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Valorization of transgenic Tomaffron-based products enriched in saffron apocarotenoids

Maria Lobato-Gómez [a], José L. Rambla [b], Jaime Zacarías-García [c], Luis Noguera-Artiaga [d], Jose Pérez-Beser [a], Diego Orzáez [a], Lourdes Gómez-Gómez [e], Antonio Granell [a,*]

[a] Instituto de Biología Molecular y Celular de Plantas, CSIC-Universidad Politécnica de València, 46022 Valencia, Spain
[b] Departamento de Biología, Bioquímica y Ciencias Naturales, Universitat Jaume I, 12071 Castellón de la Plana, Spain
[c] Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), 46980, Valencia, Spain
[d] Instituto de Investigación e Innovación Agroalimentaria y Agroambiental, Universidad Miguel Hernández de Elche, 03312 Orihuela, Alicante, Spain
[e] Instituto Botánico, Universidad de Castilla-La Mancha, 02006 Albacete, Spain

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ABSTRACT

Saffron spice (*Crocus sativus* L.) is highly valued for its bioactive apocarotenoids, including crocins, picrocrocin, and safranal, but its commercial production is limited due to labor-intensive cultivation. In this study, Tomaffron, a transgenic tomato-based platform enriched with saffron apocarotenoids, has been processed into different tomato products to assess their suitability as carriers of these valuable apocarotenoids. Their composition, antioxidant capacity, and sensory attributes were analyzed, with particular attention paid to saffron apocarotenoid stability during processing and storage. Tomaffron-processed products retained or increased the levels of saffron apocarotenoids, with crocin accumulation remaining stable during storage. Picrocrocin and safranal levels remained stable or increased, and sensory tests confirmed that these products replicated the saffron-like flavor. This work highlights the potential of Tomaffron for delivering saffron apocarotenoids in stable, ready-to-consume food matrices. These findings support the feasibility of developing functional food prototypes enriched in valuable plant secondary metabolites using metabolic engineering.

1. Introduction

Saffron spice, derived from the dried stigmata of *Crocus sativus*, owes its sensory and biological properties to specific apocarotenoids, crocins, picrocrocin, and safranal (Ríos et al., 1996), produced by the oxidative cleavage of zeaxanthin via carotenoid cleavage dioxygenase 2 (*Cs*CCD2) (Ahrazem et al., 2016). Crocins, which provide saffron's color, vary in the sugar moieties at the end of the molecule (crocin 1: glucose-OH, crocin 2: glucose-glucose, crocin-2′: gentibiose-OH, crocin-3: gentibiose-glucose, crocin-4: gentibiose-gentibiose), and exist in *cis-* and *trans-* isomeric forms (Tarantilis et al., 1995). Picrocrocin, responsible for saffron's taste, results from glycosylation of 3-OH-β-cyclocitral (HTCC), while safranal, which gives the aroma, is formed during spice processing (López et al., 2021).

Saffron cultivation requires extensive manual labor, with up to 470 h of work needed to produce one kilogram of dried saffron stigmata, making it the most expensive spice (Winterhalter & Straubinger, 2000). Due to saffron's high price, its biological activities cannot be exploited on a large scale, and it is primarily used for culinary purposes (Gresta et al., 2009). Consequently, various plant heterologous platforms have been explored to produce these valuable apocarotenoids, including *Nicotiana* species, potato, and tomato (Ahrazem et al., 2022; Gómez Gómez et al., 2022; Martí et al., 2020). Among the heterologous platforms for producing and accumulating saffron apocarotenoids, transgenic tomato fruit expressing the *Cs*CCD2 gene, named Tomaffron, has been shown to be the most suitable due to its targeted production in the fruit during ripening with no effect on plant growth (Ahrazem et al., 2022).

Tomaffron ripe fruits accumulate 1.5 % crocins and 0.3 % picrocrocin in dry weight and also accumulate safranal. These metabolically engineered fruits exhibit higher antioxidant activity than non-transgenic fruits (Ahrazem et al., 2022). Given that tomato fruits are perishable, processing Tomaffron fruits into stable products offers a promising strategy to store and preserve these bioactive compounds. Tomato paste and canned tomato are the two main tomato processing products for long-term storage and availability of tomato products

* Corresponding author.
E-mail address: agranell@ibmcp.upv.es (A. Granell).

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(Tomato News, 2024). During the manufacturing process of tomato products, high temperatures are employed, and the effects on antioxidant compounds, including lycopene, vitamin C, and phenolic compounds, have been evaluated (Seybold et al., 2004; Tomas et al., 2017; Wu et al., 2022). Disruption of the tomato matrix by mechanical homogenization or heat treatment during the production of processed products has been reported to increase lycopene bioavailability, but it also leads to its oxidation and thermal degradation at high temperatures and extended processing times (Colle et al., 2010). Vitamin C, the main water-soluble antioxidant in tomato fruit, has been shown to be unstable during tomato processing (Giannakourou et al., 2021).

In this context, it is also important to consider that saffron apocarotenoids are sensitive to high temperatures. Their thermal instability at high temperatures has been reported across various platforms, including *C. sativus* (Fallahi et al., 2021), *Gardenia jasminoides* (Li et al., 2020), and aqueous solutions (Sánchez et al., 2011). In saffron spice, post-harvest practices such as drying and storage further influence apocarotenoid content (Yao et al., 2018).

In this study, we aimed to develop Tomaffron-processed products and evaluate the stability of saffron apocarotenoids during processing and storage. We also investigated other tomato compounds that contribute to the health and sensory value of tomato and the antioxidant capacity of these products. We also conducted sensory analyses to assess their saffron-like organoleptic properties. Our findings highlight Tomaffron products as an effective platform for producing and preserving saffron apocarotenoids, replicating the characteristic flavor of saffron while overcoming the limitations of traditional saffron cultivation. As a transgenic variety, Tomaffron could be used as a food product if approved for commercialization, or alternatively, these products could serve as a platform for storing these compounds, which can be extracted

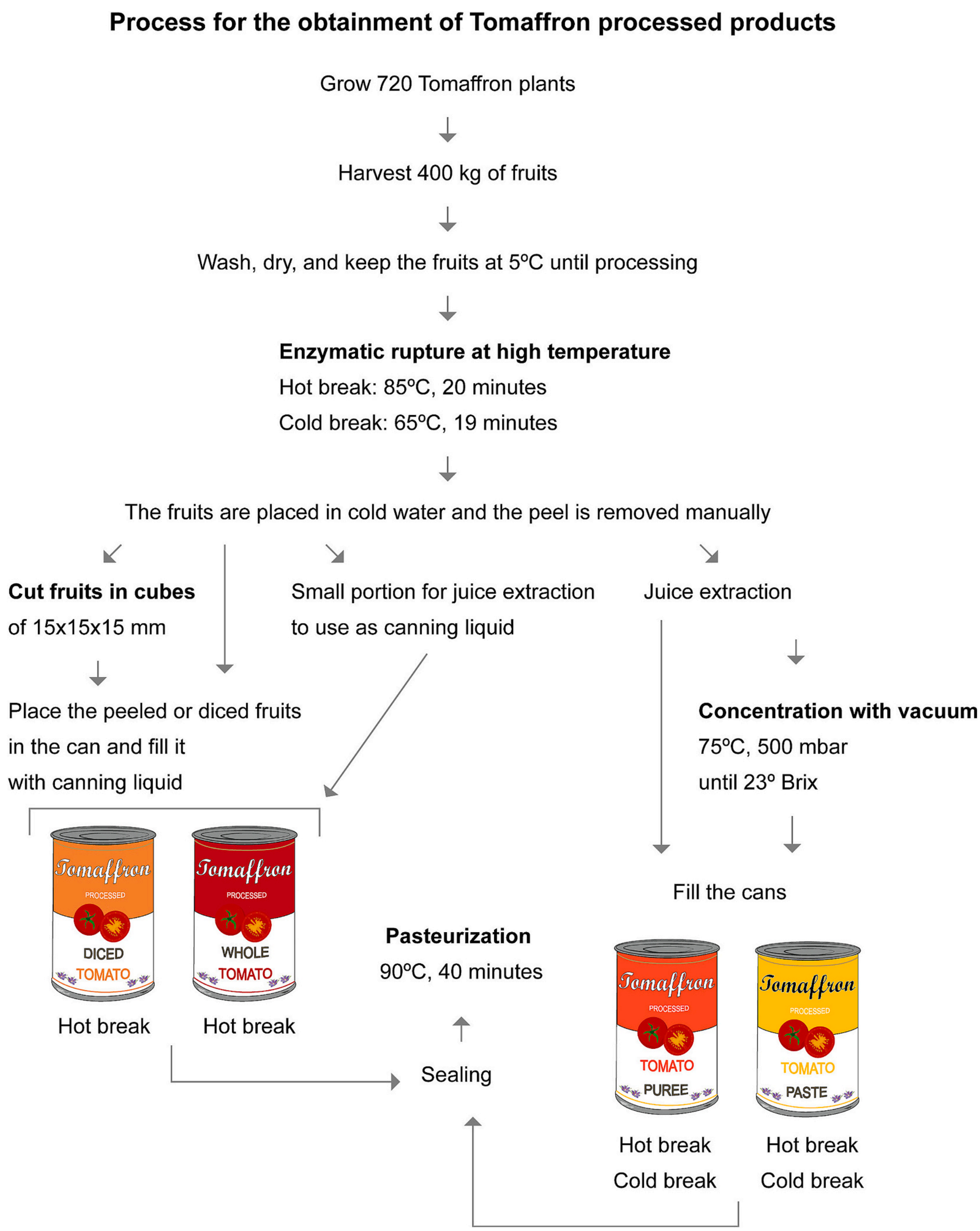


Fig. 1. Schema of the process to obtain the different Tomaffron processed products.

for other applications. This study provides a foundation for the industrial production of Tomaffron, offering a sustainable and cost-effective alternative to deliver the bioactive compounds and culinary value of saffron to consumers and the industry.

