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Proceedings

Odesa, ONUT
2023

THE EFFECT OF DIFFERENT STORAGE TEMPERATURES AND LIGHT EXPOSURE ON CAROTENOID STABILITY OF MARIGOLD FLOWERS (*Tagetes erecta* L.)

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Abstract. *Marigold (Tagetes erecta L.) is a medicinal and ornamental plant widely known for its health-promoting properties and its coloring effect on poultry skin and egg yolk. These properties result from the high content of bioactive ingredients, i.e. carotenoids, which are sensitive to environmental factors such as temperature, light, and oxygen. The aim of the study was to investigate the effect of temperature (4, 25 and 40 °C) and light exposure on the content and degradation of total carotenoids (TC) in two marigold meals during storage period of 77 days. The initial TC content in meal B (13.63 mg/g DM) was higher than the initial TC content in meal A (10.35 mg/g DM) due to the dilution effect of the leafy parts (high chlorophyll concentration). The lowest TC content was found in both meals (A and B) in the samples stored at 40 °C (6.09 mg/g DM and 5.88 mg/g DM, respectively). Consequently, the highest TC degradation percentages and rates were obtained in both meals when stored at 40 °C (41.15 vs. 56.95% and 0.0065 vs. 0.0099 day^{-1}, respectively). Exposure of the meals to light contributed to TC degradation. The optimal conditions for storage of marigold flowers were found to be 4 °C and 25 °C without exposure to light. Although the recommended storage temperatures for carotenoid sources are close to 0 °C, the results suggest that storage at 25 °C without exposure to light could be a cost-effective alternative.*

Keywords: *marigold flower (Tagetes erecta L), total carotenoids, degradation, storage conditions*

I. INTRODUCTION

The use of edible flowers as food supplements dates back to ancient times, as they positively affect the organoleptic properties of traditional foods and have a decorative effect [1-3].

The most commonly used were ornamental flowers such as mallow (*Malva sylvestris*), acacia (*Robinia pseudoacacia*) [1], dahlia (*Dahlia mignon*), calendula (*Calendula officinalis*), and marigold (*Tagetes erecta* L.) [1,3]. Over the past few decades, due to the great interest in gastronomic innovations and functional foods [1,3], numerous scientific studies have been conducted on the nutritional and health-promoting properties of edible flowers. Plant tissues, including flowers, are rich in bioactive compounds such as polyphenols, carotenoids, vitamins, and essential oils [1-3], which have shown antioxidant [1,3,4], hypoglycemic [3,4], antimicrobial [3,5,6], anticancer [8,9] and anti-inflammatory properties [10], beneficial effect on degenerative retinal diseases [7], and other health-related benefits.

The content of these phytochemicals in plant flowers depends on the plant species. Anthocyanins, a subclass of the polyphenol family, for example, are characterized as blue, purple, and reddish pigments [1,3,11], which are abundant in blue peas (*Clitoria ternatea*) [11]. On the other hand, carotenoids are recognized as yellow and orange pigments [1,3] found in citrus fruits, vegetables (corn, watercress, carrot, sweet potato, tomato) [13] as well as ornamental flowers such as chrysanthemum (*Chrysanthemum crassum*) and marigold [14].

Marigold is an ornamental annual plant native to Mexico [15] and its flowers are considered an important source of carotenoids, especially lutein, zeaxanthin and β-carotene [15-17]. According to literature data [14,18], lutein and its esters comprise more than 80% of carotenoids found in marigold petals. Therefore, marigold is widely used in poultry nutrition as an affordable feed additive that improves the colour of poultry skin [19] and egg yolk [17,19-26]. Furthermore, due to the higher content of carotenoids (especially lutein) in eggs, consumption of these enriched eggs may also reduce the risk of age-related macular degeneration [19,20,27,28], cataracts [26-28], and damage of eye photoreceptors caused by blue light [19].

One of the carotenoid properties is their sensitivity to light, heat, presence of oxygen [14,29,30], acidity, water content [30] and enzymatic factors [30] during storage and post-harvest processes. In order to achieve the full nutritional potential of marigold flowers, the stability of their carotenoids during storage should be investigated.

