Ascorbic acid-induced degradation of liposome-encapsulated acylated and non-acylated anthocyanins of black carrot extract

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Abstract

BACKGROUND: In the presence of ascorbic acid, the degradation of acylated (sinapic, ferulic and p-coumaric acid derivatives of cyanidin-3-xylosylglucosylgalactoside) and non-acylated anthocyanins of black carrot extract (BCE) encapsulated in liposomes was studied. BCEs (0.2% and 0.4% w/w) were encapsulated in liposomes using different lecithin concentrations (1%, 2% and 4% w/w).

RESULTS: The liposomes were prepared with particle diameters of less than 50 nm and zeta potentials of about −21.3 mV for extract-containing liposomes and −27.7 mV for control liposomes. The encapsulation efficiency determined using high-performance liquid chromatography (HPLC) showed that increasing lecithin levels increased the efficiency to 59% at the same extract concentration. The concentrations of total anthocyanins and individual anthocyanins were determined for ascorbic acid (0.1% w/w)-degraded extract and liposomes (containing 0.2% w/w extract). Anthocyanin quantification of both liposomal and extract samples was performed by HPLC using cyanidin-3-O-glucoside chloride as standard. Five anthocyanins in the extract and encapsulated liposomes were quantified during 24 h (0–24 h): cyanidin-3-xylosylglucosylgalactoside 1.0–0.51 and 0.82–0.58 mg g−1, cyanidin-3-xylosylgalactoside 2.5–1.1 and 2.2–1.7 mg g−1, cyanidin-3-xylosyl(sinapoylglucosyl)galactoside 0.51–0.14 and 0.35–0.28 mg g−1, cyanidin-3-xylosyl(feruloylglucosyl)galactoside 1.37–0.41 and 1.06–0.98 mg g−1, and cyanidin-3-xylosyl(coumaroylglucosyl)galactoside 0.28–0.08 mg g−1 for extract and 0.27–0.26 mg g−1 for liposomes, respectively.

CONCLUSIONS: This study demonstrates the potential beneficial effect of liposomal encapsulation on individual, particularly acylated, anthocyanins after addition of ascorbic acid during a storage time of 24 h. © 2021 The Authors. Journal of The Science of Food and Agriculture published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

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Keywords: black carrot; anthocyanins; ascorbic acid; liposomes; acylation; encapsulation

INTRODUCTION

Anthocyanins, which are glycosylated polyhydroxy and methoxy derivatives of flavylvium salts,1 are widely used as natural food colorants due to growing concerns about the potential adverse health effects of synthetic colorants. Depending on the pH value, anthocyanins are responsible for red to bluish colors, and they are relatively stable in their original sources; however, after juice or extraction processes, they lose their color stability. Encapsulation processes can protect anthocyanins against the degradation effect of light, oxygen, humidity and the presence of other compounds.2, 3 In particular, acylation with phenolic acids offers greater stability for anthocyanins. Under neutral or slightly acidic conditions, anthocyanins without acyl groups are converted into their colorless form by hydration at the C-2 and/or C-4 position.1, 4

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Specifically, co-pigmentation is a phenomenon that increases the brightness and stability of anthocyanins as a result of interaction with organic substances, even at relatively high pH values. In principle, co-pigments are colorless molecules that increase the stability of anthocyanin chromophores in sufficient quantities. Some investigations suggest that the co-pigmentation of anthocyanins with other substances is the key mechanism for the stabilization of color in plants. Such intermolecular co-pigmentation is due to another molecule; however, covalently bound acyl groups and flavylum cations define intramolecular co-pigmentation. Black carrot anthocyanins are examples of intramolecular co-pigmentation due to their mono-acylated anthocyanins (cyanidin-3-sinapoylxylosylglucosylgalactoside, cyanidin-3-feruloylxylosylglucosylgalactoside and cyanidin-3-coumaroylxylosylglucosylgalactoside) and there are also two non-acylated anthocyanins (cyanidin-3-xylosylglucosylgalactoside and cyanidin-3-xylosylgalactoside). The physical effects of acylation on anthocyanins are lower polarity, altered molecular geometry and increased molecular size. Molecular sizes of anthocyanins are increased by acylation, especially with aromatic acyl groups, due to their structural properties that lead to a steric barrier effect that prevents ions from interfering with anthocyanins in aqueous media. In addition, an altered conformation may occur due to long chains or large groups positioned over the pyrullium ring. These attached acyl groups create a sandwich-type structure which protects the C-2 and C-4 positions of the aglycone from attack of water molecules. Di-acylated anthocyanin stabilization is subjected to sandwich-like stacking, reducing the formation of the pseudo-base; however, mono-acylated anthocyanins are stabilized via one-side protection.

