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ORIGINAL RESEARCH PAPER

DEVELOPMENT OF AN INNOVATIVE FROZEN DAIRY PRODUCT FORTIFIED WITH CARROT EXTRACT

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Abstract
The development of frozen yogurt supplemented with an oily extract from carrot was the main focus of this study. The research aim converged from the fact that *Daucus carota* L. are among the worldwide most consumed vegetables, exhibiting numerous health benefits. Both analysed variants (the control sample and the fortified sample) presented high total carotenoids, *β*-carotene, and lycopene contents. The addition of the oily extract from carrot caused a significant increase of the total carotenoids, respectively the *β*-carotene contents. Also, these functional dairy products registered a slight increase in the antioxidant activity compared to the control sample, the fortified variant having the highest value (93.95±0.050%). The total polyphenols content varied depending on the analysed variant, from 0.122±0.006 to 1.712±0.008 mg/g dry weight (DW) while the content of total flavonoids varied from 0.065±0.001 to 0.780±0.002 mg/g DW. The carrot and blueberries' HPLC profiles also highlighted the abundance of the bioactive compounds. The confocal microscopy analysis of the fortified frozen yogurt revealed a complex microstructure due to the diversity of the biochemical compounds, a microstructure that preserved the probiotic strain very well and comprised functional properties to the final product. The frozen dairy product enriched with carrot extract can be considered an innovative functional product as it was designed to provide the consumer with various beneficial effects based on its high content of biologically active compounds.

Keywords: frozen yogurt, blueberry, carrot, anthocyanins, carotenoids, capsules, encapsulation

Introduction

In the past years, the world's tendency regarding the production of functional foods has been focused on promoting healthy and nutritive products based on bioactive compounds (Vulić *et al.*, 2019). The expansion of the food industry leads to the

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creation of new products, economically viable, supported within the 'bioeconomy' concept (Gil-Chavez *et al*., 2013; Ravindran and Jaiswal, 2016).

Yogurt is the most popular dairy product around the world, a food product that plays several roles in human health because it delivers a high amount of bioactive compounds and improves microflora with probiotic strains and other lactic acid bacteria (LAB) (Fazilah *et al*., 2018). Yogurt is known to be the most consumed food product containing a large number of probiotic cells for therapeutic effects. The probiotic effects are influenced both by the ability of the organism to survive in the host and also in the product. For obtaining a beneficial effect, the amount of LAB in the product has been prescribed to be 10^7 cfu/ml (Krasaekoopt *et al*., 2006).

There are numerous health benefits of probiotic bacteria described in research studies that can link them to the incorporation into a wide range of food products, such as yogurt, ice cream, etc. (Anal and Singh, 2007). The probiotics definition by Health Canada (2009) in their guidelines for foods is that they can be considered as being ''live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Sandoval-Castilla *et al*., 2010). According to the studies of Sandoval-Castilla *et al*. (2010), it would be better for probiotics to be entrapped within a limited range of bead sizes for satisfying incorporation into foods, for minimization of problems such as cell viability or food texture.

According to the research of Taksima *et al.* (2015), more than 70% of the functional food products in the market, are present in the form of a fermented dairy product. Mollakhalili Meybodi *et al*. (2020) reported in their study that if consuming yogurt, humans can develop a higher tolerance against food pathogens, it improves their immune system and the absorption of lactose and essential minerals. The main focus in the field of dairy products is on the addition of new ingredients, in order to create new yogurt variants with higher properties compared to traditional yogurt (Šeregelj *et al*., 2021).

Frozen yogurt (or yogurt ice cream) is a dairy type product, consumed as a healthful alternative to conventional ice cream, due to its bioactive compounds content and the presence of lactic acid bacteria. Frozen yogurt is as the name suggests, a frozen dessert, that can contain different food additives, etc. (FAO/WHO, 2003), and is present worldwide in any type of market at all times (Skryplonek *et al*., 2019).

According to the research studies of Terpou *et al*. (2019), the most used lactic acid bacteria (LAB), in the obtaining process of dairy products is *Lactobacillus casei*. Due to the *in vitro* and *in vivo* studies, we know that they can survive along the gastrointestinal tract, adhering to the intestine and also resisting during storage at lower temperatures (Terpou *et al*., 2019). Bosnea *et al.* (2017) used specifically in their studies, the strain *L. casei* ATCC 393 for the production of yogurt, Farias *et al*. (2019) for the production of ice cream, Abdel-Hamid *et al.* (2019), and Terpou *et al.* (2017a) for the fermented milk, and Terpou *et al*. (2017b) for the obtainment of cheese.

In order to provide consumers with frozen products such as ice-cream, with many beneficial effects on human health, frozen desserts can be supplemented with extracts of fruits or vegetables, with lactic acid bacterias, or a combination of both (Terpou *et al*., 2019).

The objectives of the study were the valorification of carrot by encapsulating its bioactive compounds in alginate beads using the electrostatic extrusion technique, and also the development of a new type of frozen yogurt. Furthermore, to highlight their functionality, the bioactive contents, and the digestibility of the obtained product were evaluated during the frozen yogurt shelf-life.