2. Material and methods

2.1. Plant material and growth

Fruits of transgenic line 9 A accumulating saffron apocarotenoids and dubbed Tomaffron were used in all the experiments (Ahrazem et al., 2022). Tomaffron plants were grown, and the fruits were harvested and processed at the Centro Tecnológico Nacional Agroalimentario in Badajoz, Spain (CTAEX) facilities.

Tomato plantlets were grown from seeds and transferred to the greenhouse (38.90°N, 6.86°W) and cultivated at a plant density of 1.6 plants/m^2. Standard hydroponic cultivation practices normally used in commercial greenhouses were used during cultivation from February to July 2023. The irrigation system was controlled using sensors for electrical conductivity, temperature, ambient humidity, and substrate moisture. The total irrigation applied per plant was 0.13 m^3. Additionally, three applications of phosphoric acid were performed via the drip irrigation system for pH control.

A total of 404.54 kg of Tomaffron fruits were harvested at the mature red ripe stage and immediately taken to the pilot plant for processing.

The handling of transgenic plants and their fruits complied with EU and Spanish regulations. These activities were approved by the National Council on Biosafety and the Junta de Extremadura on April 17, 2023, following a positive report from the Center for National Biosafety (reference A/ES/22/I-61 on activities A/ES/22/142)

2.2. Tomaffron processed products

An outline of the processes for obtaining the various Tomaffron-derived products is summarized in Fig. 1 and corresponds to those usually found for canned products in the tomato industry but performed at the pilot scale level in dedicated facilities at CTAEX. Briefly, preliminary steps for all canned products include washing with a rod washer to remove debris, drying with an air-blowing system to prevent fungal growth, and storing the tomatoes under refrigeration until processing. Tomatoes undergo enzymatic breakdown through heat treatment, either with the hot break treatment at 85 °C for 20 min (applied to all Tomaffron products) or the cold break treatment at 65 °C for 19 min (used for Tomaffron puree and paste). After heat treatment, the tomatoes are cooled in water, and the skins are manually removed.

For whole peeled tomatoes, the tomatoes were peeled and canned, while for diced tomatoes, peeled tomato fruits were cut into 15 mm and canned. A portion of the tomatoes was juiced through a fine strainer to create the canning liquid used to cover and fill both whole and diced tomatoes.

For tomato puree, the juice was extracted from batches of 100 kg of peeled tomatoes using a strainer and two sieves (3 mm and 1 mm mesh sizes). The juice was collected, and the skin and seeds were discarded. To obtain tomato paste, the same steps as for tomato puree were followed, and the juice was concentrated by vacuum at 75 °C until the final product reached 23°Brix value.

After filling the cans (400 mL/g), all products were hermetically sealed and pasteurized at 90 °C for 40 min using an industrial can autoclave. Throughout the process, pH and °Brix values were monitored, and the addition of citric acid was not needed.

2.3. Sampling and processing of Tomaffron canned products

A total of six different canned Tomaffron products were obtained: whole tomato, diced tomato, tomato puree hot break (HB), tomato puree cold break (CB), tomato paste HB, and tomato paste CB. Samples from all products were collected right after canning (T0) and at three different times of storage at RT: two (T1), four (T2), and six (T3) months after canning. At each time point, three cans of each product were randomly chosen and processed. The processing involved homogenization of the contents of each can for 10 s using a blender and freezing directly an aliquot of the material in N_2 (l), freeze-drying another aliquot of it. All frozen fresh and freeze-dried samples were stored at −80 °C, protected from light.

2.4. Metabolite extraction

Polar metabolites, including crocins, HTCC, picrocrocin, flavonoids, and ascorbic acid, were extracted from 10 mg of freeze-dried sample. Genistein was used as the internal standard (IS), following the protocol described in (Perez-Fons et al., 2022). The polar fraction was injected into the LC-MS under the conditions specified in (Ahrazem et al., 2022). Carotenoids were extracted from 5 mg of freeze-dried sample, spiked with canthaxanthin as IS, analyzed using HPLC-PDA as outlined by (Barja et al., 2021), and quantified using standard curves.

Volatiles were captured from 50 mg of freeze-dried sample and analyzed following the protocol on (Rambla & Granell, 2020). Briefly, samples were resuspended in 450 μL of water in a glass vial, incubated at 37 °C for 10 min, and sonicated for 5 min with 500 rpm agitation. Subsequently, 1 mL of the resulting mixture was transferred to a screw-cap headspace vial. Samples were pre-incubated at 50 °C for 10 min, and volatiles were extracted using a 65 μm PDMS/DVB SPME fiber for 20 min under the same conditions. The adsorbed volatiles were then desorbed for 1 min at 250 °C in the injection port of a 6890 N into a gas chromatograph for analysis. A Combi-PAL (CTC Analytics) was used for automated volatile extraction and injection. Chromatographic and detection conditions were similar to those described in (Rambla et al., 2017)

2.5. Measurement of antioxidant capacity

The hydrophilic antioxidant capacity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical assay, following the methodology outlined by (Rey et al., 2020). Absorbance was measured at 517 nm with a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Scientific, Madrid, Spain). DPPH scavenging activity was calculated as the percentage decrease in absorbance relative to the control. The hydrophilic antioxidant capacity was further evaluated using the FRAP (ferric reducing antioxidant power) assay, based on the protocol by (Benzie & Strain, 1996), with minor modifications. The FRAP reagent was prepared by mixing 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine, Sigma), 20 mM $FeCl_3·6H_2O$, and 300 mM acetate buffer (pH 3.6) in a 1:1:10 ratio (*v*/v/v), followed by incubation at 37 °C for 10 min. Absorbance was measured at 595 nm in a 96-well plate. Both DPPH and FRAP assays were performed in duplicate, with an ascorbic acid standard curve for calibration. Results are expressed as milligrams of ascorbic acid equivalents per 100 g of dry weight.

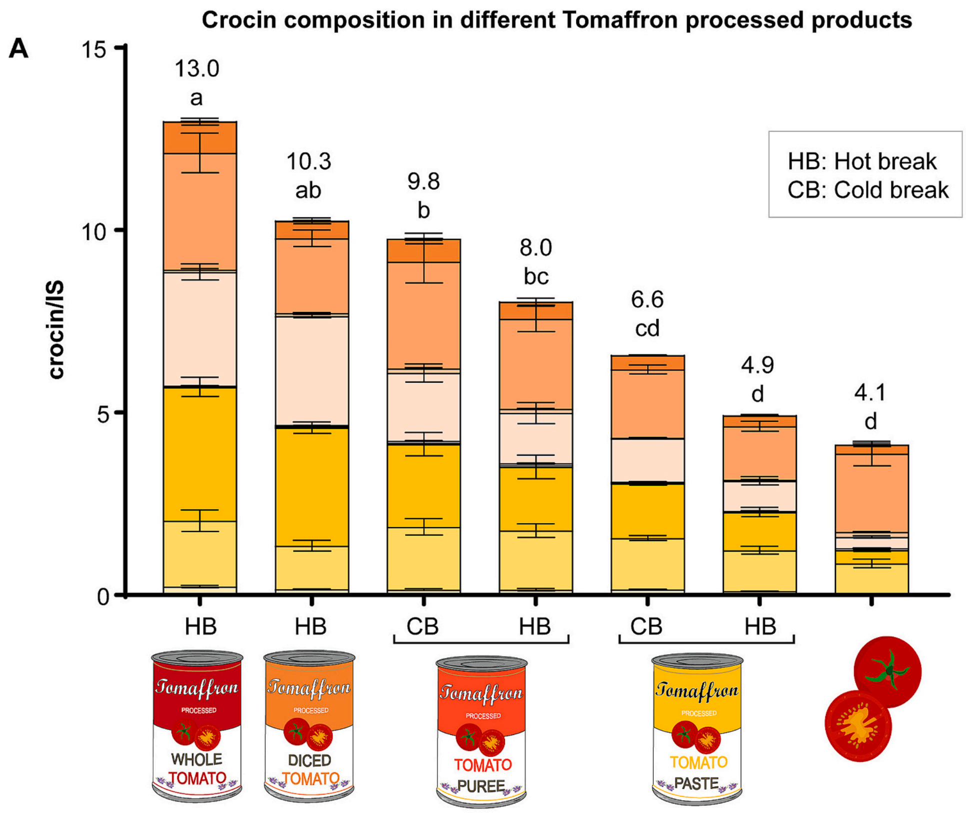
The ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay was conducted to account for both hydrophilic and lipophilic antioxidants in the same extract as described by (Zacarías-García et al., 2022). Absorbance was recorded at 730 nm using the UV/Vis microplate spectrophotometer, and results were expressed as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma, Madrid, Spain) equivalents per 100 g of dry weight.

2.6. Sensory evaluation

The sensory evaluation of canned Tomaffron products focused on general appearance, color, odor, and flavor without ingestion. Two panels conducted the evaluation: a trained sensory panel and a panel of chefs. The analyzed samples included HB Tomaffron puree and paste, which were compared to commercial tomato puree and paste, with and without added saffron. Saffron threads were added to the commercial samples to match the crocin concentration of the Tomaffron products.

The descriptive sensory analysis by the trained panel involved 10 panelists (50 % female, 50 % male), each with over 1000 h of experience in the sensory analysis of fruits and vegetables. A lexicon for the evaluation was developed during ten 1-h sessions (Hongsoongnern & Chambers IV, 2008; Predieri et al., 2021). Attributes were rated on a scale from 0 (no intensity) to 10 (extremely high intensity), with increments of 0.5.