II. LITERATURE ANALYSIS

Growing consumer awareness about the negative health impact of synthetic food supplements (colorants, antioxidants, flavour enhancers) led scientists to explore the possibility of using ornamental and medicinal plants as a source of carotenoids in human and animal nutrition. Many studies have been conducted to determine the composition of carotenoids in different fruits, vegetables and cereals [27,31,32]. However, there is a scarcity of data in literature on determining the carotenoid composition of ornamental plants.

Marigold is one of the few ornamental flowers whose carotenoid profile has been thoroughly investigated [33,34]. Nevertheless, little attention has been given to the influence of harvest and postharvest processes on carotenoid retention; only a few studies could be found in the available literature. Kurniawan et al. (2019) investigated the effects of several drying methods on the content of all-*trans* isomers of lutein and zeaxanthin: freeze drying (-45 °C), sun drying (39 °C), vacuum oven (40 °C) and conventional oven drying (40 and 100 °C) methods [35]. The authors showed that drying treatments at high-temperature caused carotenoid degradation due to the isomerization. Higher lutein and zeaxanthin contents were observed in marigold flowers dried using lower – temperature methods (freeze drying and vacuum oven), while sun drying proved to be an affordable alternative method. Similar results were observed in the study by Siriamornpun et al. (2012) [17], where freeze drying (-40 °C) and far-infrared radiation with hot air convection (40 °C for 60 min) resulted in highest lutein and lycopene content. Hot air drying (40 °C for 4 h) resulted in the highest

content of β-carotene. These results indicate that not only the different drying methods lead to changes in carotenoid content, but also that temperatures during storage could cause the degradation of carotenoids.

According to the available literature data, the effect of storage conditions on the carotenoid composition of marigold flowers is a poorly explored area. Akshaya et al. (2017) [16] investigated the effect of three different storage temperatures (-20 °C, 4 °C and 25 °C) on the retention of carotenoids during 60 days of storage. The study revealed that carotenoid degradation was lowest when marigold flowers were stored at -20 °C, followed by 4°C and 25 °C, regardless of variety. In general, further analyses of the effects of temperature and other storage conditions (e.g. exposure to light) on marigold flowers are required. Therefore, aim of the present study was to investigate the effect of temperature (4, 25 and 40 °C) and exposure to light on the content and degradation of total carotenoids (TC) in two marigold flower meals during storage of 77 days.

III. OBJECT, SUBJECT, AND METHODS OF RESEARCH

Two batches of marigold flower meals (*Tagetes erecta* L.) were obtained from the manufacturer TRAKO-AGROLUDBREG d.d. (Ludbreg, Croatia). Marigold flower meals differed in the parts of the plant used for their production – by grinding the whole flowers (A) or by grinding only the petals (B; Figure 1.). Regardless of which part of the plant was used for production, they were air-dried and ground after collection.

Figure 1. Two marigold flower meals used in the study prepared from whole flowers (A) and only from the petals (B)

Sample preparation and storage conditions. Each batch of marigold flower meal was thoroughly mixed and diveded into 12 parts. About 50 g of each part was sampled in a plastic container with a screw on cap; in total, there were 24 containers.

The prepared containers with meals were stored at four different conditions during 11 weeks:

- 4 °C in the dark (stored in the refrigerator),
- 25 °C in the dark (sample wrapped with aluminum foil, stored in a incubator without a light source),
- 25 °C exposed to light (stored in a incubator with a light source),
- 40 °C in the dark (stored in a incubator without a light source)

For each marigold meal, there were three replicates (three containers) for each storage condition (2 marigold meals × 4 storage conditions × 3 replicates), and the experiment was set up according to the completely randomized design. From each plastic container, samples for analysis were taken on day 0, 7, 14, 21, 28, 35, 49, 56, 63 and 77 of the experiment.

The analysis was carried out immidiately after sampling. All samples were ground using the ball grinder (MM 200, Retch, Germany) and the moisture content was determined by drying at 103 °C for four hours.