In the USA, a wide range of ascorbic acid fortification (0.04–1.4% w/w) to powdered beverage samples has been reported. In food processing, ascorbic acid was utilized to stabilize nutrients. In a recent study, the protection effect of co-pigments (i.e. polysaccharide and proteins) on ascorbic acid-related degradation of anthocyanin color was explained by stronger hydrogen bonding between anthocyanin and co-pigment than that for ascorbic acid. In addition to co-pigmentation, encapsulation of anthocyanins is another way to increase their stability. Anthocyanins have been encapsulated with liposome systems in previous studies. The study presented here involved an investigation of the system stability to a degradation factor as in the use of ascorbic acid and a discussion of the protective effect of liposome systems on individual anthocyanins.

In the study reported here, black carrot extract (BCE) was chosen to investigate the effects of both intramolecular co-pigmentation and encapsulation on ascorbic acid-related anthocyanin degradation. As a hypothesis, it was postulated that the protective effect of liposomal encapsulation would increase with the degree of acylation of anthocyanins. In this regard, the difference in anthocyanin loss among acylated and non-acylated anthocyanins was explored. We therefore analyzed the degradation effect of ascorbic acid on the major anthocyanins in free and liposomal-encapsulated BCE solutions over time using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS
Materials
Black carrot (Daucus carota ssp. sativus) extract (HF2.10592, 64–72° Bx, 11.3 mg g⁻¹ expressed as cyanidin-3-O-glucoside chloride) was provided by Döhler GmbH (AG Darmstadt, Germany). BCE contains five major anthocyanins: cyanidin-3-xylosylglucosylgalactoside (0.79 ± 0.02 mg mL⁻¹), cyanidin-3-xylosylgalactoside (4.33 ± 0.03 mg mL⁻¹), cyanidin-3-sinapoylxylosylglucosylgalactoside (1.82 ± 0.06 mg mL⁻¹), cyanidin-3-feruloylxylosylglucosylgalactoside (3.54 ± 0.03 mg mL⁻¹) and cyanidin-3-coumaroylxylosylglucosylgalactoside (0.79 ± 0.02 mg mL⁻¹). Soy lecithin (Lipoid AG, Lipoid S75, Ludwigshafen, Germany) was used, which contained 69.3% phosphatidylcholine, 9.8% phosphatidylethanolamine and 2.1% lysophosphatidylcholine.

Cyanidin-3-O-glucoside chloride (52976), trifluoroacetic acid (302031; 100 mL) and Sephadex® G-50 (G50300; 100 g) were purchased from Sigma-Aldrich Co. (Steinheim, Germany). L-(+)-Ascorbic acid (3525.2), anhydrous sodium acetate (6773.3), acetonitrile (7330.2) and acetic acid glacial (3738.5) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Deionized double-distilled water was used in all experiments.

Preparation of extract solutions and liposomal solutions
Solutions of BCE were prepared at different concentrations (0.2% and 0.4%) in pH 3.5 acetate buffer (250 mmol L⁻¹). Three independent replicates for each concentration were used. Liposomes were prepared from lecithin solutions by dissolving different amounts of lecithin (1%, 2% and 4%) in a 3.5 pH acetate buffer (250 mmol L⁻¹) or in the prepared extract solutions (0.2% and 0.4%) via two-stage homogenization as described in a prior study. First, the solutions were homogenized with a high-shear disperser (Heidolph Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 20 000 rpm for 5 min. Next, solutions were homogenized with a high-pressure homogenizer (Microfluidizer Processor M-110EH, Microfluidics, Newton, MA, USA; 22 500 psi, 5 passes). The microfluidizer was cooled with ice during homogenization. In addition, each liposome sample was prepared with three replicates.

