NOTE

Effect of an Emulsified Formulation on Vegetable Carotenoid Bioaccessibility
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Abstract: We investigated the effect of neutral lipids, polar lipids, and an emulsified formulation (EMF) on carotenoid bioaccessibility in an in vitro digestion assay of vegetables. These reagents enhanced carotenoid bioaccessibility. Contrary to our previous report, they also exhibited effects on lutein. Bile extracts/pancreatin concentrations also participated in the bioaccessibility. The EMF, which consisted of lower amounts of oil, had the same effect on lutein as rapeseed oil. These reagents also showed effects in the aging model, with more reduced bile extract/pancreatin concentrations, suggesting that lipids and EMF contributed to carotenoid bioaccessibility in bile/pancreatic juice secretions due to aging and disease.

Key words: bile, bioaccessibility, carotenoid, emulsified formulation, vegetable oil

1 Introduction
Among carotenoids in fruits and vegetables, lutein (Fig. 1) is implicated in eye health as it specifically accumulates in the retina. Carotenoids also exhibit low bioavailability. After ingestion, they are liberated from the food matrix, dissolved in coexisting fats/oils to form emulsions with bile, and further formed into mixed bile salt micelles by pancreatic juice. The measure of how much ingested carotenoids are solubilized as mixed bile salt micelles, i.e., bioaccessibility, is an important factor for bioavailability.

In our previous study, several neutral lipids enhanced β-carotene bioaccessibility but not lutein with higher polarity. In an in vitro digestion assay, bile was essential in solubilizing fat-soluble components. However, even under experimental conditions without added bile extract, the bioaccessibility of polyphenols, which are water-soluble components, was high enough, and even in the case of carotenoids, lutein had higher bioaccessibility when compared with β-carotene, according to a report. In our previous report, lutein bioaccessibility was high even under oil-free conditions. The concentration of bile extract and pancreatin for the in vitro digestion assays for carotenoids was 5.0% (w/v) and 0.8% (w/v), respectively. Alternatively, most reports used bile extract concentrations of 0.3%–2.4% (w/v) and 0.2%–0.4% (w/v) pancreatin. In other words, concentrations in our previous study were more than twice as high as those reported. This high concentration may have been the main reason why the effects of fats and oils on lutein bioaccessibility were not reported. In this study, bile extract and pancreatin concentrations in an in vitro digestion assay were adjusted to 2.5% (w/v) and 0.4% (w/v), respectively, and the effects of various lipids, including polar lipids, on vegetable carotenoid bioaccessibility were investigated.

Abbreviations: EMF; emulsified formulation, HPLC; high-performance liquid chromatography, MCT; medium-chain fatty acid triacylglycerol, lysoPC; lysophosphatidylcholine, PC; phosphatidylcholine

Fig. 1 Chemical structures of lutein and β-carotene.
bility were examined.

Although it was recommended that fats and oils are consumed together to increase carotenoid absorption, fat consumption is also of concern in terms of increased calorie intake. If some neutral lipids could be replaced with polar lipids or sugar alcohols, the same weight with lower calories may produce the same or better effect as neutral lipids. We also examined the effect of an emulsified formulation (EMF), which had the same weight and lower calorie content when compared with neutral lipids (Table 1). If bile and pancreatic juice secretion is reduced because of aging or disease, the bioavailability of fat-soluble components will be reduced\(^{12–14}\). We simulated such a model with a much lower concentration of bile and pancreatic juice and investigated the effect of EMF on hyposecretion. The main objective of this study was to determine the effects of EMF in comparison to the effects of various lipids on the carotenoid bioaccessibility.