Carotenoid extraction. The procedure was performed using hot saponification and subsequesnt extraction of the carotenoids with hexane. Briefly, 50 mg of the sample was weighed into a 15 mL glass tube. Then, 300 μL of ethanol containing 12% of the antioxidant BHT (2,6-di-tert-butyl-4-methylphenol) and 200 μL of the solution of the internal standard trans-β-apo-8′-carotenal (Sigma Aldrich, Germany) were pippeted in each tube. After mixing, 6 mL of methanol (containing 6% of KOH) was pippeted into each tube and the mixture was homogenized (T10, Ika, Germany; 30s at maximum speed of rotation). The samples were then incubated at 60 °C in water bath (Waterbath, 5L, Cole Parmer, USA) for 15 min. Immidiately after incubation, the tubes were placed in ice bath. To each tube, 3.5 mL of 10 % NaCl was added, followed by 2.5 mL of hexane. The mixture was vortexed for 30 s and the tubes were centrifuged (5 min, 4000 rpm; Centric 322A, Tehtnica, Slovenia). The upper hexane layer was separated into a separate tube and the extraction procedure with hexane was repeated until the hexane layer had discoloured (typically 11 times). After extraction, the combined hexane extracts were evaporated using a rotary vacuum concentrator (RVC 2-25CD plus, Martin Christ, Germany). The dry residue was dissolved in 200 μL of acetonitrile:methanol:dichloromethane (45:20:35, v/v/v).

Carotenoid quantification. The separation and quantification of carotenoids were carried out according to RP HPLC method described by Kurilich and Juvik (1999) [36]. The column system was composed of two sequentially connected C18 reversed-phase columns, Vydac 201TP54 (5 μm; 4.6 × 150 mm; Hichrom, Reading, UK) and Zorbax RX-C18 (5 μm; 4.6 × 150 mm; Agilent Technologies, USA). The columns were protected by Supelguard Discovery C18 guard column (5 μm; 4 × 20 mm; Supelco, USA) by a C18 protection column. The SpectraSystem HPLC instrument (Thermo Separation Products, Inc., USA) consisted of a quaternary gradient pump (P4000), a degassing system (SCM 1000), an autosampler (AS3000), a column heater and a UV/VIS (UV2000) detector. The mobile phase consisted of acetonitrile:methanol:methylene chloride (75:20:5, v/v/v) containing 0.05% triethyl-amine and 0.1% BHT. The mobile phase flow was set to 1.8 mL/min at room temperature, while the samples were injected using a 20-μL loop. The carotenoids were detected at 450 nm, and the total run was 40 min.

The sum of areas of all peaks separated during the run was identified as total carotenoids, and the content of total carotenoids was quantified as β-carotene equivalents by external standardization with calibration curve of β-carotene (Sigma-Aldrich, Germany; $r^2 = 0.99$). Each replicate was anlysed in duplicate and the mean value was taken as the result.

Kinetics of Carotenoid Degradation and Data Analysis. Kinetics of carotenoid degradation during storage was calculated by first order kinetics (Hidalgo and Brandolini (2008) [31] according to the equation:

$$C=C_0 \times e^{-kt}$$

where C is defined as a concentration of total carotenoids at the time (t) during storage (day 0, 7, 14, 21, 28, 35, 49, 56, 63 and 77), C_0 as the initial or carotenoid content at the beginning of storage, and k is degradation rate (day^{-1}).

SAS statistical software (version 9.4) [37] was used for the nonlinear curves data processing. Nonlinear parameters of carotenoid degradation that follow the first order kinetics, i.e. initial content and degradation rate of carotenoids, were determined using NLIN statistical procedure. Marquardt method (iterative curve adjustment procedure) was used for determination of the least sum of squares of the residual associated with the regression model.

Statistical analysis. The experiment was carried out as a randomized complete experiment with three replicates. Statistical analysis of the obtained results was performed using SAS statistical software [37], with the results analyzed as repeated measurements with fixed effect of meal type, storage conditions and their interactions using the MIXED procedure with SP(POW) as the covariance structure for time intervals and Tukey's test to assess the significance of multiple comparisons differences. Mean values and standard error were determined using the LSMEANS procedure, while mean differences were determined using the PDIFF procedure. The threshold for statistical significance was defined as $P < 0.05$.

IV. RESULTS

The initial TC content was higher in meal B (13.63 mg/g DM) than in meal A (10.35 mg/g DM) (Fig.2). These results can be explained by the different composition of carotenoids in petals, leaves and stems [33,38,39]. Namely, leaves and stems contain high amounts of chlorophyll, whereas the petals contain a high amount of the carotenoids xanthophylls (lutein, β-cryptoxanthin, zeaxanthin) which are responsible for the colour of the petals [33,38,39]. Considering that meal A was prepared by milling the whole marigold flowers, the leafy parts of the flower demonstrated diluting effect, and consequently, caused a lower initial TC content in meal B. According to Park et al. (2017) [40], the qualitative and quantitative carotenoid composition of plants depends not only on the proportion of leaves/petals, but also on different biological (cultivar/variety, developmental stage), environmental (agricultural) and post-harvest processes. Moreover, their interactions should be taken into account.