The analyses were conducted in a standardized tasting room (UNE-EN ISO 8589) with a capacity for 25 sensory judges, equipped with both natural and artificial lighting, and maintaining an ambient temperature of 24 °C. All products were analyzed over three tasting sessions, with six samples evaluated per session. Each sample was assessed in triplicate. Samples were labeled with unique 3-digit codes to ensure blind testing. Twenty grams of each sample were served in odor-free blue glass beakers (COI/T.20/Doc. No 5/Rev. 2) covered with glass lids. Before tasting, samples were heated to 60 °C for 10 min and maintained at 40 °C during evaluation (4P Tasting Heater, Bunsen S.A., Madrid, Spain). Panelists were provided with unsalted crackers and osmosis water for



B Percentage of each crocin in the different Tomaffron processed products

	cis-crocin-1			*cis*-crocin-3			*cis*-crocin-4			%*cis*-crocin		
	Mean	SD		Mean	SD		Mean	SD		Mean	SD	
HB	1.82	0.22	bc	13.81	0.77	d	28.34	1.01	b	43.97	0.98	c
HB	1.55	0.06	c	11.61	0.62	d	31.55	0.86	a	45.05	0.70	abc
CB	1.53	0.04	c	17.66	0.48	c	23.21	0.46	c	42.82	0.75	c
HB	1.81	0.14	bc	20.25	0.78	b	21.61	0.60	c	44.34	0.71	bc
CB	2.36	0.10	a	21.38	0.46	ab	22.76	0.26	c	46.92	1.01	a
HB	2.17	0.25	ab	22.81	1.19	a	21.23	0.85	c	46.44	0.15	ab
	0.43	0.06	d	20.67	0.28	ab	8.82	0.61	d	30.15	0.29	d

	trans-crocin-2			*trans*-crocin-2'			*trans*-crocin-3			*trans*-crocin-4			%*trans*-crocin		
	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
HB	24.08	1.12	b	0.50	0.11	d	24.51	1.80	c	6.63	0.07	a	56.03	0.98	b
HB	29.10	1.27	a	0.82	0.11	c	19.95	0.97	d	4.63	0.45	b	54.95	0.70	bcd
CB	19.12	0.62	c	1.29	0.03	b	29.79	1.03	b	6.43	0.37	a	57.18	0.75	b
HB	17.02	0.75	c	1.39	0.03	b	30.80	0.95	b	5.80	0.24	a	55.66	0.71	bc
CB	18.04	0.31	c	0.35	0.01	d	28.43	0.91	b	5.87	0.10	a	53.08	1.01	d
HB	16.73	0.94	c	0.53	0.13	d	29.82	1.03	b	6.05	0.16	a	53.56	0.15	cd
	7.40	0.44	d	3.29	0.06	a	52.15	0.49	a	5.86	0.31	a	69.85	0.29	a

Fig. 2. Crocin accumulation after the processing (time 0) measured by LC-MS using genistein as internal standard. **A**: total crocin accumulation and composition in Tomaffron fruit and Tomaffron processed products. **B**: percentage of each crocin in the different Tomaffron processed products. The bars display the mean values along with their standard deviation (SD). The letters above the bars denote statistically distinct groups, identified through ANOVA followed by Tukey's post-hoc analysis. HB: Hot break; CB: Cold break.

palate cleansing between samples. This study was approved by the Research Ethics and Integrity Committee of the Vice-Rectorate for Research and Transfer of the Miguel Hernández University of Elche (Ref. PRL.NA.AACB.240405).

The sensory evaluation by chefs was conducted at Fundació Alícia in Barcelona, Spain. Seven evaluators assessed all products, which were either previously heated to 55 °C or served without heating. Evaluations focused on the intensity of saffron color and aroma, with saffron threads used as a reference. The order of sample presentation was randomized for each round of evaluation.

All panelists and evaluators voluntarily participated in the sensory evaluation and provided their informed consent prior to the study.

2.7. Statistical analyses

All statistical analyses were conducted in R. Group comparisons were performed using ANOVA, with post-hoc grouping based on the Tukey test results. PLS-DA was carried out using the mixOmics package (Florian et al., 2017), with data scaling applied. The Pearson correlation matrix was visualized using the pheatmap package.

3. Results

3.1. Tomaffron processed products

The Tomaffron tomato was successfully processed into various canned tomato products, including whole peeled tomatoes, diced tomatoes, tomato puree, and tomato paste, without displaying any anomalous behavior or encountering difficulties beyond those commonly observed with conventionally processed tomatoes at CTAEX (Supplementary Fig. 1 A). The pH of the processed products remained below 4.5, eliminating the need to lower the pH by citric acid addition in the case of Tomaffron products. The tomato paste reached a °Brix value of 23 compared to the 4.5°Brix value of tomato puree (Supplementary Fig. 1B).

3.2. Effect of the different processing steps on crocin and carotenoid composition

The total accumulation and composition of crocins were evaluated in all processed Tomaffron products and compared to non-processed Tomaffron fruit (Fig. 2) using freeze-dried powder. Interestingly, all Tomaffron products showed higher crocin accumulation per dry weight than the non-processed fruit. However, within the processed Tomaffron products, total crocin levels decreased as the degree of processing increased. Notably, the most processed product, tomato paste, exhibited similar total crocin levels than those of fresh fruit in dry weight (Fig. 2A). However, crocin content by fresh weight is significantly higher in tomato paste, with °Brix values of 23 compared to the average of 4.5 in the other products. This results in an 8-fold increase in crocins in tomato paste relative to fresh Tomaffron fruit (Supplementary Fig. 2).

Among the products subjected to cold and hot break treatments for enzymatic breakdown, the total crocin accumulation was higher in those processed products where cold break treatment was used. However, the differences were not statistically significant (Fig. 2A).

As it is known that heat and light may affect the specific profiles of crocins in saffron spice, the crocin composition was also analyzed to see if this was also the case for Tomaffron and their processing products (Fig. 2B). All Tomaffron products exhibited an increase in *cis*-crocins and a decrease in *trans*-crocins compared to fresh fruit. Tomato paste showed the highest percentage of *cis*-crocins, driven mainly by increases in *cis*-crocin-1 and *cis*-crocin-3, followed by diced tomato, which showed a relatively higher increase in *cis*-crocin-3 compared to the other products. In contrast, the percentages of *trans*-crocin-2′ and *trans*-crocin-3 decreased by threefold and twofold, respectively, with the most dramatic reduction observed for *trans*-crocin-3, which is the predominant crocin in fresh Tomaffron fruit. Strikingly, *trans*-crocin-2 significantly increased across all products, particularly in diced tomato, while *trans*-crocin-4 percentage remained stable (Fig. 2B).

3.3. Stability of saffron apocarotenoids and carotenoids during storage of Tomaffron processed-products

The stability of saffron apocarotenoids during storage was evaluated after analyzing the impact of processing on canned Tomaffron products. No significant differences were observed in crocin levels across processed products at different time points, highlighting their stability after processing and storage up to 6 months in the cans at room temperature (Fig. 3A).

However, a distinct trend was observed for the saffron apocarotenoid molecules derived from the end cyclic structure of zeaxanthin following cleavage by *Cs*CCD2. HTCC levels decreased dramatically from T0 to subsequent time points in all canned Tomaffron products, consistently remaining lower than those found in fresh fruit (Fig. 3B). In contrast, picrocrocin, the glycosylated derivative of HTCC, exhibited an overall increase during storage. While this increase was statistically significant in whole and diced tomatoes and cold-break tomato puree (CB), other products showed a similar trend without statistical significance (Fig. 3C).

Safranal, the volatile apocarotenoid responsible for the characteristic flavor and aroma of the saffron spice, displayed stable levels throughout the storage period, with a general tendency toward an increase in all products. In hot-break (HB) and cold-break (CB) tomato purees, this increase was statistically significant from T0 to later time points (Fig. 3D). Notably, the levels of picrocrocin were comparable to those in fresh fruit, while safranal levels were significantly higher in all processed products compared to the fresh Tomaffron fruit.

3.4. Changes in other health-related tomato compounds in Tomaffron fruit after processing

Tomato metabolites contributing to the high antioxidant activity of tomato fruit were also evaluated in this experiment. Zeaxanthin and lutein, the direct substrates of *Cs*CCD2 for crocin biosynthesis, were not detected in fresh Tomaffron fruit or its processed products. Instead, the two main carotenoids identified were lycopene and β-carotene, along with trace amounts of phytoene (Fig. 4). Total carotenoid accumulation in the processed products did not correlate with their level of processing; the least and most processed products exhibited carotenoid levels similar to each other and fresh Tomaffron. In contrast, diced tomatoes and tomato puree displayed slightly lower total carotenoid levels (Fig. 4A). Lycopene was the carotenoid most affected by processing; its percentage decreased across all products, from approximately 90 % in fresh Tomaffron to 60–73 % in processed products (Fig. 4B). Conversely, β-carotene levels increased in all processed products, rising from 10 % in fresh Tomaffron to 25–28 % in the processed products (Fig. 4A,B).

In addition to safranal, other volatile apocarotenoids are produced through the cleavage activity of tomato endogenous CCDs. Notably, tomato puree CB exhibited significantly higher levels of most of these apocarotenoids than tomato puree HB, except for geranial and α-cyclocitral. However, no significant differences were observed between CB and HB in tomato paste. Furthermore, none of the endogenous apocarotenoids displayed a consistent pattern of change following processing (Fig. 4C,D).