Materials and methods

Materials

Fresh *Daucus carota* sp. and fresh *Vaccinium myrtillus* L., were purchased from the local market, Galati, Romania in June 2021. Formic acid, methanol, ethyl acetate, acetonitrile, sodium carbonate, aluminium chloride, sodium nitrite, sodium hydroxide, $ABTS^{+}$ (2,20 azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), and Folin-Ciocalteu's reagent were purchased from Sigma-Aldrich Chemical Co. (USA). The carotenoids standards (β-carotene, lycopene, lutein), were purchased from Sigma-Aldrich Chemical Co. (China). The anthocyanins standards (delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-arabinoside, cyanidin-3-glucoside, petunidin-3-galactoside, petunidin-3-glucoside, peonidin-3-galactoside, petunidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside, malvidin-3-arabinoside) were acquired from Sigma-Aldrich Chemical (Germany) and Extrasynthese (France). Gallic acid and catechin standards were acquired from Sigma-Aldrich Chemical (Germany).

Microbial culture

A *Lactobacillus casei* strain (DSMZ, Braunschweig, Germany) was used for the frozen yogurt manufacture. *L. casei* was grown at 37°C in a MRS liquid medium (De Mann, Rogossa and Sharpe, Sigma Aldrich, GermanyMerck,) for 48 - 72 h under anaerobic conditions (7% CO_2 and 11.5% O_2, Sanyo, MCO-18M, Japan, Sakata). The obtained *L. casei* biomass was harvested by centrifugation (Hettich, Universal 320R, Germany) at 5000 rpm for 10 min at 4°C. A 10^{12} CFU/mL inoculum was prepared and added to the encapsulation step.

Preparation of oily carrot extract and encapsulation in beads

The procedure used for obtaining the carrot extract was performed according to Šeregelj *et al*. (2021). In short, the fresh carrot was mixed with sunflower oil at 25°C (1:10 w/v) by stirring with a ProBlend Crush blender for 20 min (Philips, Romania), applying repeated cycles of 5 min blend and 2 min pause, in order to avoid heating. Afterward, the oily extract was centrifuged at 6000 rpm for 10 min (Hettich, Universal 320R, Germany), the supernatant was recovered and stored in a dark glass bottle for further use.

The encapsulation step of the oily carrot extract was performed according to Šeregelj *et al*. (2021), using alginate as the carrier. A 2% sodium alginate solution was prepared using ultrapure water under magnetic stirring at 700 rpm (IKA RCT basic, Germany). The proportion of the carrot extract added to the alginate solution was 25%. To the final emulsion, it was added the *L. casei* culture obtained previously, which stayed under magnetic stirring at 300 rpm (IKA RCT basic, Germany) for another 5 minutes, in order to allow good incorporation. The obtained alginate/carrot extract/LAB emulsion was extruded through a 0.5-mm blunt stainless-steel needle using a syringe pump (LKB Bromma 12000 Varioperpex peristaltic pump, Sweden) under a constant flow rate. The carrot extract beads were maintained in a calcium chloride solution for one hour in order to finalize their formation. At last, the obtained carrot extract beads were washed using ultrapure water and stored at ambient temperature before freeze-drying (Alpha CHRIST 1-4 LD Plus, Germany). Freeze-dried carrot extract beads were stored at 20°C until further analyses.

Preparation of frozen yogurt samples

In order to prepare the samples, a standard yogurt (as control and the basis for the fortified product) was acquired from the local market Galati, Romania. Briefly, 140 g of yogurt, 100 g mascarpone, 150 g of blueberries, 3 mL of vanilla extract, and 10 g of vanilla sugar were homogenized, and the packaging step of yogurt was continued. The yogurt was filled in plastic containers and kept overnight at -20°C in a freezer, in order to obtain frozen yogurt (control sample - CFY).

In the case of fortified frozen yogurt (FFY), carrot extract beads containing LAB were added to the yogurt, before the packaging step. The fortified variant was also transferred into plastic containers and kept overnight at -20°C in a freezer.

For all the analysis, the fortified sample (FFY) and the control sample (CFY), were prepared in triplicate.

Characterization of fortified frozen yogurt

Digestibility

For the analysis of the *in vitro* digestibility of the frozen yogurt samples, the method described by Barbu *et al*. (2020) was used. The gastric digestion simulation was made using a mixture that contained 20 mg pepsin from porcine gastric mucosa (Sigma-Aldrich, USA) and 20 mL 0.1M HCl, with the pH set at a -value of 2.0. The aforementioned mixture that contained simulated gastric juice and also 10 mL of sample (500 mg of sample dissolved in 10 mL 20 mM Tris buffer with a pH of 7.7) was incubated at 37°C on an orbital shaker (Incubated shaker SI-300R, Korea) at 150 rpm, aliquots being taken and analyzed every 30 min. The mixture used for simulating the intestinal digestibility consisted of 40 mg of pancreatin from porcine pancreas (Sigma-Aldrich, USA) and 20 mL of 0.9M $NaHCO_3$, with the pH set at a value of 7.0. The mixture that contains intestinal simulated juice and also 10 mL of sample (10 mL taken from the remaining simulated gastric juice) was incubated (Incubated shaker SI-300R, Korea) using the same conditions as in

the case of the gastric simulation. The determination of carotenoids and anthocyanins contents was assessed as previously described.