Measurement of particle diameter and zeta potential
Freshly prepared liposomes were analyzed using a dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worcester, UK) for particle diameter and zeta potential. The liposomes were diluted to a lecithin concentration of 0.1% (w/w) to avoid multiple scattering effects. In the measurements, refractive indices of 1.44 and 1.33 were used for the lecithin and aqueous part, respectively. The z-average diameter and standard deviation of the measurements were obtained from three independent samples using three measurements per sample.

Sephadex® gel filtration
For the removal of the non-encapsulated fraction from the liposome solution, a Sephadex® gel filtration was used. Sephadex® gels were prepared using the same method as previously described. Liposome samples (1 mL) were passed through a Sephadex® gel (height: 3 cm)-filled cartridge (volume: 5 mL). Then the liposomes were centrifuged at 3000 rpm for 10 min (Hermle, type Z32 HK, Germany). The gel-filtered samples were collected after centrifugation for further analysis.

Efficiency of liposome encapsulation using HPLC
The encapsulation efficiency (EE) of BCEs entrapped in liposomes was measured using HPLC as a ratio of anthocyanin content in the liposome solutions before and after Sephadex® gel filtration. Before the HPLC analyses, gel-filtered and unfiltered liposomes
were broken down with ethanol using a method similar to that of a recent study.\(^2\) The extract, including gel-filtered and unfiltered liposomes, was diluted (1:2 v/v) with ethanol and centrifuged twice at 21,380 \(\times g\) for 10 min. The lecithin-free upper part was filtered through a 0.45 \(\mu\)m membrane filter and analyzed using an Agilent HPLC 1100 Series (Agilent, Waldbronn, Germany) equipped with ChemStation software. A Phenomenex\(^a\) (Torrance, CA, USA) Hydro-RP 80 A-Synergi (250 \(\times 4.6\) mm; 4 \(\mu\)m) LC column with a SecurityGuard\(^b\) guard column (4.0 \(\times 0.2\) mm) was used at an operating temperature of 40 °C. The mobile phase consisted of solvent A (Milli-Q water with 0.1% (v/v) trifluoroacetic acid) and solvent B (acetonitrile with 0.1% (v/v) trifluoroacetic acid). A linear gradient, as described in a previous study,\(^8\) was used. The anthocyanin detection was performed at 520 nm as cyanidin-3-O-glucoside chloride as described by Kammerer et al.\(^2\)\(^7\) Identification was achieved based on the retention times and characteristic spectra, and quantification was done using external standard curves.\(^8\) The total anthocyanin content was determined as the sum of the areas of each compound class. The ratio of total anthocyanin content before and after gel filtration, as a percentage, was determined for the EE of liposomes. The anthocyanin content of the free extract (\(AC_{\text{free-\text{extract}}}\)) is the difference between the unfiltered liposomal sample (\(AC_{\text{total-\text{extract}}}\)) and gel-filtered liposomal sample (\(AC_{\text{gel-filtered}}\)):

\[
\text{EE} \% = \frac{AC_{\text{gel-filtered}}}{AC_{\text{total-\text{extract}}} - AC_{\text{free-\text{extract}}} - AC_{\text{gel-filtered}}} \times 100
\]

**HPLC determination of extract and anthocyanins**

The liposome sample containing 0.2% extract and 4% lecithin was chosen for the degradation study due to its high EE. Before ascorbic acid (0.1%) addition to the extract and liposome solutions, the liposome sample was gel-filtered to omit the amount of non-encapsulated extract from the anthocyanin content. The extract solution (0.2%) was also diluted with 3.5 pH acetate buffer (250 mmol L\(^{−1}\)) (5:9 v/v) according to EE (approximately 56%) to fix the initial anthocyanin content in both the extract and liposomal solutions. Both extract and liposomal solutions were stored in the dark at room temperature for 24 h. Samples were taken at 0, 3, 6, 9, 12, 18 and 24 h and analyzed immediately via HPLC using the above-mentioned procedure. Figure 1 shows the procedure of the analysis. Each treatment was replicated three times.