During 77 days of storage, the TC content decreased in both meals (Fig. 2; $P<0.001$). However, the changes in TC content during storage under different conditions differ between meal samples. In meal A, differences between sampling times were determined form 21st day of storage ($P<0.05$). On the 77th day of storage, the highest TC content was determined in samples stored at 25 °C without exposure to light (9.04 mg/g DM), followed by storage at 4 °C (8.86 mg/g DM) and at 25 °C with exposure to light (8.07 mg/g DM). The lowest TC content in meal A was determined

in samples stored at 40 °C (6.09 mg/g DM). Similarly to the presented results, Song et al. (2018) [32] reported the lowest TC in samples of dehydrated pumpkins stored at 40 °C in study investigating the effect of storage temperatures (4, 25 and 40 °C) on TC content after 210 days of storage.

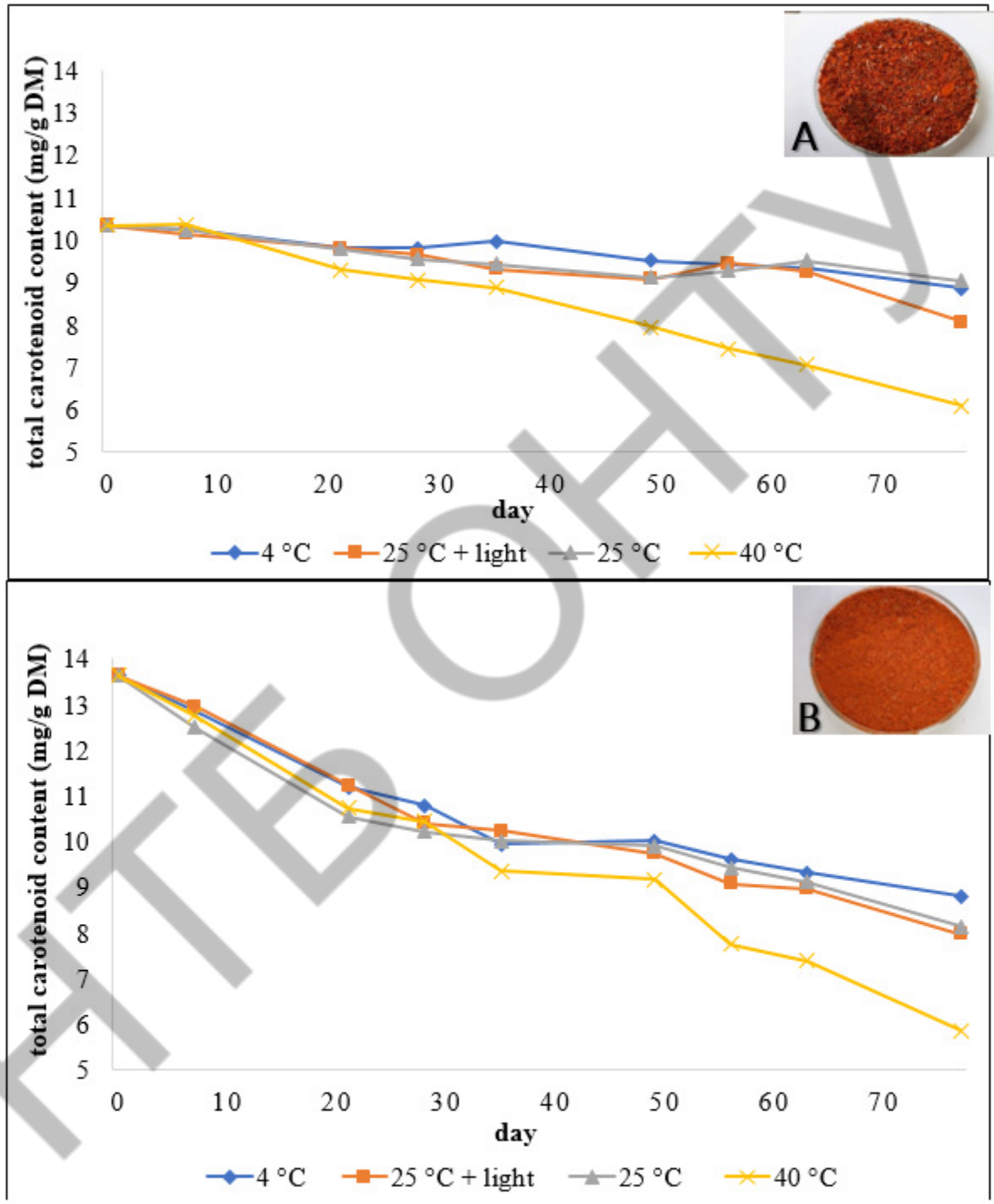

Figure 2. The effect of four storage conditions on the total carotenoid content (mg/g DM) in a meal prepared from whole marigold flowers (A) and in a meal prepared only from marigold petals (B) during 77 days of storage.

In contrast to meal A, changes in TC content in meal B began to decrease immediately after the beggining of storage ($P<0.05$) resulting in a steeper decrease in TC content during the storage period (Fig. 2). After 77 days of storage, the highest TC content was determined in the samples stored at 4 °C (8.81 mg/g DM), while the lowest TC content was found when stored at 40 °C (5.88 mg/g DM). There was no significant difference in TC between meals B stored at 25 °C with exposure to light (7.99 mg/g DM) and without exposure to light (8.15 mg/g DM). Different patterns of decrease in the TC content can be attributed to the absence of the leafy-parts (high chlorophyll content) in meal A, which in some manner reduce the degradation of carotenoids due to their dilution effect in meal B. Furthermore, as can be seen in Fig.1, meal B (prepared by milling only the petals) contained smaller particles that allowed greater exposure to oxygen. Results similar to the decrease in TC