Flavonoid content was also analyzed as it contributes to the health value and the water-soluble antioxidant properties of tomatoes. Most flavonoids generally exhibited an increasing trend during processing. Whole tomatoes, diced tomatoes, and tomato puree CB displayed similar flavonoid levels, whereas the other three products showed higher accumulations of all flavonoids. Notably, for each flavonoid analyzed, at least one Tomaffron product retained levels comparable to those found in the fresh fruit (Fig. 5). Naringenin chalcone, naringenin hexose 1, and

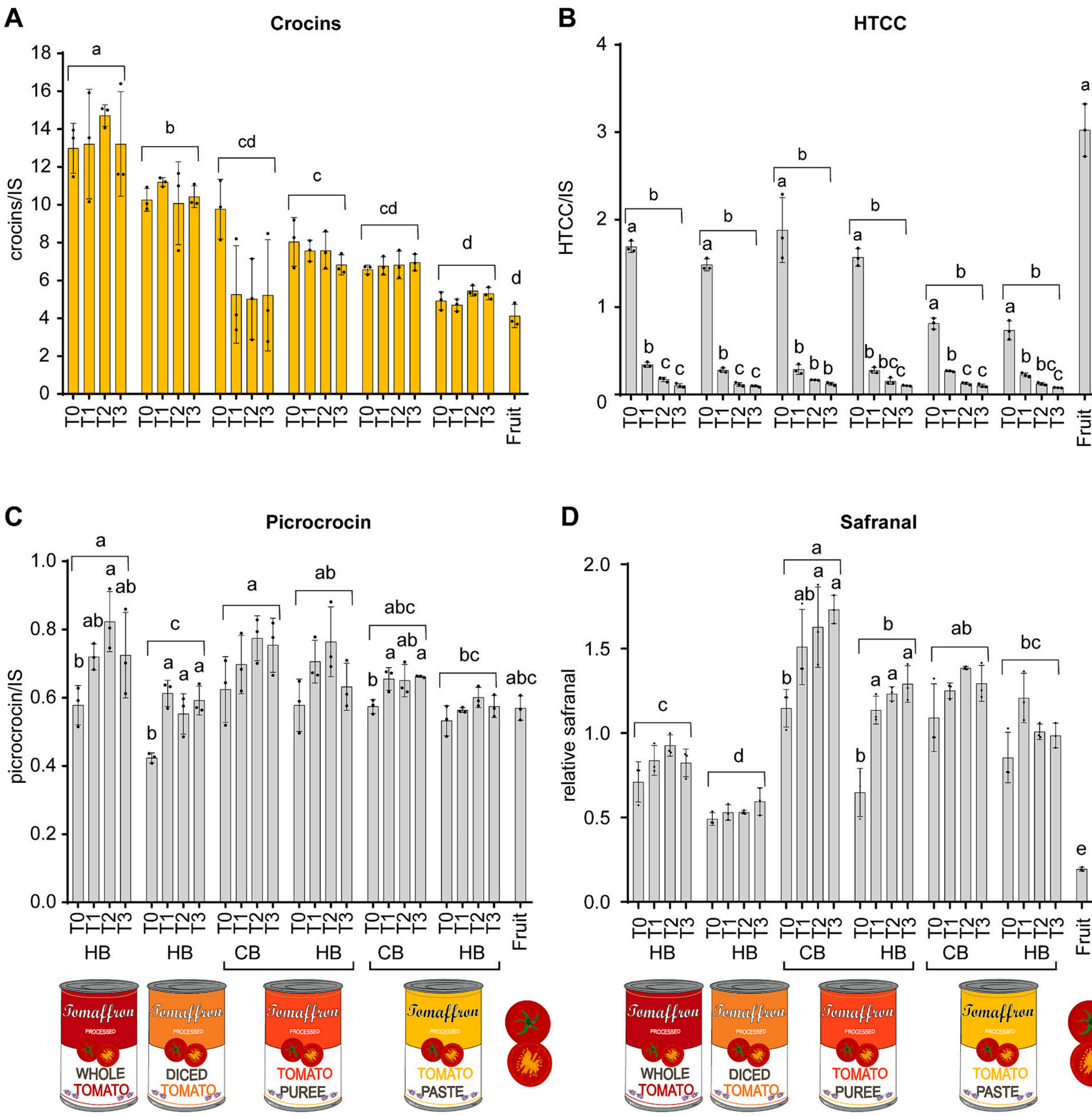


Fig. 3. Changes of saffron apocarotenoids in the Tomaffron processed products over time. Crocins (**A**), HTCC (**B**), and picrocrocin (**C**) were analyzed by LC-MS using genistein as internal standard. Safranal (**D**) was measured by GC–MS relative to a reference line. The bars display the mean values along with their standard deviation (SD). The letters above the bars denote statistically distinct groups, identified through ANOVA followed by Tukey's post-hoc analysis. HB: Hot break; CB: Cold break. HTCC: 3-OH-β-cyclocitral.

eriodictyol chalcone could not be found in any of the processed tomatoes, while they were easily detected in the non-processed fruit (Fig. 5), likely due to their accumulation in the skin.

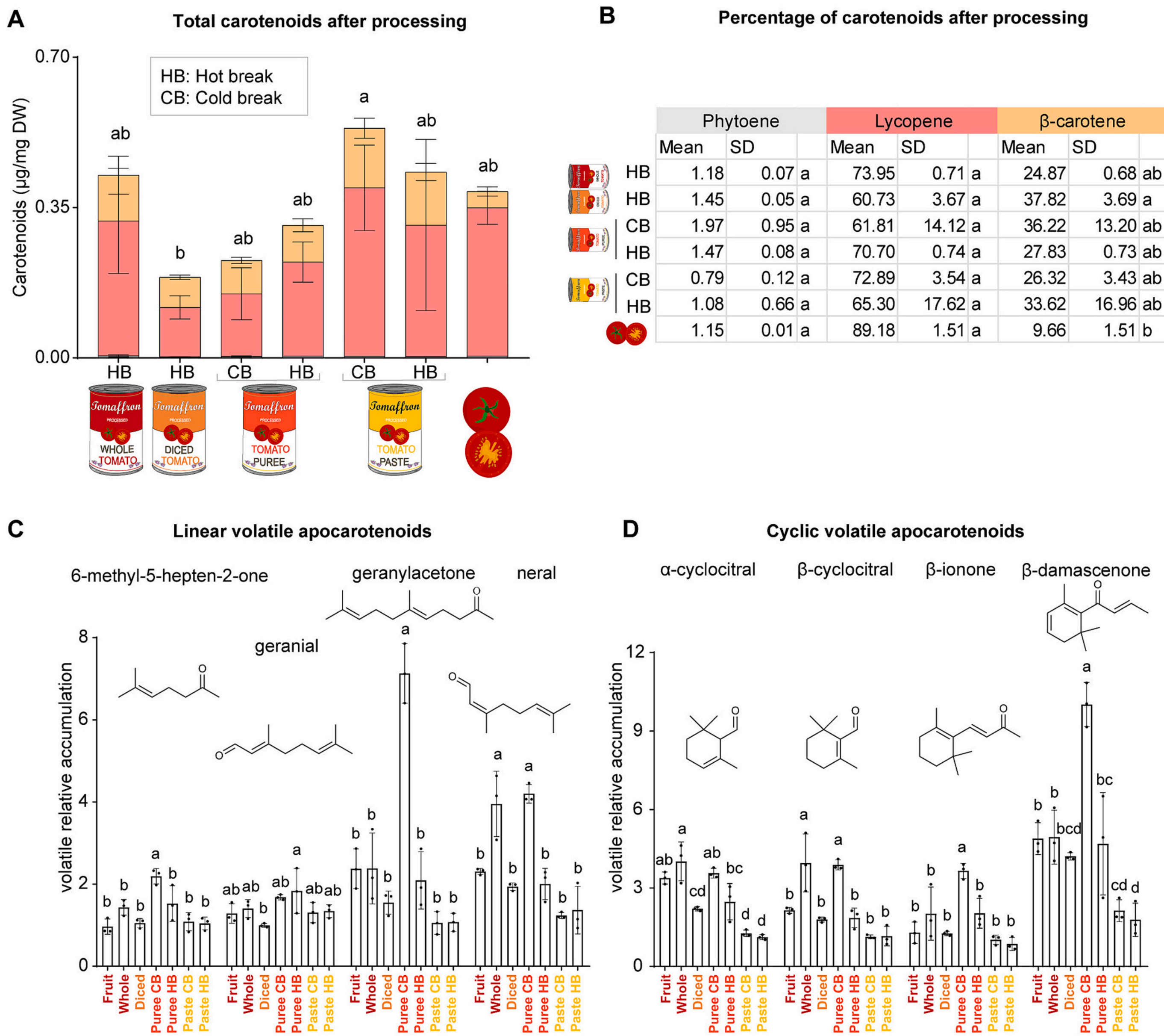
In contrast, a clear negative effect of processing was observed on the ascorbic acid content of the samples, with the lowest levels detected in the most processed products. However, the levels of dehydroascorbic acid remained unchanged throughout the processing stages (Fig. 5).

3.5. Antioxidant capacity of the Tomaffron processed products

After the stability of the primary metabolites that confer antioxidant activity in tomato was evaluated, the actual antioxidant capacity of canned Tomaffron products was assessed in both hydrophilic and lipophilic fractions.

In the hydrophilic fraction, where the crocins and picrocrocin accumulate, none of the Tomaffron products showed a decrease in the antioxidant capacity in any of the three analyses performed. Instead, CB and HB tomato paste presented higher ABTS values compared to fresh fruit and the other products (Fig. 6A). Regarding the antioxidant capacity of the lipophilic fraction, where tomato carotenoids accumulate, the fresh fruit exhibited the largest value, as a significant decrease in the antioxidant capacity has been observed in all Tomaffron products compared to Tomaffron fruit (Fig. 6B).

