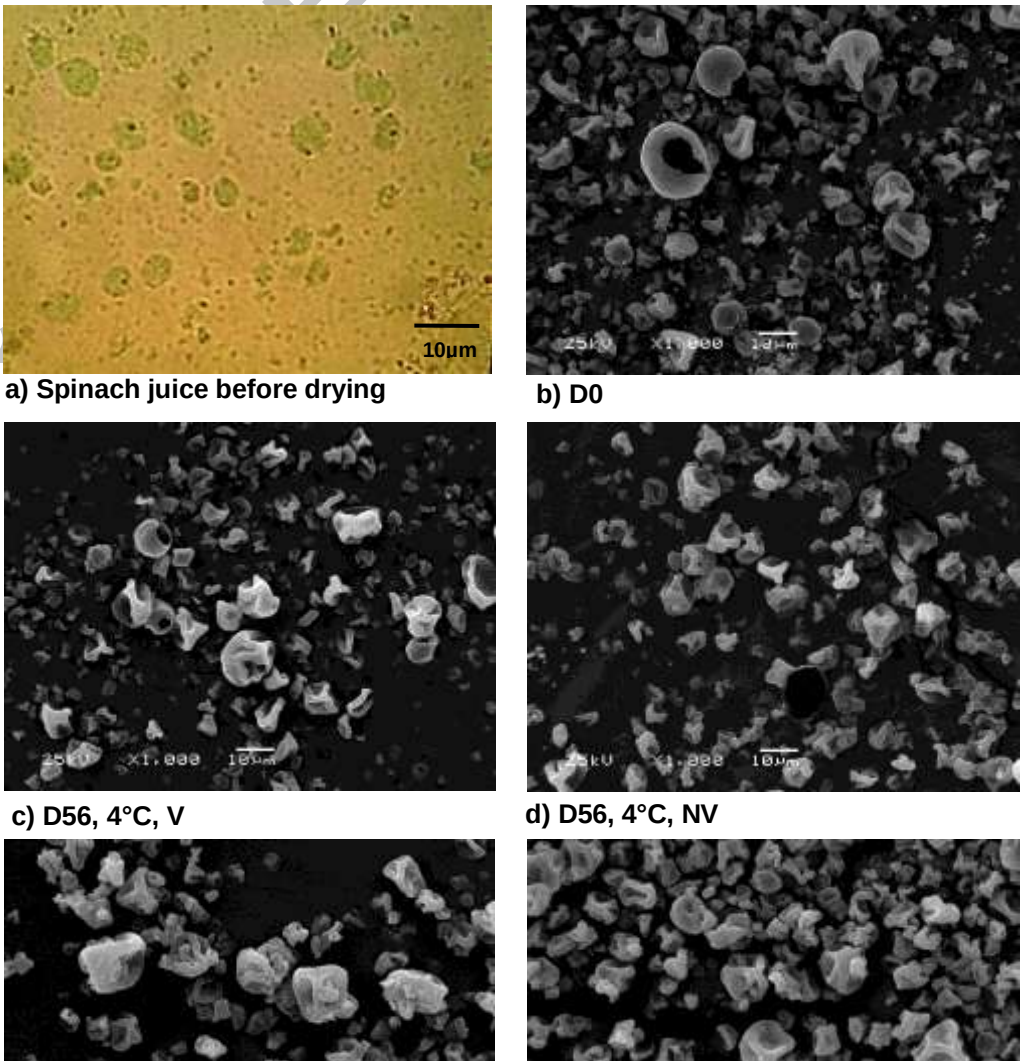

Figure 3

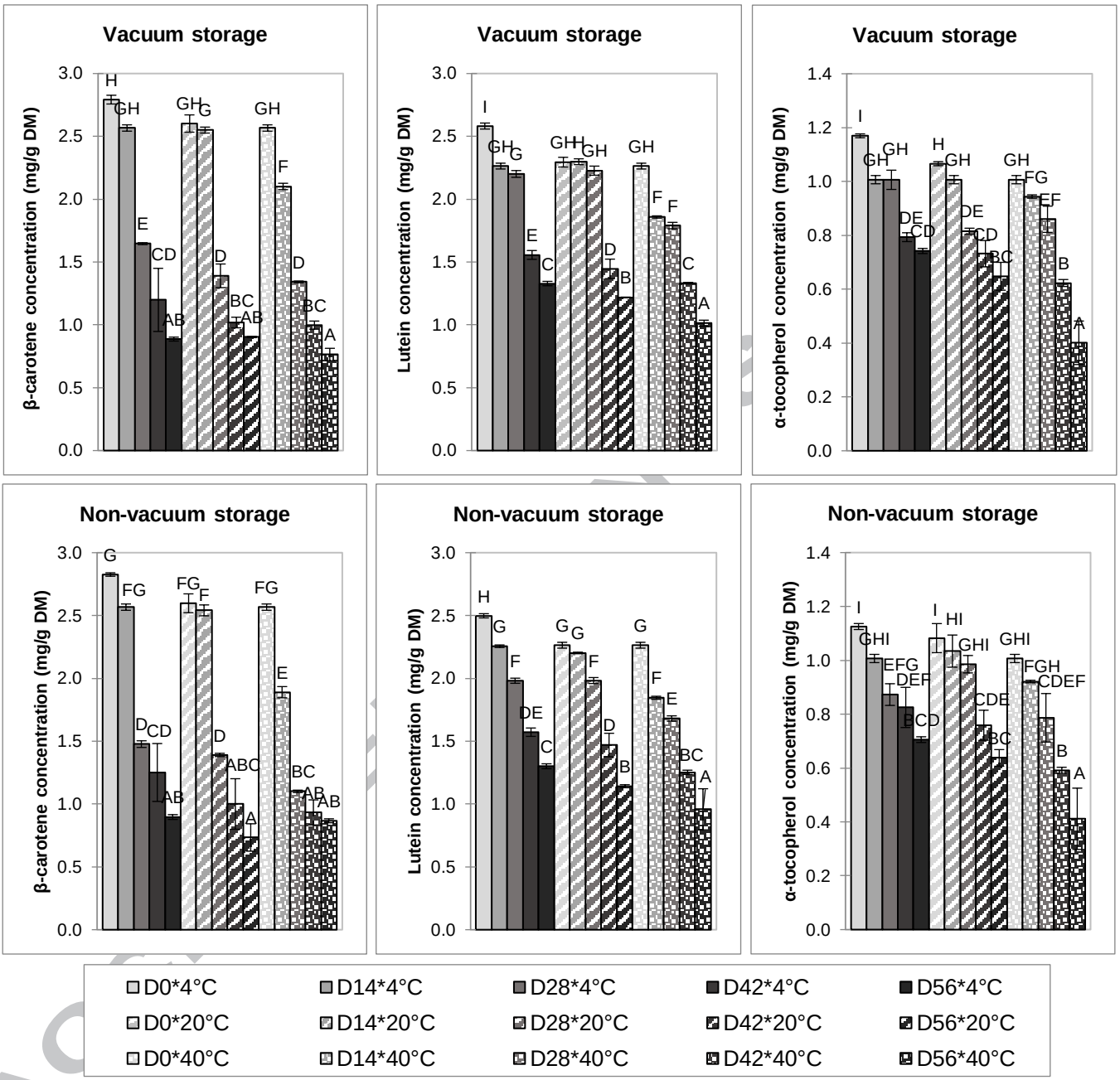

Figure 3: The effect of storage temperature (4, 20 and 40˚C) in vacuum and non-vacuum packaging on degradation of β-carotene, lutein and α-tocopherol in spray dried spinach powder.

Both types of packaging materials excluded light from the samples. Mean values with different letters indicate statistically significant differences according to Tukey-test at $p<0.05$.
(D: Day)