content in meal B were obtained in the study by Akshaya et al. (2017) [16]. The authors reported higher TC content in the extract of marigold petals stored at 4 °C compared to extract stored at ambient temperature after 60 days. Due to the polyene chain and terminal groups in their structure [41], carotenoids are, sensitive to oxidation, exposure to light and elevated temperatures [39,41]. These effects lead to the isomerization of carotenoids [39,41], the accumulation of mono- and di-epoxides, carbonyls, alcohols, etc. [39] and consequently to a lower TC content. According to Schweiggert et al. (2006) [41], isomerization can also occur during extraction and saponification of the carotenoids.

In agreement with obsererved differences in TC content between meal types during storage, meal type affected degradation percentage at all timepoints during storage ($P<0.001$; Table 1.). Furthermore, after 21st day of storage, the differences in TC degradation percentage in both meals were detected between storage conditions ($P<0.05$). The degradation precentage of TC was lower in meal A than in meal B ($P<0.001$), regardless of the storage conditions and the duration of storage. More so, a twofold to even threefold higher TC degradation was observed in a meal A at 40 °C compared to the other storage conditions from 49th to 77th day of storage. At the end of the experiment, the highest degradation percentage was determined in samples of meal A stored at 40 °C, followed by storage at 25 °C with exposure to light, and storage at 25 °C without exposure to light and storage at 4 °C, with the two later storage conditions showing no significant difference between them. Likewise, a storage temperature of 40 °C proved to be the most damaging for TC in meal B. Opposite to meal A, a storage temperature of 4 °C proved to be the most effective in protecting TC in meal B.

Table 1. The effect of four storage conditions on the degradation of total carotenoids (%) in a meal prepared from whole marigold flowers (A) and in a meal prepared only from marigold petals (B) during 77 days of storage.