Confocal Laser Scanning Microscopy

The images were obtained by using a Zeiss confocal laser scanning system (LSM 710) equipped with different lasers (a diode laser, Ar-laser, DPSS laser and HeNe-laser) of the new functional nutraceutical product based on an oily carrot extract and LAB. In order to observe in detail, the *L. casei* cells and the vegetal microstructures present in this functional product, a Live/Dead stain kit (Molecular Probes, Eugene, OR, USA) was used, following the manufacturer's instructions.

A Zeiss Axio Observer Z1 inverted microscope equipped with a 40× apochromatic objective (numerical aperture 1.4) was used for the analysis of the samples. The 3D images were processed using the ZEN 2012 SP1 Black edition software. The evaluation was made on a minimum number of twenty fields, with each assay being performed in triplicate.

Phytochemical analysis

Carotenoids determination and the encapsulation efficiency

Total carotenoids (TC), β-carotene (BC), and lycopene (LC) contents in beads were determined after they were dissolved in 2% sodium citrate solution in a ratio of 1:7 w/v for 15 min, with vigorous stirring on Vortex (BenchMixer, Taiwan) to allow chemical dissolution. After dissolving, the carotenoids were extracted with a mixture of hexane:acetone (1:3, v/v), and their contents were determined spectrophotometrically at a wavelength corresponding to the maximum absorption of each of the carotenoids with a Biochrom Libra S22 (Holliston, MA) equipment:

Equation 1 was further used (Mihalcea *et al*., 2021):

$$\text{Bioactive compounds content (mg/g DW)} = \frac{A \cdot M_W \cdot Df}{\varepsilon \cdot L} \qquad (1)$$

where A is the absorption at 450 nm for total carotenoids, at 470 nm for *β*-carotene, and at 503 nm for lycopene, D_f is the sample dilution rate, L is the cell diameter of the spectrophotometer (1 cm).

To quantify the total carotenoids (TC), *β*-carotene (BC), and lycopene (LC), the molecular weights (MW) and molar extinction coefficients (ε) of the representative compounds were undertaken *i.e.* total carotenoids (ε = 2590 L/mol cm^{-1} in hexane:acetone, M_W = 536.88 g/mol), *β*-carotene (ε = 2500 L/mol cm^{-1} in hexane:acetone; M_W = 536.88 g/mol) and lycopene (ε = 3450 L/mol cm^{-1} in hexane:acetone; M_W = 536.88 g/mol).

The encapsulation efficiency (EE%) of the *β*-carotene, was calculated according to the equation below (Šeregelj *et al*., 2021), where β-CarI represents initial *β*-carotene added to the emulsion and *β*-CarB represents β-carotene content in the beads:

$$EE(\%)=(\beta\text{-CarB}/\beta\text{-CarI})\times 100 \qquad (2)$$

Total monomeric anthocyanin content

In order to determine the total monomeric anthocyanin content (TMA) the AOAC (2005.02) official method was used and the TMA content was expressed as mg of cyanidin 3–glucosides equivalents (CGE) per g dry weight (DW), based on the method described by Oancea *et al*. (2018). The results were expressed as mg of cyanidin-3-glucoside equivalents (C3G) per gram dry weight (DW).

Total Phenolic Content

The total phenolic content (TPC) was determined as stated by Barbu *et al*. (2020), by using the Folin-Ciocalteu reagent. By adding 200 µL of extract over 15.80 mL of ultrapure water and 1000 µL of Folin-Ciocalteu reagent was obtained a mixture that was maintained 10 minutes in the dark to react. After the reaction time, 3000 µL of 20% Na_2CO_3 was added, and the final mixture was maintained in the dark at room temperature for 60 minutes in order to react. The absorbance was measured at a wavelength of 765 nm. The results were expressed as mg of gallic acid equivalents (GAE) per gram dry weight (DW).

Total flavonoids content

The total flavonoids content (TFC) was determined using the colorimetric method described by Oancea *et al*. (2018), and the results were expressed as mg catechin equivalents (CE) per gram dry weight (DW). In order to determine total flavonoids, a mixture was prepared that contained 250 µL of extract, 1.25 mL of ultrapure water, and 75 µL of 5% $NaNO_2$, which was allowed to react for 5 minutes. Over this mixture 150 µL of 10% aluminum chloride solution which was also let to react for 6 minutes. Finally, 500 µL of 1M NaOH solution and ultrapure water until 3mL final volume were added. The absorbance was measured immediately at a wavelength of 510 nm.

Antioxidant Activity

The antioxidant capacity (AA) was employed as described by Gheonea *et al.* (2021), by using the 2,20 azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$, Sigma Aldrich, Steinheim, Germany) radical's method. The $ABTS^{\cdot+}$ antioxidant activity of the samples was expressed as mM Trolox equivalents/g DW based on the calibration curve. The percent inhibition of $ABTS^{\cdot+}$ was calculated as follows:

$$\text{Antioxidant activity } (\%) = \frac{\text{Abs. of the blank} - \text{Abs. of the sample}}{\text{Abs. of blank}} \cdot 100 \qquad (3)$$

Identification of biologically active compounds

Carotenoids found in the fresh carrot sample were identified using the chromatographic analysis, as described by Ursache *et al*. (2017).