Figure 4

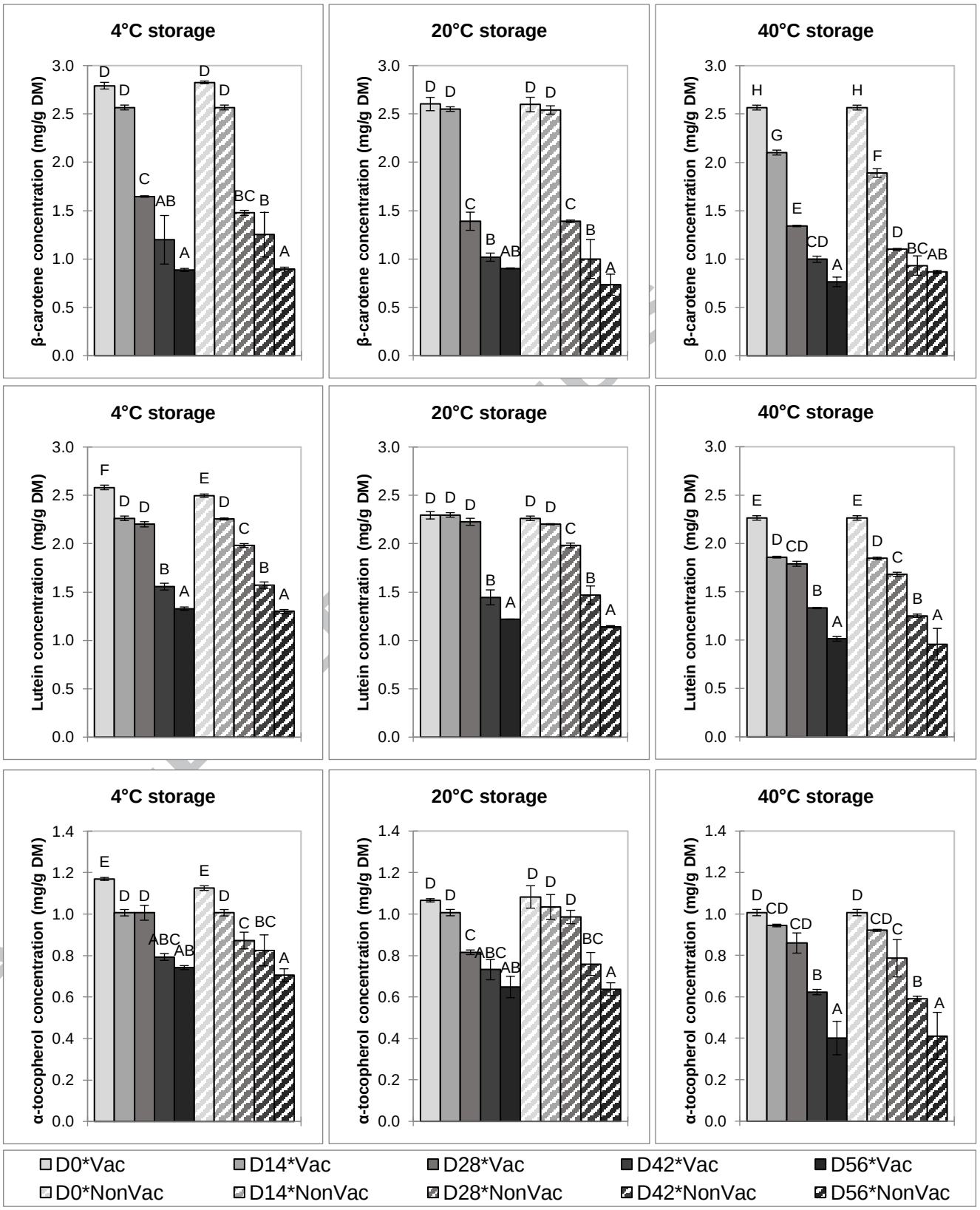

Figure 4: The effect of vacuum and non-vacuum packaging on degradation of β-carotene, lutein and α-tocopherol in spray dried spinach powder at different e temperatures during storage.

Both types of packaging materials excluded light from the samples. Mean values with different letters indicate statistically significant differences according to Tukey-test at $p<0.05$.
(D: Day, Vac: Vacuum, NonVac: Non-Vacuum)