Day of storage	Meal	Storage conditions			
		4 °C	25 °C + light	25 °C	40 °C
		%			
7	A	$1.07^{a,A}$	$2.03^{a,A}$	$0.98^{a,A}$	$0.78^{a,A}$
	B	$5.58^{a,B}$	$4.98^{a,A}$	$8.35^{a,B}$	$6.44^{a,B}$
21	A	$5.27^{a,A}$	$5.19^{a,A}$	$5.43^{a,A}$	$10.29^{b,A}$
	B	$17.86^{ab,B}$	$17.55^{a,B}$	$22.58^{c,B}$	$21.28^{bc,B}$
28	A	$5.2^{a,A}$	$6.84^{ab,A}$	$7.79^{a,A}$	$12.5^{c,A}$
	B	$20.79^{a,B}$	$23.63^{ab,B}$	$25.06^{c,B}$	$23.44^{ab,B}$
35	A	$3.62^{b,A}$	$10.07^{a,A}$	$8.95^{a,A}$	$14.21^{c,A}$
	B	$27.11^{a,B}$	$24.88^{a,B}$	$26.54^{a,B}$	$31.43^{b,B}$
49	A	$8.13^{b,A}$	$12.31^{a,A}$	$11.92^{a,A}$	$23.21^{c,A}$
	B	$26.50^{a,B}$	$28.48^{a,B}$	$27.34^{a,B}$	$32.73^{b,B}$
56	A	$8.88^{a,A}$	$8.75^{a,A}$	$10.3^{a,A}$	$28.24^{b,A}$
	B	$29.42^{b,B}$	$33.41^{a,B}$	$30.81^{ab,B}$	$43.08^{c,B}$
63	A	$9.81^{a,A}$	$10.63^{a,A}$	$8.15^{a,A}$	$31.85^{b,A}$
	B	$31.63^{a,B}$	$34.14^{a,B}$	$33.11^{a,B}$	$45.85^{b,B}$
77	A	$14.39^{b,A}$	$22.08^{a,A}$	$12.72^{b,A}$	$41.15^{c,A}$
	B	$35.35^{b,B}$	$41.41^{a,B}$	$40.20^{a,B}$	$56.95^{c,B}$

^{a,b,c}Values superscribed with the different lowercase letters present statistically significant difference ($p<0.05$) among storage conditions within the same meal type and storage time according to the Tukey's test.

^{A,B}Values superscribed with the different uppercase letters present statistically significant difference ($p<0.05$) among meals within the same storage conditions and storage time according to the Tukey's test.

The rate of degradation of TC in the samples was determined for each month of storage and for the entire storage period. The first approach showed that the highest TC degradation rate was observed in the first month of storage of meal B, regardless of the storage conditions. On the other hand, the rate of degradation of TC in meal B remained constant during all three months of storage, except for the third month of storage at 25 °C with exposure to light and at 40 °C. The highest TC degradation rates were found in both meals almost during all three months for the samples stored at 40 °C.

Table 2. The effect of four storage conditions on the degradation rate (k / day^{-1}) of total carotenoid content in a meal prepared from whole marigold flowers (A) and in a meal prepared only from marigold petals (B) at the end of the first, second and third month of storage.

Month	Meal	Storage conditions			
		4 °C	25 °C + light	25 °C	40 °C
		k / day^{-1}			
1	A	$0.0022^{a,A}$	$0.0025^{a,A}$	$0.0030^{a,A}$	$0.0054^{b,A}$
	B	$0.0087^{a,B}$	$0.0096^{b,B}$	$0.0104^{b,B}$	$0.0102^{b,B}$
2	A	$0.0028^{a,A}$	$0.0010^{a,A}$	$0.0021^{a,A}$	$0.0084^{b,A}$
	B	$0.0012^{a,A}$	$0.0054^{ab,B}$	$0.0025^{ab,A}$	$0.0072^{b,A}$
3	A	$0.0031^{a,A}$	$0.0077^{b,A}$	$0.0016^{a,A}$	$0.0074^{b,A}$
	B	$0.0042^{a,A}$	$0.0063^{ab,A}$	$0.0068^{b,B}$	$0.0133^{c,B}$

^{a,b,c}Values superscribed with the different lowercase letters represent statistically significant difference ($p<0.05$) among storage conditions within the same meal type and month according to the Tukey's test.
^{A,B}Values superscribed with the different uppercase letters represent statistically significant difference ($p<0.05$) among meals within the same storage conditions and month according to the Tukey's test.

The second approach in determination of TC degradation rate was used to evaluate the entire storage period. Meal B showed a higher degradation rate in samples stored under the tested storage conditions compared to meal A, in accordance with the greater decrease in TC content depending on the presence of the leafy-parts and the smaller particles in the meal. The results are consistent with research by Hidalgo and Brandolini (2008) [31], who also found lower TC degradation rates in samples containing leafy carotenoids. For both meals, the highest degradation rates were reported for samples stored at 40 °C. Furthermore, the degradation rates of TC in samples stored at 4 and 25 °C without light exposure were similar for both types of meal, despite the monthly difference in TC degradation rates between these two storage conditions in meal A.