The changes in the antioxidant activity over time have also been evaluated. Overall, the hydro- and lipophilic antioxidant capacities of the different Tomaffron processed products remained moderately stable or with minor changes. The hydrophilic phase antioxidant capacity in whole tomato and tomato puree CB decreased significantly by 23 % and 33 %, respectively, starting from 4 months after canning. In tomato puree HB, both the hydrophilic and lipophilic antioxidant capacities evaluated by ABTS were stable during the storage period, while the FRAP was reduced between 28 and 44 % over time. Some changes were



	Phytoene			Lycopene			β-carotene		
	Mean	SD		Mean	SD		Mean	SD	
HB	1.18	0.07	a	73.95	0.71	a	24.87	0.68	ab
HB	1.45	0.05	a	60.73	3.67	a	37.82	3.69	a
CB	1.97	0.95	a	61.81	14.12	a	36.22	13.20	ab
HB	1.47	0.08	a	70.70	0.74	a	27.83	0.73	ab
CB	0.79	0.12	a	72.89	3.54	a	26.32	3.43	ab
HB	1.08	0.66	a	65.30	17.62	a	33.62	16.96	ab
	1.15	0.01	a	89.18	1.51	a	9.66	1.51	b

Fig. 4. Carotenoid and volatile apocarotenoid accumulation after processing (time 0). **A**: total carotenoid accumulation and composition measured by HPLC-PDA. **B**: percentage of each carotenoid in each product. **C**: accumulation of linear apocarotenoids measured by GC–MS relative to a reference line. **D**: accumulation of cyclic apocarotenoids measured by GC–MS relative to a reference line. The bars display the mean values along with their standard deviation (SD). The letters above the bars denote statistically distinct groups, identified through ANOVA followed by Tukey's post-hoc analysis.

also observed in diced tomato in FRAP and lipophilic ABTS; however, these were not correlated with time (Supplementary Fig. 6).

The correlation between antioxidant capacity parameters and various metabolites was analyzed. The ABTS assay for the lipophilic fraction showed a significant correlation with both detected carotenoids, β-carotene and lycopene, being the first one more correlated to the antioxidant capacity. The assays evaluating hydrophilic antioxidant capacity positively correlated with flavonoids; however, they exhibited low or negligible correlation with crocins and ascorbic acid (Supplementary Fig. 7). This observation may be attributed to the fact that highly processed Tomaffron products, such as tomato puree and paste, have the lowest levels of crocins but the highest levels of the detected flavonoids.

3.6. *Effect of processing and storage on substrates and enzymes that generate volatiles in Tomaffron processed products*

The volatile organic compounds (VOCs) from reconstituted freeze-dried Tomaffron fruit and canned products were analyzed to identify markers associated with processing steps.

Immediately after canning, all Tomaffron products showed a decrease in fatty acid-derived VOCs, including (*E*)-hexenal, (*E*)-octenal, and (*E*)-pentenal, as well as volatile benzenoids, such as guaiacol and methyl salicylate, compared to fresh Tomaffron. In contrast, processed products exhibited increased sulfurous compounds relative to fresh fruit. Whole and diced tomatoes and tomato puree showed an increase in ester compounds and branched-chain amino acid (BCAA)-related VOCs compared to fresh fruit and tomato paste and puree (Fig. 7A).

Differences in the volatile profiles were observed between products processed with hot break (HB) or cold break (CB) treatments during the enzymatic cleavage step. These differences were evident in tomato

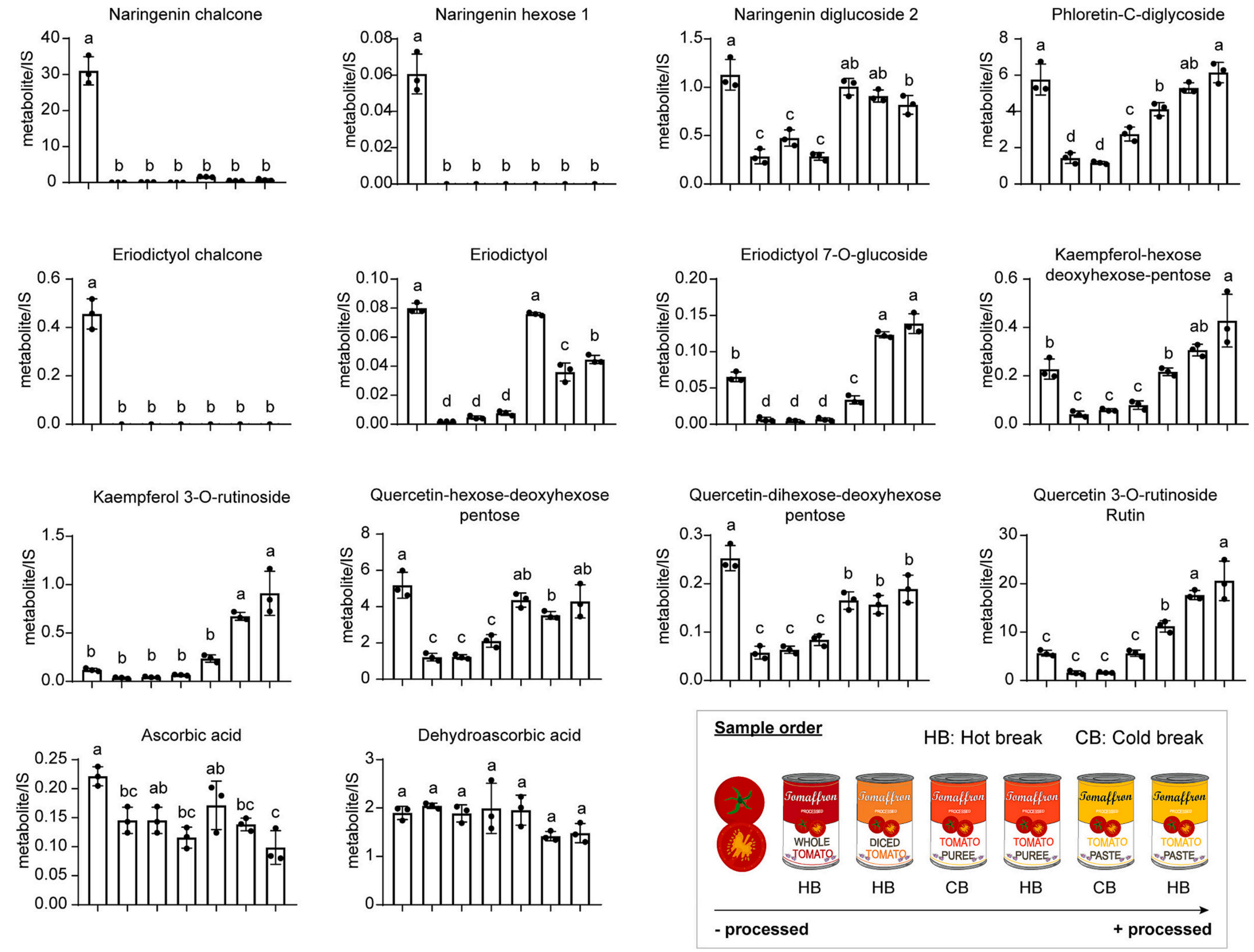


Fig. 5. Flavonoid, ascorbic acid, and dehydroascorbic acid accumulation after processing (time 0) measured by LC-MS using genistein as internal standard. The bars display the mean values along with their standard deviation (SD). The letters above the bars denote statistically distinct groups, identified through ANOVA followed by Tukey's post-hoc analysis.

puree, where CB-treated products exhibited a higher accumulation of VOCs overall compared to HB-treated products. Notably, the VOCs that showed no differences between CB and HB tomato puree were those that uniformly decreased in all Tomaffron products relative to fresh fruit (Fig. 7A). In tomato paste, however, these VOCs were similarly reduced in both CB and HB treatments, with no significant differences observed between the two heat treatments (Fig. 7B).

The evolution of VOC profiles in freeze-dried Tomaffron products was also evaluated over time. Most VOCs that declined during storage were fatty acid-derived, except for the apocarotenoid β-damascenone, which also showed a consistent decrease. Conversely, 3-methylbutanoic acid and safranal levels increased in nearly all products as storage time progressed (Fig. 7C, Supplementary Fig. 4, Supplementary Fig. 5).

3.7. Tomaffron puree and paste are identified as tomato products with saffron organoleptic properties

After one year of storage at RT, a sensory evaluation was performed with Tomaffron puree and paste HB. The biotechnological products were compared to tomato puree and paste supplemented with saffron threads to achieve the same typical yellow color levels of the Tomaffron products and with commercial standard tomato puree and paste.

In the first sensory analysis, performed by a trained panel, both Tomaffron products showed the highest intensities for saffron identification and the lowest for tomato identification in aroma and flavor. In the tomato puree, the product scored as the one with the most pungent flavor was tomato with added saffron instead of Tomaffron. In contrast, in the tomato paste, the Tomaffron product scored as the one with the highest bitter taste (Fig. 8A,B).

In a culinary and gastronomic evaluation test of Tomaffron puree and paste performed by a panel of chefs, both products were preferred as the top choice for a saffron-containing preparation based on their aroma, outperforming conventional tomato puree and paste with added saffron threads. Notably, Tomaffron paste was consistently selected as the first option when the products were preheated before serving. Across all other products and conditions, Tomaffron products were chosen 71.4 % of the time. The second preference consistently went to the products with added saffron threads (Fig. 8C,D).