Figure 5

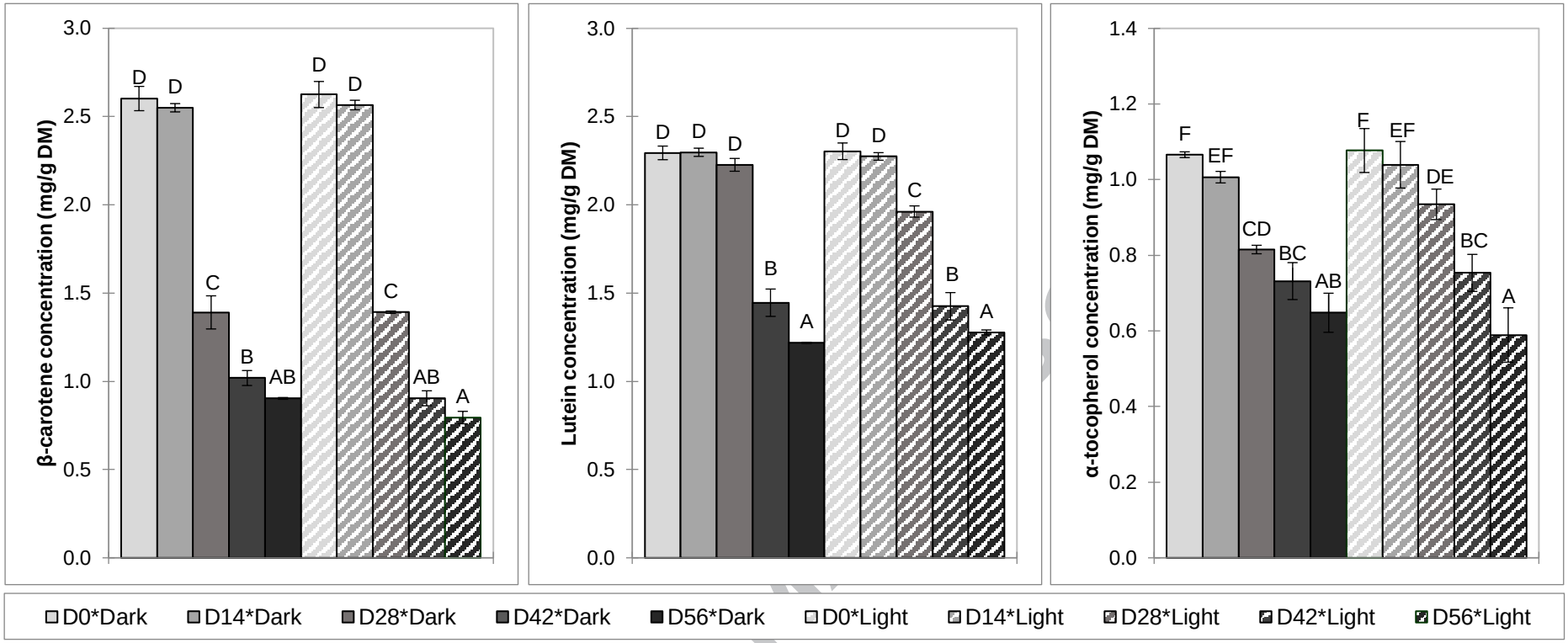

Figure 5: The effect of the presence of light on spray-dried juice powder properties stored at 20°C and vacuum for 56 days. Mean values with different letters indicate statistically significant differences according to Tukey-test at $p<0.05$

Table 1

Table 1: Kinetic and thermodynamic parameters of β-carotene, lutein and α-tocopherol during degradation derived from experimental data.

Compounds	Temperature (°C)	Vacuum	Light	k (day^{-1})	R^2	$t_{1/2}$ (day)	Ea (kJ/mol)	R^2
β-carotene	4	Yes	No	2.2×10^{-2}	0.9461	31.62	0.665	0.5355
	20	Yes	No	2.1×10^{-2}	0.9201	31.99		
	40	Yes	No	2.3×10^{-2}	0.9852	30.62		
	4	No	No	2.2×10^{-2}	0.9391	32.08	1.089	0.0755
	20	No	No	2.1×10^{-2}	0.9201	31.99		
	40	No	No	2.1×10^{-2}	0.9111	33.68		
	20	Yes	Yes	2.5×10^{-2}	0.9309	28.84	-	-
Lutein	4	Yes	No	1.2×10^{-2}	0.9247	56.96	2.650	0.8584
	20	Yes	No	1.2×10^{-2}	0.8192	56.08		
	40	Yes	No	1.4×10^{-2}	0.9459	49.98		
	4	No	No	1.2×10^{-2}	0.9747	58.25	4.923	0.9430
	20	No	No	1.2×10^{-2}	0.8192	56.08		
	40	No	No	1.5×10^{-2}	0.9346	45.63		
	20	Yes	Yes	1.1×10^{-2}	0.9156	58.94	-	-
α-tocopherol	4	Yes	No	0.8×10^{-2}	0.9245	84.43	13.893	0.9118
	20	Yes	No	1.0×10^{-2}	0.9377	73.44		
	40	Yes	No	1.6×10^{-2}	0.8279	42.45		
	4	No	No	0.8×10^{-2}	0.9360	85.37	14.142	0.945
	20	No	No	1.0×10^{-2}	0.8614	70.94		
	40	No	No	1.6×10^{-2}	0.8257	42.34		
	20	Yes	Yes	1.1×10^{-2}	0.8646	63.19	-	-

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Highlights

1. A high product recovery of spray-dried juice without encapsulant is achievable.
2. Excluding low light intensity and air did not improve the stability of micronutrients.
3. The rate of β-carotene, lutein and α-tocopherol loss displayed first order reaction kinetic.
4. The *Ea* of β-carotene, lutein and α-tocopherol is 0.7, 2.7 and 13.9 kJ/mol for vacuum-pack sample.
5. The nutrients in the dried spinach juice has low half-life and low activation energy.