Briefly, the carrot extract was prepared by mixing 0.1g of fresh sample with 10 mL of hexane, and then ultrasonication for 30 minutes (MRC Scientific Instruments), followed by a centrifugation step at 6000×g, at 4°C for 5 min. The obtained supernatant was filtered through 0.2 µm nylon membranes and then injected into the Surveyor HPLC system (Finnigan Surveyor LC, Thermo Scientific, Waltham, USA) equipped with a Lichrosorb RP-18 (5 µm) Hibar RT 125-4 column (Phenomenex, Torrance, USA). Compounds of interest were detected at a wavelength of 450 nm.

Anthocyanins found in the fresh blueberry sample were identified using chromatographic analysis, based on the method described by Condurache *et al.* (2021).

The blueberry extract was prepared from 1.0 g of fresh blueberry and 10 mL of 80% methanol followed by ultrasonication for 30 minutes (MRC Scientific Instruments). The sample was centrifuged and filtered before injection, by using 0.22 µm syringe filters (Bio Basic Canada Inc., ON, Canada). A Hypersil BDS C18 column (150x4.6 mm, 5 µm) was used, maintained at a temperature of 30°C. The mobile phase consisted of 100% pure methanol (A) and 10% formic acid (B), whereas the injection volume was 20 µL, at a 1 mL/min flow rate. The detection was specifically set at a wavelength of 520 nm.

Statistical Analysis of Data

All the data reported in this research study represents the averages of a triplicate analysis and was reported as mean ± standard deviation. The analysis of variance (ANOVA) ($p < 0.05$) was carried out to assess the significant differences between the obtained values.

Results and discussion

Confocal Laser Scanning Microscopy

The confocal analysis of the frozen fortified yogurt revealed a complex microstructure due to the diversity of the biochemical compounds that are part of the product. With a fairly high frequency (approximately 70%), it could be seen several well-preserved fragments of the plant tissue in the root parenchyma of *Daucus carota*. As it can be seen in Figure 1, the cells present almost intact cell walls (shown in yellow) being full of chromoplasts (in green) that contain carotenoids with a fluorescence emission in the range of 500-550 nm. Around these tissue fragments, with the help of the Live/Dead stain kit, dense masses of lactic acid bacteria (*L. casei* microcolonies) were highlighted, in a proportion of 80-90% alive (colored in green) and 10-20% dead (colored in red). The chosen method of microencapsulation of the biologically active compounds from carrot root, as well as the preparation of the frozen fortified yogurt, are original methods that preserved the probiotic strains very well and gave functional properties to the product.

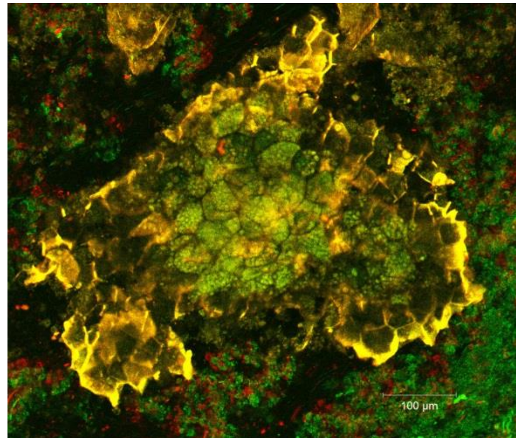

Figure 1. CLSM images of fortified frozen yogurt microstructure.

Carotenoids Quantification, Total Polyphenols Content, Total Flavonoids Content, and Antioxidant Activity

The contents of the bioactive compounds and the antioxidant activity differed significantly ($p < 0.05$) when comparing the values obtained for the CFY sample and the FFY variant. As such, the total carotenoids content, *β*-carotene content and lycopene content in the CFY sample (control sample) presented the following values 0.005 ± 0.001 mg/g DW, 0.016 ± 0.004 mg/g DW, and 0.006 ± 0.0 mg/g DW, respectively (Table 1). The FFY variant displayed significantly ($p < 0.05$) higher TC, BC, and LC contents compared to the control sample 89.885±4.429 mg/g DW, 78.901±3.417 mg/g DW, and 24.911±1.698 g/g DW respectively (Table 1).

The microencapsulation method was used in this research study to obtain high-quality beads, by minimizing oxidative degradation of carotenoids during the freeze-drying process. The encapsulation efficiency (EE) of β-carotene was obtained by dividing the amount of β-carotene in the product with that from the extract. The encapsulation efficiency was 23.36 ± 0.13%, implying a loss of bioactive compounds during freeze-drying, which may be caused by the mass loss. Gheonea *et al*. (2021) reported an encapsulation efficiency of lycopene of 83.6±0.020%, Jia *et al*. (2020) reported an encapsulation efficiency and an encapsulation yield of 94% and 86% for lycopene microencapsulation in whey protein isolate conjugated with xylo-oligosaccharides via Maillard reaction. Sampaio *et al*. (2019) reported a maximum lycopene encapsulation efficiency of 100%. The EE of β-carotene reported by Mihalcea *et al*. (2018), was 36.23±1.58%. Mihalcea *et al*. (2017) reported a significantly higher EE of β-carotene from the sea buckthorn (SBT) extract obtained using supercritical CO_2 46.18 ± 0.13%. Rodríguez-Huezo *et al*. (2004) reported an EE varying from 25.6% to 87.5%. Ursache *et al*. (2018) reported a higher value of 56.16±1.24% for microencapsulation of carotenoids extracted from SBT using conventional methods.