The presence of the compounds conferring saffron aroma, safranal, and saffron taste, picrocrocin, was confirmed in the cans (Fig. 8E), and the results of both sensory evaluations confirmed that Tomaffron products show the organoleptic and culinary characteristics of saffron, even at a greater extent than a tomato product with added saffron that had a similar coloring effect.

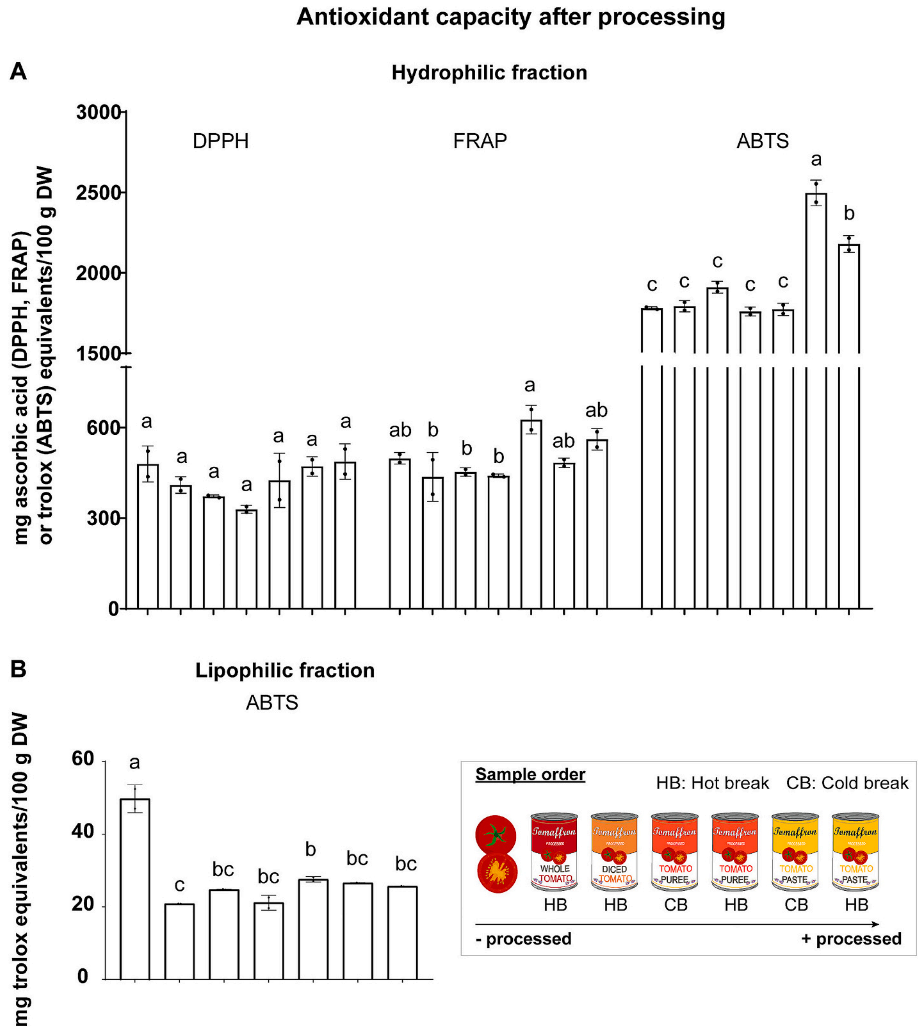


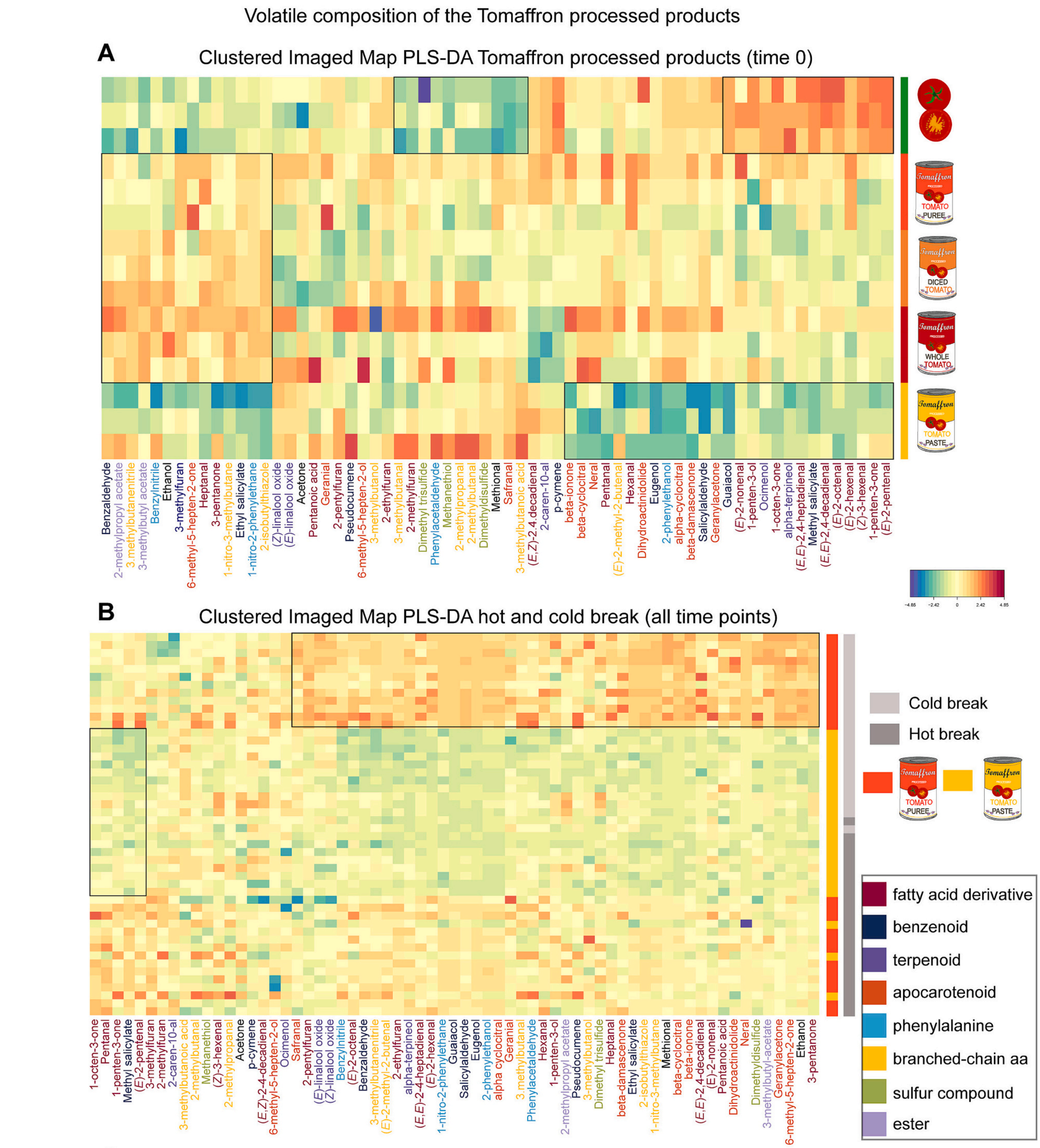
Fig. 6. Antioxidant capacity of the hydrophilic (**A**) and the lipophilic (**B**) fractions of the non-processed and Tomaffron processed products after processing (time 0). The bars display the mean values along with their standard deviation (SD). The letters above the bars denote statistically distinct groups, identified through ANOVA followed by Tukey's post-hoc analysis. DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

4. Discussion

4.1. Saffron apocarotenoids conferring saffron color, aroma, and taste are stable after the processing steps

Tomaffron processed products exhibited higher crocin concentration than fresh Tomaffron fruits, with the highest levels on dry material observed in the less-processed products, and the highest levels on fresh material were on tomato paste when considering the °Brix values of the products (Fig. 3A, Supplementary Fig. 2). To produce saffron spice, the moisture content must be reduced to very low levels to ensure product stability, with drying temperature and time playing a critical role in determining saffron quality (Fallahi et al., 2021). The accumulation of crocins has not been shown to increase under drying protocols involving temperatures below 40 °C, likely due to the prolonged drying periods leading to extended enzymatic degradation of crocins (Raina et al., 1996). Higher temperatures for extended durations result in thermal degradation of the pigments. In contrast, drying processes involving shorter times at temperatures up to 90 °C have been reported to yield higher crocin levels compared to non-processed samples (Carmona et al., 2005; Gregory et al., 2005; Rocchi et al., 2018). The increase of crocins in the absence of *Cs*CCD2 substrate, zeaxanthin, could be due to a higher extractability of the compounds after the processing steps involving cell disruption and high temperatures, or a potential inactivation of β-glucosidases in plants at temperatures above 60 °C (Gregory et al., 2005). The inhibition of crocin degradation may explain the higher crocin levels observed in saffron dried at high temperatures, as well as in our tomato products, which underwent enzymatic disruption at 65 °C during cold break or 85 °C during hot break. The decrease in crocin content throughout the processing workflow can likely be attributed to additional steps that cause an alteration of the integrity of the tomato fruit, such as slicing tomatoes or juice extraction, which have been shown to impact ascorbic acid in conventional processed tomato products (Gahler et al., 2003; Giannakourou et al., 2021). However, this effect is less pronounced for crocin accumulation. All Tomaffron processed products exhibited reduced ascorbic acid levels compared to fresh Tomaffron fruit (Fig. 5), while a significant decrease in crocin content was only observed when comparing less-processed to more-processed products, but not when comparing with the fresh fruit (Fig. 3A).