2 Materials and Methods

2.1 Materials

Soybean phosphatidylcholine (PC), soybean lysosphatidylcholine (lysoPC), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, and porcine bile extract were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyrogallol was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Egg PC (PC-98N) and egg lysoPC (LPC-1) were obtained from Kewpie Co. (Tokyo, Japan). Soybean oil was purchased from Clea Japan Inc. (Tokyo, Japan). Rapeseed oil was purchased from MP Biomedicals (Santa Ana, CA, USA). These oils were purified as outlined in our previous report\(^ {13}\). Medium-chain fatty acid triglyceride (MCT) oil was purchased from the Nishin OilliO Group (Tokyo, Japan). \(\beta\)-Carotene and lutein standards were prepared as previously described\(^ {15}\). All other chemicals and solvents were of reagent grade.

2.2 EMF

EMF composition, which is the same as that sold under the trade name Enap 100 (Kewpie Co.), is shown (Table 1). The major fatty acids of the lipids that make up this composition were C16:0 (5.0%), C18:0 (2.1%), C18:1n-9 (62.4%), C18:2n-6 (18.9%), C18:3n-3 (8.1%), and C20:4n-6 (0.1%).

2.3 Preparation of vegetable homogenates

Spinach (Spinacia oleracea) and carrot (Daucus carota) were purchased at a supermarket in Tsukuba City, Japan. Homogenates were prepared using a modification of our previous method\(^ {16}\). Briefly, spinach roots and carrot peel were discarded and the edible components were boiled and cooled at room temperature. Water was then added at a ratio of 1:2 by weight. This water contained pyrogallol at 10% of the vegetable weight. The mixture was homogenized on ice using a Polytron blender (PT2500E, Kinematics AG, Malters, Switzerland).

2.4 Carotenoid content in vegetable homogenates

Carotenoids were first extracted from 1.5 g homogenate using 1.5 mL ethanol, 1.5 mL ethyl acetate, and 1.5 mL hexane. For second and third extractions, 1.5 mL ethyl acetate and 1.5 mL hexane were used. The organic solvent fractions were pooled, dried in a centrifugal evaporator, and subjected to high-performance liquid chromatography (HPLC) analysis (see below).

2.5 In vitro digestion assay

Gastrointestinal digestion was simulated on the basis of our previous report, with modifications\(^ {13}\). To investigate the effect of neutral lipids, polar lipids, and EMF on vegetable carotenoid solubilization during digestion, these reagents were added to homogenates and mixed well. The mixture was mixed with 3 mL 0.5% pepsin in a buffer\(^ {17}\). The pH was adjusted to 2.0 with HCl, and then, the mixture was shaken at 120 rpm in the dark at 37°C for 1 h under nitrogen. After incubation, the pH was increased to 5.0 with NaHCO\(_3\) and 3 mL of pancreatin and bile extract in 0.1 mol/L NaHCO\(_3\) was added. After the pH was further increased

| Nutrients | Major materials | Weight (g) | Energy (kcal) |
|-----------|----------------|------------|---------------|
| fat       | vegetable oil  | 9.0        | 81.0          |
|           | egg-yolk oil   |            |               |
| carbohydrate | reduced sugar syrup | 4.3 | 17.2          |
|           | dextrin, etc.  |            |               |
| protein   |                 | 0          | 0             |
| others    | salt, etc.     | 2.7        | 1.8           |
| Total     |                 | 16         | 100           |
to 7.5 with NaOH, the mixture was shaken at 120 rpm in the dark at 37°C for 2 h under nitrogen. Afterward, the digest was centrifuged at 2900 g for 20 min at 4°C, and part of the supernatant was passed through a 0.2 µm filter to generate a solubilized carotenoid fraction. We regarded this filtrate as a solubilized fraction. Bioaccessibility (%) was defined as the ratio of carotenoid in the filtrate (solubilized fraction) to carotenoid in the vegetable homogenate. For lutein analysis in spinach, the filtrate was diluted in nine volumes of dichloromethane: methanol (1:4, v/v). An aliquot was then subjected to HPLC. Since the amount of β-carotene solubilized from carrots was low, carrot β-carotene was extracted from the filtrate as previously described and subjected to HPLC.

2.6 HPLC analysis

Carotenoids were analyzed using HPLC, as previously described. However, LC-10AD and SPD-10A UV-VIS were used for the pump and detector, respectively.