**Data analysis**

All experiments were carried out at least three times for each liposomal sample. The average and standard deviation of the measurements were calculated using Microsoft Excel for Mac (version 15.32). Statistical analyses were performed using one-way analysis of variance, followed by the Duncan post hoc test using SPSS software (version 21.0, SPSS, Chicago, IL, USA) and the Tukey post hoc test using Minitab software (version 16.0, Minitab Inc.).

**RESULTS AND DISCUSSION**

**Characterization of liposomes**

It has been previously reported that encapsulation may enhance the color stability of anthocyanins against ascorbic acid-related degradation.\(^2\)\(^8\) In this study, the bleaching of the red color of BCE by the effect of added ascorbic acid was investigated, and the anthocyanin stability of encapsulates was examined. For the present purpose, BCE, liposomes without BCE and BCE-encapsulated liposomes were prepared in various extract (0.2–0.4%) and lecithin (1%, 2% and 4%) concentrations. After two-step homogenization, transparent red-colored liposomal solutions were generated. The results of z-average particle diameter and zeta potential measurements are presented in Table 1. The particle size of both unloaded liposomes and BCE-loaded liposomes was between 44 and 46 nm. The particle diameter (z-average) did not significantly differ between the liposomal samples, which can be classified as small unilamellar vesicles (20–100 nm).\(^2\)\(^9\)\(^,\)\(^3\)\(^0\) In the present study, the zeta potentials of liposomes were between −20 and −29 mV (Table 1). In addition, the change in lecithin concentration slightly altered the zeta potentials of the BCE-loaded liposomes between −19.6 and −22.9 mV. However, the increasing extract concentrations in the liposomes resulted in significantly lower zeta potentials than the control liposomes without extract (mean: −27.7 mV; \(P < 0.05\)) (Table 1). This shift can be a consequence of the adsorption of the positively charged flavylum cations on the liposome surface.

**Encapsulation efficiency**

EE was calculated to determine the ratio of extract entrapped inside the liposomes using HPLC data. Before the HPLC analyses, the liposome solutions were diluted with ethanol to break down the liposomes, as explained in detail in our previous study.\(^2\)\(^8\) In this way, unencapsulated extract and liposomes containing extract were separated using a Sephadex\(^a\) gel-separation method.
and lecithin was removed from the solutions by centrifugation before the samples were subjected to HPLC. According to the EE of the liposomal samples presented in Table 1, the extract and lecithin concentration significantly affected the entrapment efficiency of the liposomal system. Increasing lecithin concentrations increased the EE at the same extract concentration; however, increasing extract concentration reduced the EE of the liposomes at the same lecithin concentration. As a result, 57% higher EE was reported from samples with 0.2% ascorbic acid. 

**Table 1.** Zeta potentials, particle diameters, polydispersity indices and encapsulation efficiencies of liposomal controls and samples with BCE according to the content of the sum of five anthocyanins (expressed as cyanidin-3-O-glucoside chloride) determined using HPLC.