After processing, all Tomaffron products exhibited significantly lower levels of HTCC and higher levels of safranal compared to fresh Tomaffron fruit, while picrocrocin levels remained stable (Fig. 3B,C,D). Fresh saffron stigmata have a low odor, and most flavor compounds, including safranal, are generated during incubation due to thermal, chemical, or enzymatic degradation of picrocrocin (Carmona et al.,



C VIP values PLS-DA of each tomaffron processed product at different times

Volatile	HB (Whole tomato)	HB (Diced tomato)	CB (Tomato puree)	HB (Tomato puree)	CB (Tomato paste)	HB (Tomato paste)
3-methylbutanoic acid	2.02	1.82	1.78	1.90	2.23	1.89
Eugenol	0.83	1.82	0.87	1.45	0.70	1.79
1-nitro-3-methylbutane	1.22	1.67	0.25	1.69	0.10	0.72
Safranal	1.85	1.14	1.75	1.83	1.74	0.10
Dimethyldisulfide	0.28	0.60	1.52	1.62	1.32	1.46
beta-damascenone	2.61	1.59	1.96	1.77	2.03	1.46
(*E*)-2-hexenal	2.06	1.64	1.78	1.95	0.92	0.22
2-methylfuran	1.57	1.01	0.61	1.30	1.66	0.55
(*E*,*E*)-2,4-decadienal	0.07	1.67	1.62	1.18	0.80	0.54
Hexanal	0.64	0.84	1.36	1.88	2.32	0.51

Increase over time
Decrease over time
No change over time

Fig. 7. Volatile composition in Tomaffron processed products measured by GC–MS using a reference line to calculate the relative accumulation. **A**: Clustered Imaged Map (CIM) of the PLS-DA analysis of the non-processed Tomaffron and the Tomaffron processed products after processing (time 0). **B**: CIM of the PLS-DA analyses of cold and hot break Tomaffron processed products at all time points. **C**: VIP values of the PLS-DA analyses of each Tomaffron processed product over time. VIP scores higher than 1 are considered significant. The bar graphs showing the tendency are shown in Supplementary Fig. 4 and Supplementary Fig. 5. HB: Hot break; CB: Cold break.

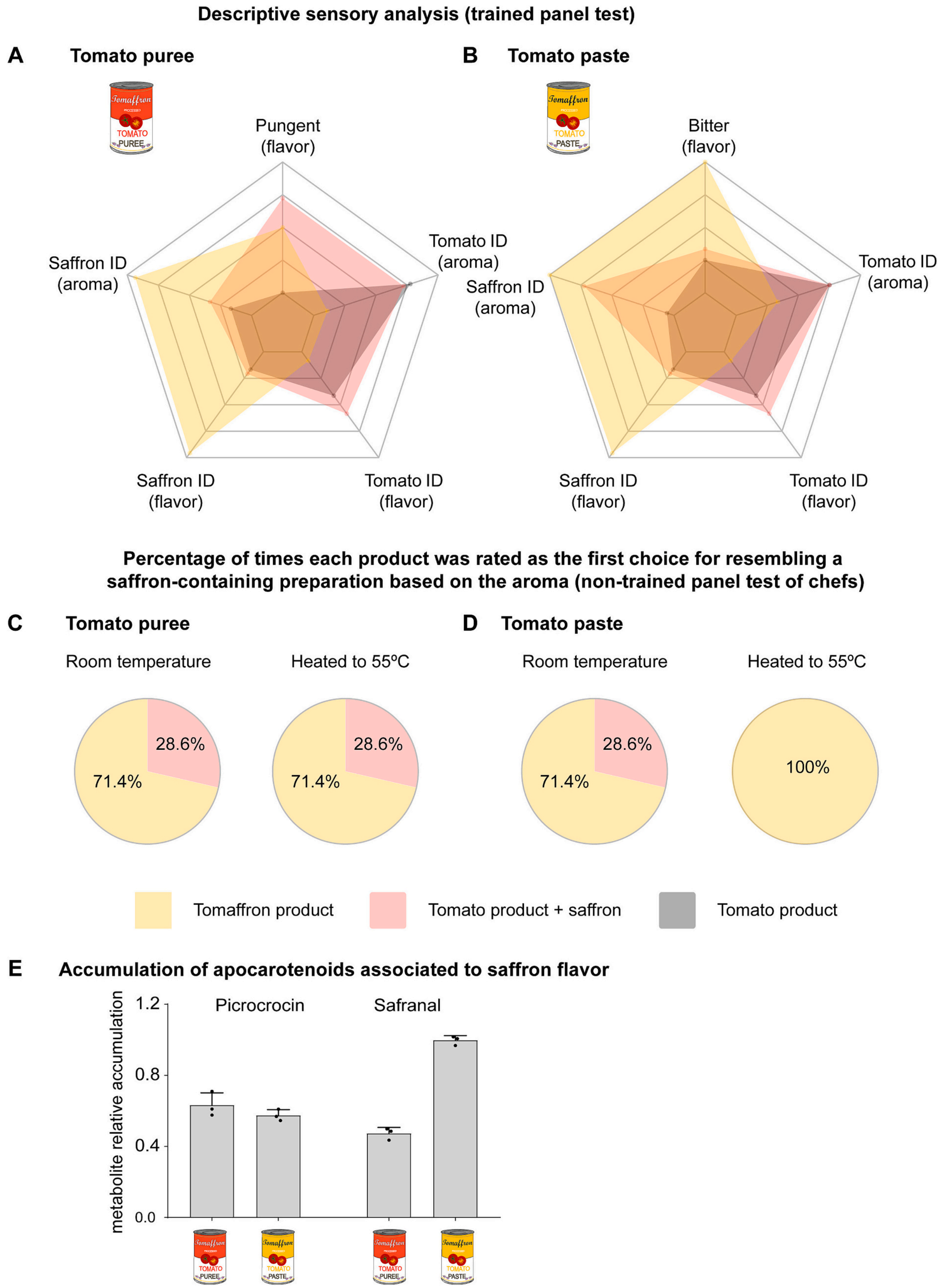


Fig. 8. Descriptive sensory evaluation of Tomaffron-based products. Performed by a trained panel test with Tomaffron puree HB (**A**) and Tomaffron paste HB (**B**). The parameters that showed a significant difference between the samples are represented. Performed by a non-trained panel of chefs with Tomaffron puree HB (**C**) and Tomaffron paste HB (**D**). **E**: accumulation of volatile compounds in fresh material from the cans used for the panel test.

2005; Raina et al., 1996). Safranal can be produced through the dehydration of HTCC or by the thermal, acidic, or two-step enzymatic/dehydration hydrolysis of picrocrocin (Sánchez et al., 2011). Our results suggest that in Tomaffron products, safranal is primarily formed from HTCC, as HTCC showed a drastic decrease following processing. Similar results were reported by (Gregory et al., 2005), where HTCC levels decreased 50-fold, picrocrocin levels declined by 5 %, and safranal content increased significantly in samples treated at temperatures above 80 °C, while minimal changes were observed at lower drying temperatures (43–58 °C).

4.2. Crocin composition and accumulation remain stable after storage of Tomaffron products

cis-Crocin percentage increased significantly in all Tomaffron products compared to fresh Tomaffron (Fig. 2), and the crocin composition remained stable during storage (Supplementary Table 1, Meilgaard, Carr, & Carr, 2006; Pickering & Demiglio, 2008). *Trans*-crocin-3 was the predominant crocin in fresh Tomaffron and exhibited the most pronounced decrease in processed products. Similarly, a significant reduction in *trans*-crocin-3 was reported when the saffron spice was dried at 90–100 °C (Carmona et al., 2005). In *G. jasminoides*, *cis*-crocin increase was observed after treating *Gardenia* fruit at temperatures ranging from 60 to 100 °C, with this increase being more pronounced at higher temperatures, and *trans*-crocin-3 was the most affected crocin (Li et al., 2020). Among the different saffron drying processes, only toasted saffron subjected to high temperatures for a short duration displayed a higher proportion of *cis*-crocin (Rocchi et al., 2018).

In all Tomaffron products, crocin levels remained stable throughout the analyzed storage period (Fig. 3A). This contrasts with saffron spice, where crocin content has been shown to decrease by an average of 13 % and 26 % after three and six months of storage, respectively, when dried at 40 °C. Samples with lower initial moisture content exhibited delayed crocin degradation (Raina et al., 1996). Other studies have also reported a negative correlation between crocin content and saffron storage duration (Bolandi & Ghoddusi, 2006; Fallahi et al., 2021; Sereshti et al., 2018). The observed differences between Tomaffron products and saffron spice may be attributed to the enzymatic inactivation occurring during tomato processing, which does not occur in saffron drying at moderate temperatures or shade drying. In studies on accelerated aging of saffron through heat, a decrease in *trans*-crocin-3 and *trans*-crocin-4 was observed alongside an increase in *cis*-crocin (Carmona et al., 2005). However, these changes may be more closely related to heat exposure than aging, as the same study reported similar changes after high-temperature drying. Likewise, we observed comparable results following high-temperature processing but not during room-temperature storage. These findings underline the significance of the matrix in producing and preserving metabolites of interest, with various factors influencing their stability during processing and storage (Mishra et al., 2024). Tomato has been proven to be a suitable matrix to produce and store crocins, and the levels of crocins can be further increased by using carotenoid mutants that result in higher levels of crocins in the initial fresh fruit (Lobato-Gómez et al., 2024).