The content of total monomeric anthocyanin content (TMA) in the CFY sample was 0.013±0.001 mg cyanidin-3-glucoside (C3G)/g DW (Table 1) whereas the FFY sample had a content of TMA of 0.774±0.015 mg C3G/g DW. It can be stated that the addition of the selected fruits (blueberries) caused a significant increase in the content of the bioactive compounds.

Because of the low temperature, air presence, and light, the oxidation reactions are limited, therefore the bioactive content in the product is substantially preserved (Cui *et al*. 2008). In the case of carrots, the antioxidant activity is usually correlated with the *β*-carotene content, whereas in the case of blueberries it is correlated with the total anthocyanins and polyphenolic contents. Moreover, it is known that processed fruits and vegetables have lower antioxidant activity compared to raw or fresh due to vitamin C degradation during processing (Lau *et al*., 2018).

The FFY sample registered an inhibition of 93.95±0.050%, compared to the CFY sample that exhibited a 30.71±0.014% inhibition (Table 1). These results can be correlated with the higher contents of both anthocyanins and carotenoids, but also with other compounds, besides the analyzed ones, that contribute to the antioxidant activity of the samples. Purkiewicz *et al*. (2020) concluded that the antioxidant activity of the orange and yellow carrot juices did not differ, whether using DPPH (28.54±0.35 µmol Trolox/mL for orange carrot juices and 29.12±0.59 µmol Trolox/mL for yellow carrot juices) or ABTS assays (3.28±0.01 µmol Trolox/mL for orange carrot juices and 3.15±0.01 µmol Trolox/mL for yellow carrot juices). Bystrická *et al*. (2015) managed to determine an antioxidant activity varying from 6.88±0.92% for the Jitka carrot variety, to 9.83±0.62% for the Koloseum carrot variety. Their results are similar to those obtained by Algarra *et al*. (2014) who determined an antioxidant activity between 1.4 – 17.6%, and Bembem *et al.* (2014) who determined an antioxidant activity in carrots of 11.2%.

Table 1. Phytochemical features of the CFY and the FFY samples.

	FFY	**CFY**
TMA (mg/g DW)	0.774±0.015[a]	0.013±0.001[b]
TC (mg/g DW)	89.885±4.429[a]	0.005±0.001[b]
BC (mg/g DW)	78.901±3.417[a]	0.016±0.004[b]
LC (mg/g DW)	24.911±1.698 [a]	0.006±0.00[b]
TPC (mg GA/g DW)	1.712±0.008 [a]	0.122±0.006[b]
TFC (mg CE/g DW)	0.780±0.002 [a]	0.065±0.001[b]
AA (%)	93.95±0.050 [a]	30.71±0.014[b]

For each tested biologically active compounds content, the values from the same row that do not share a letter are statistically different at $p < 0.05$ based on the Tukey method and 95% confidence; TMA – total monomeric anthocyanin content; TC— total carotenoids content; BC—β-carotene content; LC – lycopene content, TPC—total polyphenols content, TFC-total flavonoids content, AA-antioxidant activity.

The TPC content varied from 0.122±0.006 mg gallic acid equivalents (GAE)/g DW in the case of the CFY sample to 1.712±0.008 mg GAE/g DW for the FFY sample (Table 1). According to the studies of Purkiewicz *et al*. (2020), the total polyphenolic content varied between the carrot varieties, the higher content being registered for the black carrot juices, while the lower content was registered for the orange carrot juices. Another study conducted by Bystrická *et al*. (2015) on different varieties of carrots (Jitka, Kardila, Katlen, Rubína, and Koloseum) ranged from 81.25±13.11mg/kg to 113.69±11.57mg/kg. Also, Bouzari *et al*. (2015) obtained a polyphenolic content in fresh carrots of 108 mg GAE/100 g DW.

The TFC content varied from 0.065±0.001 mg catechin equivalents (CE) in the case of CFY to 0.780±0.002 mg CE/g DW for the FFY variant (Table 1).

Chromatographic analysis of the biologically active compounds

In order to characterize the carotenoids from the fresh carrot extract, a chromatographic analysis was performed (Figure 2). The carotenoid identification was made depending on the retention time and by comparison with the available standards and the data existing already in the literature.