| Concentration of extract (%) | Concentration of lecithin (%) | Zeta potential\(^a\) (mV) | Particle diameter\(^b\) (nm) | PDI\(^c\) | Encapsulation efficiency\(^d\) (%) |
|-------------------------------|-------------------------------|--------------------------|-----------------------------|-----------|-----------------------------|
| 0                             | 1                             | -26.5 ± 0.37\(^c\)       | 45.6 ± 0.40\(^a\)           | 0.397 ± 0.0093\(^a\) | 25 ± 0.49\(^a\) |
| 0.2                           | 1                             | -19.6 ± 1.59\(^a\)       | 45.7 ± 1.95\(^a\)           | 0.322 ± 0.0539\(^c\) | 29 ± 2.0\(^a\) |
| 0.4                           | 1                             | -21.5 ± 0.44\(^a\)       | 45.1 ± 0.98\(^a\)           | 0.324 ± 0.0380\(^c\) | 41 ± 5.1\(^a\) |
|                               | 2                             | -22.9 ± 1.27\(^b\)       | 46.0 ± 0.50\(^a\)           | 0.336 ± 0.0443\(^c\) | 57 ± 4.9\(^c\) |
|                               | 2                             | -21.6 ± 0.98\(^a\)       | 43.7 ± 1.03\(^b\)           | 0.330 ± 0.0333\(^c\) | 40 ± 2.6\(^b\) |
|                               | 4                             | -19.9 ± 0.71\(^a\)       | 44.7 ± 1.95\(^a\)           | 0.310 ± 0.0423\(^c\) | 40 ± 2.6\(^b\) |
|                               | 4                             | -20.9 ± 0.62\(^a\)       | 45.1 ± 0.55\(^a\)           | 0.301 ± 0.0328\(^c\) | 51 ± 1.9\(^a\) |

\(^a\) Data represent average value ± standard deviation of three replicates from each sample. Different letters in the columns represent statistically significant differences (\(P < 0.05\)).

In several previous studies, the EE of liposomes was determined using absorbance measurements at 520–550 nm in elderberry extract as 25%, \(20\) hibiscus extract as 61% (2% lecithin and 0.2% extract) using absorbance measurements at 520–550 nm in elderberry extract as 25%, \(20\) hibiscus extract as 61% (2% lecithin and 0.2% extract) up to 72% (5% lecithin and 0.8% extract) and BCE as 32% (1% lecithin and 0.2% extract) to 48% (4% lecithin and 0.2% extract), \(28\) and by phenolic content measurements in grape seed extract as 88% (1% lecithin and 0.1% extract). \(31\) The higher EE in the present study in comparison with a recent study \(28\) was a result of the addition of lecithin directly to the extract solution before starting the pre-homogenization.

**Degradation of anthocyanins of BCE using ascorbic acid**

BCE with ascorbic acid was stored at room temperature for 24 h. The degradation rate increased with lower lecithin concentrations with the same extract amount of 0.2% as noted in previous studies, in which the highest degradation in anthocyanins was detected for samples with 0.1% ascorbic acid. \(28, 32\) Furthermore, as observed in Fig. 2, without addition of ascorbic acid, BCE solution was stable during the storage time. In addition, BCE-loaded liposomal samples with 0.1% ascorbic acid showed lower degradation during the storage period. The protective effect of liposomal encapsulation is in agreement with a previous study reporting that the degradation effect of digestive fluids on liposomal-encapsulated antioxidants was reduced. \(33\) The difference between samples with and without ascorbic acid was reduced, while lecithin concentration rose among liposomal samples. The highest lecithin concentration (4%) showed the best protective effect against ascorbic acid-related fading of the red color. \(28\) Additionally, increasing EE with increasing lecithin concentration may be the reason for higher protective ability of liposomal encapsulation with higher lecithin concentrations (Table 1). Basically, encapsulation methods may provide wall-like protection for core material against undesired external materials and environmental factors. \(34\)

**Figure 2.** Visual observations of color fading after addition of ascorbic acid (0.1%) to (a) gel-filtered liposome (0.2% BCE and 4% lecithin) and (b) diluted 0.2% BCE according to EE with time.