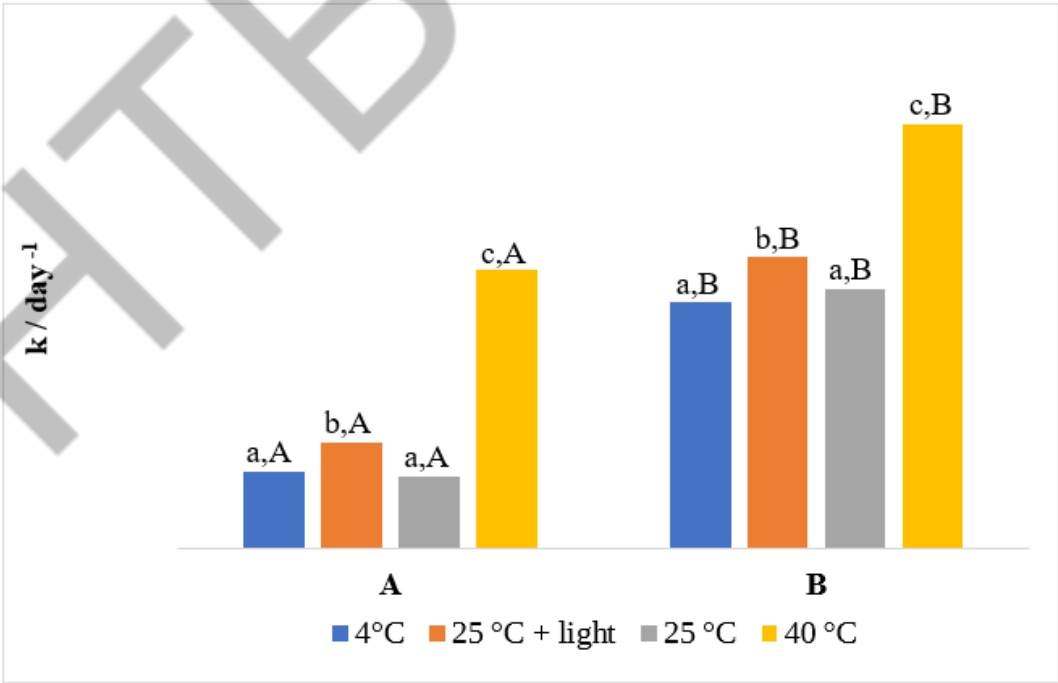

Figure. 3. The effect of four storage conditions on the degradation rate (k /day^{-1}) of total carotenoids in a meal prepared from whole marigold flowers (A) and in a meal prepared only from marigold petals (B) during the whole period of storage (77 days).
^{a,b,c} – Columns superscribed with the different lowercase letters represent statistically significant difference ($p<0.05$) among storage conditions within the same meal type according to the Tukey's test.
^{A,B} - Columns superscribed with the different uppercase letters represent statistically significant difference ($p<0.05$) among meals within the same storage conditions according to the Tukey's test.

V. CONCLUSIONS

Based on the obtained results, it can be concluded that storage conditions (temperature and light exposure) and meal type affect the content and degradation of TC in marigold flowers (*Tagetes erecta* L.). More precisely, higher temperatures (regardless of the meal type) promote the degradation of TC during storage. Consequently, the storage temperature of 40 °C has proven to be the most deleterious. The similarity of TC degradation rates between the two meals stored at 4 °C and 25 °C without light exposure was unexpected, and although recommended storage temperatures for carotenoid sources are close to 0 °C, the results suggest that storage at 25 °C without light exposure could be a cost-effective alternative. In addition, exposure to light has also been proven to be unfavorable to carotenoid retention. Furthermore, the experimental results indicate that the meal type in terms of flower parts used for preparation also affects the TC degradation. Although the initial TC content was higher in the meal prepared only from the petals compared to meal prepared from whole flower, the degradation rates are more pronounced which implies greater sensitivity of this carotenoid source. It can be concluded that, even after 3 months of storage, both types of marigold meals contain sufficient amount of carotenoids and can be used as a supplements in animal nutrition.

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