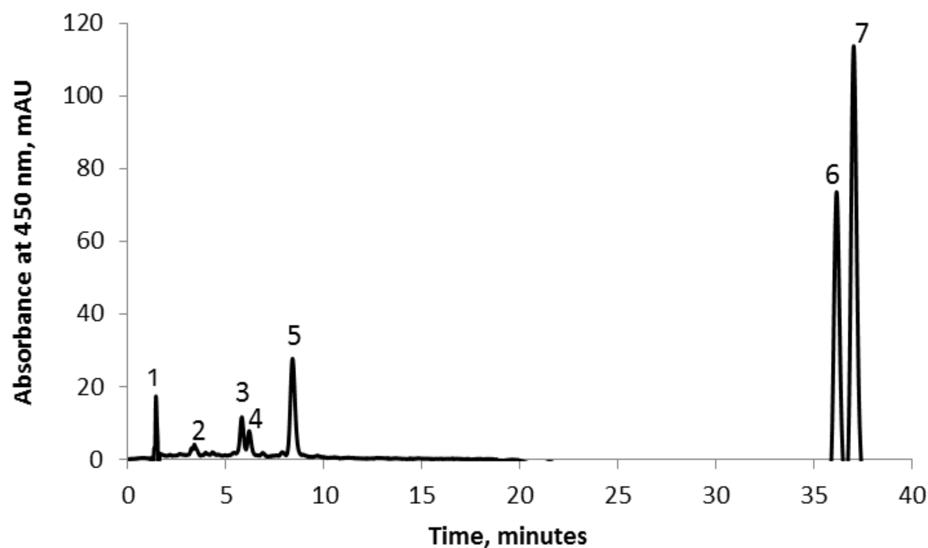

Figure 2. Chromatographic profile of fresh carrot extract. Peak 1 – 4 – unidentified, peak 5 – lutein, peak 6 – lycopene, peak 7 – β-carotene.

The carotenoid identification was made at 450 nm, and the chromatographic analysis revealed the presence of seven compounds in the carrot extract: 1 – 4 – unidentified, 5 – lutein, 6 – lycopene, 7 – β-carotene. These results are in agreement with those obtained by Lau *et al*. (2018) on carrot peel extract. The highest peak was registered for the compound β-carotene, 29.69±0.18 µg β-carotene/mL extract (Table 2).

In their research study, Purkiewicz *et al*. (2020) managed to separate and identify five different carotenoids in yellow, orange, and black juices: α-carotene, β-carotene, 13-*cis*-β-carotene, lutein, and zeaxanthin. In the orange carrot juices they highlighted that the dominant carotenoid was β-carotene, and the second dominant compound was 13-*cis*-β-carotene. Significant lower amounts of these two compounds were found in the black carrot juices (Purkiewicz *et al*. 2020).

Table 2. The detected carotenoids in the extract of fresh carrot by HPLC-DAD.

Peak	Compound	t_R	Carotenoid, μg/mL
1.	Unidentified	1.44±0.01	NQ
2.	Unidentified	3.40±0.01	NQ
3.	Unidentified	5.82±0.02	NQ
4.	Unidentified	6.19±0.02	NQ
5.	Lutein	8.14±0.01	5.04±0.02
6.	Lycopene	36.16±0.03	17.98±0.10
7.	β-carotene	37.02±0.02	29.69±0.18

*NQ - not quantified

In another study conducted by Szczepańska *et al*. (2022) on carrot juice, seven carotenoids were separated and identified: phytoene, lutein, ε-carotene, α-carotene, β-carotene, 9-Z-β-carotene, and δ-carotene. In an early study, Szczepańska *et al*. (2021) managed to separate from the fresh carrot juice the following carotenoids, namely: lutein, β-cryptoxanthin, ε-carotene, 9-Z-β-carotene, α-carotene and β-carotene, and one unidentified carotenoid.

In order to characterize the anthocyanins from blueberry extract, chromatographic separation was realized (Figure 3). The anthocyanins identification also was made depending on the retention time and by comparison with the available standards and the data existing already in the literature.

The identification of these compounds was made at 520 nm, and the chromatogram revealed the presence of thirteen compounds in the blueberry extract: 1 – delphinidin-3-galactoside, 2 – delphinidin-3-glucoside, 3 – cyanidin-3-galactoside, 4 – delphinidin-3-arabinoside, 5 – cyanidin-3-glucoside, 6 – petunidin-3-galactoside, 7 – petunidin-3-glucoside, 8 – peonidin-3-galactoside, 9 – petunidin-3-arabinoside, 10 – malvidin-3-galactoside, 11 – malvidin-3-glucoside, 12 – unidentified, 13 – malvidin-3-arabinoside, with the highest peak being represented by malvidin-3-galactoside, 1854.84±2.10 μg M3G/mL extract (Table 3).

These results are in agreement with those obtained by Skrede *et al*. (2000) on highbush blueberries who reported 12 anthocyanins separated from the Bluecrop variety.

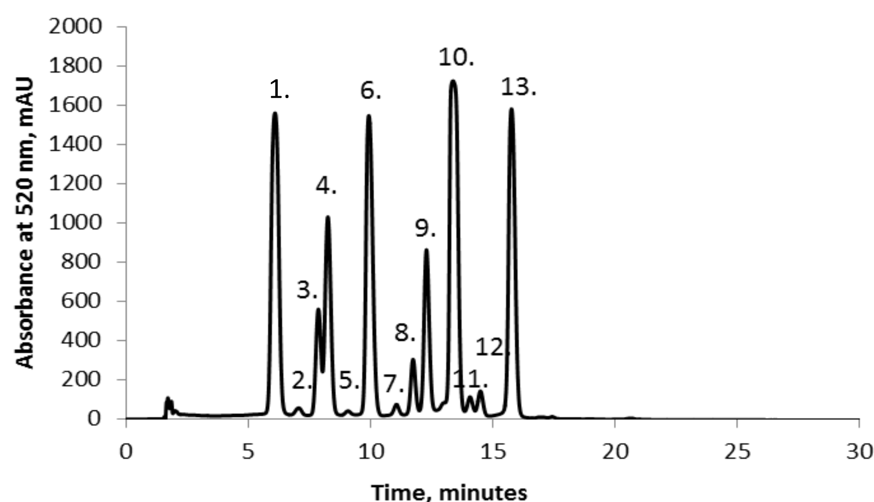

Figure 3. Chromatographic profile of fresh blueberry extract.