**Figure 3.** HPLC chromatogram obtained from 0.2% BCE at 520 nm. Peaks: 1, cyanidin-3-xylosylglucosylgalactoside; 2, cyanidin-3-xylosylgalactoside; 3, cyanidin-3-xylosyl(sinaoylglucosyl)galactoside; 4, cyanidin-3-xylosyl(feruloylglucosyl)galactoside; 5, cyanidin-3-xylosyl(coumar-oylglucosyl)galactoside.
Effect of ascorbic acid on individual anthocyanins

Previously, the structural protection of liposome encapsulation for anthocyanin color degradation in the presence of ascorbic acid has been determined.\textsuperscript{28} In this manner, further investigation was necessary to analyze in detail the influence of liposomal encapsulation on individual anthocyanins. For this purpose, the effect of ascorbic acid on the degradation of anthocyanins was analyzed using HPLC. As the appropriate liposomal system, numbers correspond to the peaks in chromatogram. The data presented includes mean ± standard deviation of three replicates from each sample. Different lower-case letters represent statistically significant differences at that time ($P < 0.05$).

**Figure 4.** Effect of 0.1\% ascorbic acid on individual anthocyanins in both gel-filtered liposome and BCE solutions during 24 h: (1) cyanidin-3-xylosylglucosylgalactoside; (2) cyanidin-3-xylosylgalactoside; (3) cyanidin-3-xylosyl(sinapoylglucosyl)galactoside; (4) cyanidin-3-xylosyl(feruloylglucosyl)galactoside; (5) cyanidin-3-xylosyl(coumaroylglucosyl)galactoside; (6) total amount of five anthocyanins expressed as cyanidin-3-O-glucoside chloride. Numbers correspond to the peaks in chromatogram. The data presented includes mean ± standard deviation of three replicates from each sample. Different lower-case letters represent statistically significant differences at that time ($P < 0.05$).
concentrations of 4% lecithin and 0.2% BCE were chosen due to the facts as mentioned above. In Fig. 2, both color-protected liposome solution and color fading of extract solutions can be detected visually.

Before HPLC analysis, lecithin was removed from gel-filteed liposomes using ethanol as previously described. Furthermore, all samples were membrane-filtered just before the HPLC analysis. Five major peaks were identified: cyanidin-3-xilosylglucosylgalactoside (cya 3-xylglcgal), cyanidin-3-xilosylgalactoside (cya 3-xylgal) and p-coumaric acid derivative of cya 3-xylglcgal (cyanidin-3-xylsyl (coumaroylglucosyl)galactoside), sinapic acid derivative of cya 3-xylglcgal (cyanidin-3-xylslysinapoylglucosyl)galactoside) and ferulic acid derivative of cya 3-xylglcgal (cyanidin-3-xylsylferuoylglucosyl)galactoside) (Fig. 3). This is in agreement with our previous results and the findings of other studies. In addition, anthocyanin quantification of both liposomes and extract samples were done using cyanidin-3-O-glucoside chloride as standard considering these previous studies.

As presented in Fig. 4, the degradation effect of ascorbic acid on the contents of individual anthocyanins and total anthocyanins was determined. These data may provide information on the type of anthocyanins that are mostly encapsulated within the liposomal system or incorporated in the liposomal membrane, which provides its protective effect. In a previous study, it was reported that 8% of the entrapped extract was located in the aqueous part inside the liposomes; however, 92% of the entrapped extract was incorporated in the liposomal membrane. Therefore, the encapsulated material may be located in the aqueous part inside the liposome, or integrated to the liposomal membrane. Although acylated anthocyanins have been reported to be stable against degradation factors such as pH, light and temperature, the results of this analysis indicated that they degrade with the addition of ascorbic acid. In a similar manner, it has been reported that, in the presence of hydrogen peroxide, the stability of acylated anthocyanins is not higher than that of non-acylated ones. Previous studies hypothesized that condensation of ascorbic acid and other bleaching agents at C4 or C2 of flavylium cation could be the possible reason for color degradation of anthocyanins due to susceptibility of those sites. Since pyrananthocyanins exhibit ascorbic acid-induced bleaching even when C4 position is blocked, it has been proved that C4 position plays a major role but is not the only site responsible for the bleaching of anthocyanins. Direct condensation of ascorbic acid to flavylium salt at C4 is represented in the supporting information (Fig. S1). Ascorbic acid could produce hydrogen peroxide that would fade red anthocyanin color especially in the presence of oxygen and iron or copper ions via Haber–Weiss reaction and subsequent Fenton reaction. Additionally, Jurd proposed oxidation of anthocyanins by a Baeyer–Villiger reaction to form o-benzoylphenylacetic acid ester product (supporting information). It is known from electron spin resonance analysis that anthocyanin degradation is mainly a radical reaction involving hydroxyl radicals. Furthermore, cyanidin-3-O-p-glucoside degradation to 6-hydroxycyanidin-3-O-p-glucoside by ascorbic acid as analyzed by HPLC-ESI-MS proved the inclusion of hydroxyl radical possibly to C6 position to form a new compound via Haber–Weiss reaction. Besides, Liu et al. proposed the formation of ascorbate and hydroxyl radicals due to the oxidation of ascorbic acid with hydrogen peroxide which can be a redox initiator system for free radical-induced reactions as well as anthocyanin degradation.