4.3. Saffron flavor changes in Tomaffron products over storage time

Safranal accumulation increases during saffron spice storage (Bolandi & Ghoddusi, 2006; Carmona et al., 2006; Fallahi et al., 2021; Gregory et al., 2005; Raina et al., 1996; Sereshti et al., 2018). In most studies, picrocrocin content decreases over time (Fallahi et al., 2021; Gregory et al., 2005; Sereshti et al., 2018), although one study reported fluctuations in picrocrocin levels during storage (Bolandi & Ghoddusi, 2006). Tomaffron products exhibited a slight increase in safranal and picrocrocin content during storage. In contrast, HTCC levels showed a significant decline two months after canning, with even lower levels observed at four and six months post-canning (Fig. 3B,C,D). The increase in picrocrocin and safranal does not appear to correlate with the decrease in HTCC. This discrepancy could be due to the degradation of safranal after its formation or an underestimation of safranal levels caused by using freeze-dried material rather than fresh material for volatile analyses. However, in fresh Tomaffron, safranal levels were low despite high levels of picrocrocin and HTCC. This indicates that the measured safranal was present in the samples and not produced from picrocrocin during volatile extraction. These findings suggest that safranal is primarily generated from HTCC in Tomaffron products.

After one year, picrocrocin and safranal remained present in Tomaffron puree and paste, providing a saffron-like flavor and aroma as confirmed by two independent panel tests, in which Tomaffron outperformed tomato with added saffron, suggesting that these valuable, costly compounds are better perceived when produced within the tomato matrix rather than added externally. Tomato paste demonstrated the most consistent results, likely due to its higher safranal content compared to tomato puree and its elevated picrocrocin levels when accounting for differences in °Brix values between the two products (Fig. 8). If approved for commercialization, Tomaffron could be used to prepare foods traditionally containing both tomato and saffron, such as paella, or recipes based on tomato sauces.

4.4. Effect of the processing on the Tomaffron matrix

The initial enzymatic rupture at high temperatures in tomato processing reduces the accumulation of hydrophilic antioxidant metabolites, including ascorbic acid and flavonoids (Koh et al., 2012; Wu et al., 2022). Many flavonoids accumulate in the fruit skin (Wu & Burrell, 1958), and tomato peel is particularly rich in chalcones and flavanones (Iijima et al., 2008). This explains the absence of naringerin and eriodictyol chalcones in Tomaffron processed products (Fig. 5), as tomato peeling is one of the first steps in the processing workflow (Fig. 1). The high temperatures in tomato processing also result in significant ascorbic acid degradation (Giannakourou et al., 2021). In our results, all Tomaffron processed products showed reduced ascorbic acid content compared to fresh fruit, with the lowest levels observed in the products subjected to higher temperatures for longer durations (Fig. 5).

Despite the loss of ascorbic acid and chalcones, the antioxidant capacity of the hydrophilic fraction remained comparable in all Tomaffron products compared to the unprocessed fruit. This could be attributed to the higher crocin accumulation or availability of the processed products. Tomato paste showed significantly higher ABTS antioxidant capacity than both fresh Tomaffron and other processed products (Fig. 6), likely due to the elevated levels of specific flavonoids, which increased in the paste but not in the other products (Fig. 5). In previous studies, the phenolic content of tomato also increased after processing to tomato paste (Tomas et al., 2017), and the phenolic compounds have been shown to be strongly correlated with the antioxidant capacity of the hydrophilic fraction (Gahler et al., 2003; Podsędek et al., 2003). It has been shown that quercetin exhibits up to five times greater antioxidant activity against ABTS compared to vitamin C (Re et al., 1999).

In previous studies, Tomaffron fruits have shown higher antioxidant capacity than wild-type tomatoes (Ahrazem et al., 2022). Here, the antioxidant capacity of Tomaffron products remained stable during storage, aligning with the observed stability of crocins (Fig. 3), flavonoids, and ascorbic acid (Supplementary Table 3) during storage. In contrast, ascorbic acid content in conventional tomato products has been reported to decrease during storage, with reductions of up to 50 % within the first days after canning (Koh et al., 2012). Although the conventional methods to measure the antioxidant capacity are not optimal for revealing the antioxidant activity of saffron extracts (Ordoudi et al., 2009), these results suggest that the accumulation of saffron apocarotenoids in Tomaffron products may enhance the stability of other antioxidant compounds within the tomato matrix.

The levels of carotenoids in tomato products vary significantly on processing conditions, tomato cultivar, and even plant growth. Although a significant amount of research has been done on how processing affects the lycopene and β-carotene content in fresh tomatoes and tomato products, the results are still inconsistent. Generally, lycopene is reported to exhibit good stability during heat treatments, while β-carotene is often found to decrease after processing in several studies (Wu et al., 2022). However, our results show a different pattern: a significant reduction in lycopene content was observed in diced tomato and tomato puree products, likely due to the specific processing conditions used (Fig. 4A,B). Certain studies suggest thermal treatments and mechanical processing can degrade lycopene through isomerization and oxidation (Shi & Le Maguer, 2000). In contrast, β-carotene content showed a noticeable increase across all Tomaffron products compared to fresh Tomaffron (Fig. 4A,B). This increase can partly be explained by enhanced extractability due to cell wall disruption during mechanical and thermal processing. In this regard, (Graziani et al., 2003) observed a significant increase in β-carotene content in unpeeled tomato puree during heating at 100 °C for up to two hours of treatment, followed by a decrease in its concentration with longer heat treatment durations.

Previous studies have demonstrated that an increase in the lipophilic antioxidant capacity of tomato extracts correlates with the increase in lycopene content and bioavailability in heat-processed tomato products, even in the presence of ascorbic acid loss (Al Maiman et al., 2021; Dewanto et al., 2002). However, all Tomaffron products exhibited similar lipophilic antioxidant capacities, with a significant reduction compared to fresh Tomaffron, despite no significant differences in total carotenoid levels between fresh Tomaffron and the processed products, except for the diced tomato product (Fig. 6B). This apparent contradiction can be attributed to several factors. Crocin aglycone, crocetin, is naturally lipophilic; however, it was not detected in the analyzed material (Fig. 2), confirming its efficient conjugation to various sugar moieties. This suggests that changes in lipophilic antioxidant capacity are likely due to alterations in endogenous non-polar metabolites rather than the presence of saffron metabolites. The antioxidant capacity of fresh tomatoes is not solely dependent on individual compounds but also on the synergistic interactions between various antioxidants (Dewanto et al., 2002). Processing may disrupt these interactions, leading to a net decrease in antioxidant capacity. Although β-carotene content increased, its antioxidant activity is approximately half that of lycopene (Bohm et al., 2002). Therefore, the increase in β-carotene may not fully compensate for the loss of lycopene in terms of overall antioxidant capacity. Additionally, fresh Tomaffron, including the rind, may contain higher levels of antioxidant compounds like tocopherols (vitamin E) than the processed products. In this context, it has been observed that short-term heating led to an increase in α-tocopherol content in tomatoes, while prolonged heating resulted in a decrease in α-tocopherol levels (Seybold et al., 2004). Thus, these compounds could contribute significantly to the lipophilic antioxidant capacity of fresh Tomaffron but may be reduced during processing.

Overall, the relationship between antioxidant capacity and carotenoid composition is complex, with processing having significant and variable effects on both. Thus, the inconsistencies observed in different studies can be attributed to factors such as variety, maturation stage, processing conditions, and storage duration.

Considering that the volatiles were measured in freeze-dried samples, our results primarily reflect the effect of processing on the substrates and enzymes responsible for producing VOCs. Notably, CB tomato puree exhibited significantly higher linear and cyclic volatile apocarotenoid levels (Fig. 4C,D). This product did not accumulate higher levels of lycopene or β-carotene compared to the other products, suggesting that the endogenous CCD enzymes were not entirely inactivated during processing in CB tomato puree. However, they were inactivated in the other products, including CB tomato puree, in which the high temperature in the concentration step compensated for the lower temperature in the enzymatic rupture.

Additionally, substantial differences were observed between the volatile profiles of CB and HB tomato puree. The CB product showed a higher accumulation of many volatiles, except for terpenoids and benzenoids, which were consistently present at lower levels in all Tomaffron products compared to fresh Tomaffron (Fig. 7). Interestingly, CB tomato puree accumulated the highest levels of safranal after canning (Fig. 3D), while picrocrocin levels were comparable across products. This observation further supports the hypothesis that the increased safranal levels are not solely due to greater substrate availability but may also be attributed to residual enzymatic activity.

5. Conclusion

In summary, Tomaffron processed products have demonstrated their potential as an effective platform for preserving saffron apocarotenoids, maintaining stability in the key metabolites responsible for the characteristic flavor and aroma of saffron. After one year of storage, these products were confirmed to be tomato-based foods enriched with saffron compounds, retaining their sensory and biochemical properties. Notably, the tomato matrix may help retain crocins over time, likely due to its antioxidant-rich nature, and a different matrix composition than saffron spice, likely due to differences in the food matrices, highlighting a significant advantage of these innovative products. Furthermore, the stability of these compounds during processing and storage underscores the feasibility of Tomaffron products with extended shelf life and enhanced nutritional value, and provides proof of concept that, if approved for commercialization, Tomaffron could serve as a promising functional food.

CRediT authorship contribution statement

Maria Lobato-Gómez: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **José L. Rambla:** Writing – review & editing, Formal analysis, Data curation. **Jaime Zacarías-García:** Writing – review & editing, Formal analysis, Data curation. **Luis Noguera-Artiaga:** Writing – review & editing, Formal analysis, Data curation. **Jose Pérez-Beser:** Writing – review & editing, Formal analysis, Data curation. **Diego Orzáez:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Lourdes Gómez-Gómez:** Writing – review & editing, Funding acquisition, Conceptualization. **Antonio Granell:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Author contributions

A.G., L.G.G., and D.O. designed the research. M.L.G, JL.R., J.Z.G., L. N.A, and J.P·B performed the experiments. M.L.G. analyzed the data and wrote the article. All authors contributed to the writing and approved the final version.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.144828.

Data availability

Data will be made available on request.

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