(peak 1 – delphinidin-3-galactoside, peak 2 – delphinidin-3-glucoside, peak 3 – cyanidin-3-galactoside, peak 4 – delphinidin-3-arabinoside, peak 5 – cyanidin-3-glucoside, peak 6 – petunidin-3-galactoside, peak 7 – petunidin-3-glucoside, peak 8 – peonidin-3-galactoside, peak 9 – petunidin-3-arabinoside, peak 10 – malvidin-3-galactoside, peak 11 – malvidin-3-glucoside, peak 12 – unidentified, peak 13 – malvidin-3-arabinoside).

Table 3. The detected anthocynins in the extract of fresh blueberry by HPLC-DAD.

Peak	Compound	tR	Anthocyanins, µg/mL
1.	delphinidin-3-galactoside	6.08	1533.013±1.91
2.	delphinidin-3-glucoside	7.05	58.13±0.11
3.	cyanidin-3-galactoside	7.86	362.99±1.54
4.	delphinidin-3-arabinoside	8.25	NQ
5.	cyanidin-3-glucoside	9.07	38.39
6.	petunidin-3-galactoside	9.92	NQ
7.	petunidin-3-glucoside	11.06	68.64±0.15
8.	peonidin-3-galactoside	11.73	NQ
9.	petunidin-3-arabinoside	12.28	NQ
10.	malvidin-3-galactoside	13.37	1854.84±2.10
11.	malvidin-3-glucoside	14.07	83.27±0.21
12.	Unidentified	14.49	NQ
13.	malvidin-3-arabinoside	15.76	1339.515±2.52

*NQ - not quantified

In another study conducted by Sun *et al.* (2012) on 11 batches of blueberry extracts, fourteen anthocyanins were also separated and identified, the highest peak being represented by delphinidin-3-galactoside, peonidin 3-glucoside, and malvidin

3-glucoside. Also, 9 compounds were identified by Cesa *et al.* (2017) in a hydroalcoholic extract of blueberry samples, namely: delphinidin-3-O-galactoside; cyanidin-3-O-galactoside; delphinidin-3-O-arabinoside; petunidin-3-O-galactoside; cyanidin-3-O-arabinoside; petunidin-3-O-arabinoside; malvidin-3-O-galactoside; malvidin-3-O-glucoside and, malvidin-3-O-arabinoside. Also, our results are according to those obtained by Aliaño-González *et al*. (2020) who managed to separate 14 anthocyanins in the blueberry extract (delphinidin 3-O-galactoside, delphinidin 3-O-glucoside, cyanidin 3-O-galactoside, delphinidin 3-O-arabinoside, cyanidin 3-O-glucoside, petunidin 3-O-galactoside, cyanidin 3-O-arabinoside, petunidin 3-O-glucoside, peonidin 3-O-galactoside, petunidin 3-O-arabinoside, peonidin 3-O-glucoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside, malvidin 3-O-arabinoside).

The quantification of the rest of the bioactive compounds identified by the HPLC chromatograms will be further examined in future studies.

Digestibility of the Frozen Yogurt Products

The *in vitro* digestibility analysis of the frozen yogurt products tried to evaluate the carotenoids and anthocyanins' behavior in both gastric and intestinal juices.

The biological activity of anthocyanins and carotenoid compounds, and their behavior in the gastrointestinal tract (Figure 4 a, b and Figure 5 a, b), in regards to their beneficial properties on human health represents a complex process so that depending on their concentration may affect their release and absorption at the gastrointestinal level.

Pinto *et al*. (2017), stated that gastrointestinal digestion contributes to the release of total polyphenols from the food matrix. However, several transformations of polyphenolic compounds under gastrointestinal conditions can lead to a reduction in their potential bioactivity. Polyphenols are considered to be among the most unstable compounds in food, thus decreasing during the digestion step.

As the recipe indicates, the CFY sample did not contain any bioactive compounds from carrot. After 90 minutes of maintaining the FFY sample in contact with the simulated gastric juice, a release of the microcapsules was observed, in a percentage of 54.70±1.06%, a value that increased by 9.38±1.44% after 120 minutes. In the case of the simulated intestinal juice, a decrease of 33.91±1.63% in the release of the carotenoids could be observed for the same sample, being afterward followed by an increase of 23.12±0.87%. Furthermore, after 60 minutes a rather constant release of β-carotene was also assessed.

In terms of TMA, the CFY sample did not contain any bioactive compounds from blueberries. For the FFY variant, a constant decrease in the anthocyanins release was observed. In the intestinal digestion step, there was an increase in the release of anthocyanins of 12.26±0.07% after 30 min, followed by a decrease of about 44.54±1.18% after 60 min for the FFY variant and an increase of 37.88±3.71% after 120 min, while for the CFY sample also a constant level of the C3G content was observed. According to Zygmantaité *et al*. (2021), there was a gradual increase in the polyphenols that were released into the gastrointestinal fluids the during

digestion of the yogurt samples. However, most polyphenols remained insoluble and were not released at all digestion times (Zygmantaité *et al*., 2021).