Possible reaction sites for ascorbic acid-induced anthocyanin degradation are shown in the supporting information (Fig. S1). However, the reactions and further condensation reactions are not yet fully understood. Nevertheless, the prevention of ascorbic acid-related degradation of anthocyanins using encapsulation in liposomes was found to be less efficient for non-acylated anthocyanins such as cya 3-xylglcgal and cya 3-xylgal in the present study. On the other hand, as shown in Fig. 4, it was found that liposomal encapsulation protected acylated anthocyanins, such as sinapic acid derivative of cya 3-xylglcgal, ferulic acid derivative of cya 3-xylglcgal and p-coumaric acid derivative of cya 3-xylglcgal, from the influence of ascorbic acid. The total anthocyanin degradation of extract and liposomal samples is shown in Fig. 4. The degradation slope of total anthocyanin contents of liposomal solutions was found to be 69% lower than that of the extract solution (data not shown). In Fig. 5, the degradation slopes of non-acylated (Fig. 5(a)) and acylated (Fig. 5(b)) anthocyanins expressed as cyanidin-3-O-glucoside chloride are shown. The liposomal encapsulation is more efficient for acylated anthocyanins with a 90% lower degradation slope in comparison to the non-acylated anthocyanins, which was found to be 57% lower than that of the extract solutions.
Similarly, the effect of encapsulation on reducing the degradation of anthocyanins was previously reported for pomegranate juice using spray drying. Acylated anthocyanins are larger molecules than non-acylated anthocyanins because of the phenolic acids attached. These results may also indicate that encapsulation and intramolecular co-pigmentation can provide an additive or synergistic effect. On the other hand, further analyses are required to identify the site of entrapment of individual anthocyanins in liposomal systems, e.g. in the inner aqueous compartment or on the bilayer membrane.

To sum up, the results of the present study indicate that the presence of acetic acid results in color loss and degradation of anthocyanins. In this study, among other protection techniques, the effect of liposome encapsulation was investigated and the results showed that degradation decreased very slowly for acylated anthocyanins as a result of liposome encapsulation. HPLC analysis of extract and liposomes indicated the possible relationship of acylation and liposomal encapsulation. However, further analyses are necessary to overcome possible concerns of the effect of acylation on entrapment efficiency in liposomes.

CONCLUSIONS
The present study investigated in particular the protective effect of liposomal encapsulation during a storage time of 24 h on the stability of acylated and non-acylated anthocyanins, known as unstable natural colorants, in the presence of ascorbic acid. The protective effect of liposomal encapsulation against ascorbic acid-induced degradation was most evident for acylated anthocyanins. This study highlighted the synergistic effect of co-pigmentation and liposomal encapsulation against ascorbic acid-induced degradation on the stability of anthocyanins. Overall, the study provided promising results especially for food and beverage manufacturers to use the liposomal system to improve color stability. Additionally, it might be exciting to investigate the interaction of liposomes and individual anthocyanin standards in future model studies. Additionally, a long-term study should be performed in combination with additional protective measures such as addition of polyphenols or metal ion chelators to minimize the reaction of ascorbic acid to hydrogen peroxide and reactive radicals.

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SUPPORTING INFORMATION
Supporting information may be found in the online version of this article.

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