2.7 Statistical analysis

Data were processed using a one-way analysis of variance, followed by the Tukey–Kramer method. A p-value of <0.05 was considered statistically significant.

3 Results

3.1 Effect of neutral lipids, polar lipids, and EMF on lutein bioaccessibility from spinach

The effect of oils and fats on lutein bioaccessibility from spinach were investigated using the neutral lipids, namely, soybean oil, rapeseed oil, and MCT oil (Fig. 2A), and the polar lipids, namely, soybean PC, soybean lysoPC, egg PC, and egg lysoPC (Fig. 2B), and were compared with EMF effects.

In these lipids or EMF, the effect on bioaccessibility tended to increase in a concentration-dependent manner. We observed no statistical differences in effects among oils or phospholipids at 15 mg. Similarly, no differences were observed between those lipids and EMF. The addition of 15 mg rapeseed oil or EMF increased lutein bioaccessibility approximately 1.5 times when compared with no addition (0 mg).

3.2 Effect of rapeseed oil and EMF on β-carotene bioaccessibility from carrots

Of the lipids tested in Fig. 2A, rapeseed oil tended to exhibit the highest effect on the bioaccessibility, although this was not statistically different. Thus, the effects of rapeseed oil and EMF on β-carotene bioaccessibility from carrots were examined and compared. As shown (Fig. 3), the addition of 15 mg rapeseed oil or EMF increased bioaccessibility by approximately four or two times, respectively.

3.3 Effect of bile extract and pancreatin concentrations on carotenoid bioaccessibility from vegetables

The effect of bile extract and pancreatin concentrations on the in vitro digestion of vegetable carotenoids was studied. Neither lipids nor EMF was added to the assay. Both spinach lutein (Fig. 4A) and carrot β-carotene (Fig. 4B) bioaccessibility decreased significantly with decreasing bile extract and pancreatin concentrations.

3.4 Effect of rapeseed oil and EMF on vegetable carotenoid bioaccessibility in a decreased bile and pancreatin secretion model

The effects of rapeseed oil and EMF when bile extract and pancreatin concentrations in the in vitro digestion assay were reduced to one-tenth of the concentration (Figs. 2 and 3), i.e., from 2.5% to 0.25% and 0.4% to 0.04%, respectively, are shown (Fig. 5). Both showed a concentration-dependent increase in spinach lutein (Fig. 5A) and carrot β-carotene bioaccessibility (Fig. 5B). We observed no differences in effects between rapeseed oil and EMF at all concentrations.
Fig. 3  Effect of rapeseed oil and emulsified formulation (EMF) on \(\beta\)-carotene bioaccessibility in carrots. Rapeseed oil and EMF at 0–15 mg each were added to carrot homogenates. Bile extract and pancreatin concentrations for the \textit{in vitro} digestion assay were 2.5\% and 0.4\%, respectively. Bars represent the mean \pm standard deviation (SD) \((n = 4)\). Values not sharing a common letter are significantly different by the Tukey–Kramer test \((p < 0.05)\).

![Fig. 3](image)

**A** Spinach \(\beta\)-carotene bioaccessibility (%)

| Bile extract | Pancreatin | Spinach \(\beta\)-carotene bioaccessibility (%) |
|--------------|------------|---------------------------------------------|
| 2.5\%        | 0.4\%      | a                                           |
| 0.25\%       | 0.04\%     | b                                           |
| 0.125\%      | 0.02\%     | c                                           |

**B** Carrot \(\beta\)-carotene bioaccessibility (%)

| Bile extract | Pancreatin | Carrot \(\beta\)-carotene bioaccessibility (%) |
|--------------|------------|-----------------------------------------------|
| 2.5\%        | 0.4\%      | a                                             |
| 1.25\%       | 0.25\%     | b                                             |
| 0.25\%       | 0.04\%     | c                                             |

Fig. 4  Effect of bile extract and pancreatin concentrations on vegetable carotenoid bioaccessibility. Bile extract and pancreatin concentrations were 0.125\%–2.5\% and 0.02\%–0.4\% in spinach homogenates (A) or 0.25\%–2.5\% and 0.04\%–0.4\% in carrot homogenates (B), respectively. Bars represent the mean \pm standard deviation (SD) \((n = 4)\). Values not sharing a common letter are significantly different by the Tukey–Kramer test \((p < 0.05)\).