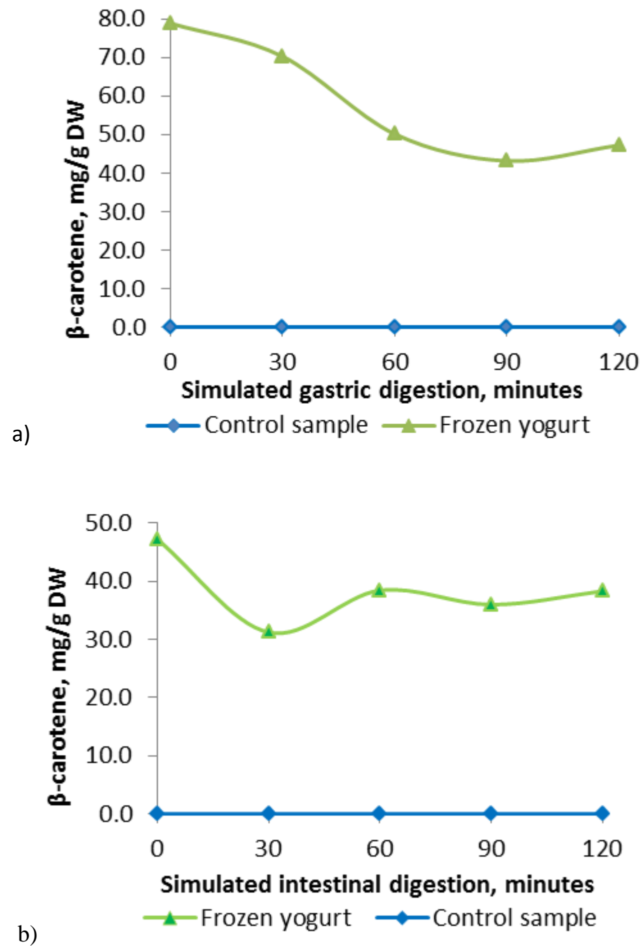

Figure 4. Effect of the simulated gastric (a) and intestinal digestion (b) on the level of *β*-carotene (BC) released from the frozen yogurt.

The findings of Zygmantaite *et al*. (2021) highlighted the relationship between the formation of complexes between milk proteins and the phenolic-rich cranberry extract (Ozdal *et al*., 2013). The changes in digestibility were positively correlated to the strength of protein–polyphenol interactions (Stojadinovic *et al*. 2013). Other researchers have highlighted similar results. Pan *et al*. (2019) stated that 1%–5% pomegranate juice rich in polyphenols decreased the *in vitro* digestibility in yogurt.

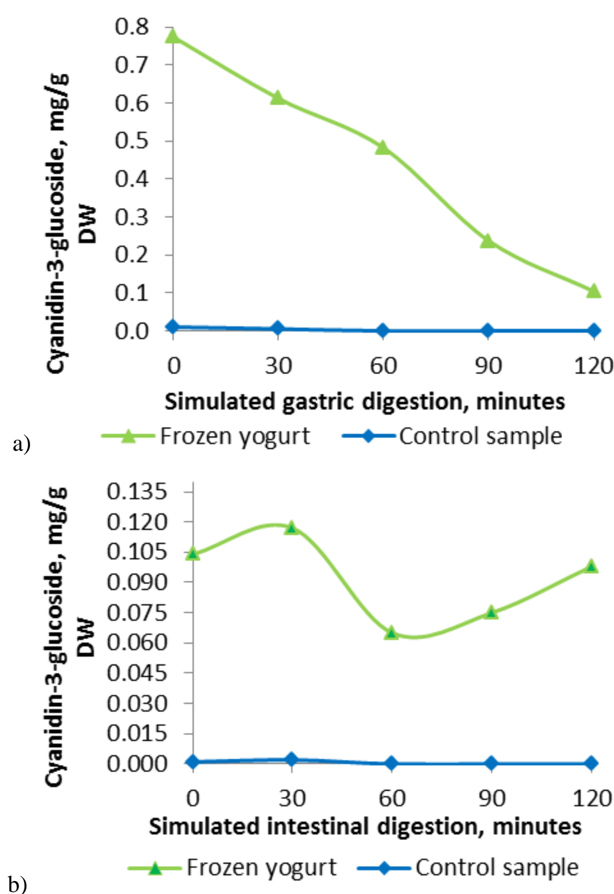

Figure 5. Effect of the simulated gastric (a) and intestinal digestion (b) on the level of total monomeric anthocyanin content (TMA) released from the frozen yogurt**.**

Conclusions

This research study aimed to use oily extract produced from carrots and fresh blueberry fruits for the fortification of frozen yogurt. The fortified yogurt as an innovative product displayed high concentrations of carotenoids, β-carotene, lycopene, total polyphenols, total monomeric anthocyanins, and high antioxidant activity, so the final product can be considered and regarded as a functionally enhanced food product. These kinds of dairy products are ready for consumption, and they also can provide a sufficient daily intake of probiotics, being a dairy product. The health benefits exhibited by such products should be further examined.

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