![Fig. 4](image)

4 Discussion

Previously, neutral lipids exerted no effects on increasing lutein bioaccessibility in vegetables\(^3\). However, these lipids showed effects when bile extract and pancreatin concentrations were reduced in the \textit{in vitro} digestion assay (Fig. 2A), suggesting the effects of neutral lipids on lutein solubilization during digestion after vegetable consumption are influenced by bile and pancreatic juice secretion. Since the concentration of bile in this study was half that in the previous report, the ability to solubilize lutein was reduced, but the reduction was reinforced by free fatty acids, which are hydrolyzed products of lipids. Therefore, the effect of lipids would have been apparent.

The fatty acid composition of the rapeseed oil used in this study was C16:0 (4\%), C18:0 (1\%), C18:1 (13\%), C18:2 (15\%), C18:3 (16\%), and C22:1 (51\%) according to the product catalog. We previously showed the effect of various free fatty acids on vegetable carotenoid bioaccessibility\(^4\), indicating that the fatty acid type produced by lipid hydrolysis affected carotenoid bioaccessibility. C18:1 was the most effective but did not differ from C22:1\(^5\). The elevated effect of rapeseed oil may have been attributed to the fact that C18:1 and C22:1 were the major constituent fatty acids in rapeseed oil. Since C18:1 is the major fatty acid in EMF, this may have been a factor contributing to its elevated effects. However, the total composition (64\%) of C18:1 and C22:1 in rapeseed oil was almost the same as that of C18:1 (62.4\%) in EMF. Hence, the fatty acid composition was different between the two; however, this difference in chain length of fatty acids would not affect their differential effects on bioaccessibility. The lysophospholipid that is hydrolyzed products of phospholipid may also affect the effect of phospholipids on bioaccessibility (Fig. 2B). The effect of EMF on \(\beta\)-carotene bioaccessibility was lower than rapeseed oil (Fig. 3). However, EMF has approximately 30\% fewer calories when compared with the same number of lipids (Table 1). If a comparison was made at the same calorie level (10 mg rapeseed oil, which is 0.90 kcal, is about equivalent to 15 mg EMF, which is 0.93 kcal), then EMF would have had a same effect (Fig. 3).

In the elderly, bile and pancreatic juice secretion is reportedly reduced\(^7\). Vegetable carotenoid bioaccessibility was decreased when bile extract and pancreatin concentrations in the \textit{in vitro} digestion assay were lowered (Fig. 4). We hypothesized this decrease may have been compensated by lipids and EMF (Fig. 5). However, the low pancreatin concentration may decrease the lipid hydrolysis rate, which reduces the effects of promoting carotenoid solubilization. Thus, rapeseed oil may not be sufficiently effective on the bioaccessibility. Alternatively, sugar alcohols/dextrin and phospholipids in egg yolk oil\(^6\), which are EMF components (Table 1), may have worked to promote solubilization even under such conditions. This mechanism requires elucidation in the future.
The intake of oils increases concerns regarding increased calorie intake; however, EMF generated the same level of carotenoid bioaccessibility at a lower calorie level when compared with vegetable oils. Alternatively, issues for the elderly include low nutrition and calorie intake rather than excessive calorie intake. In other words, a high intake of oil may be preferable, but a decrease in digestive enzyme secretion due to aging reduces carotenoid bioaccessibility. Thus, EMF consumption would be more favorable; since EMF has 30% fewer calories than vegetable oils of the same weight, there is room to add another calorie as a constituent oil of EMF in a calorie-based comparison. If this could be done, it would increase the potential of EMF in the bioaccessibility.

MCT oil reportedly increases body weight by increasing muscle mass when compared with salad oil containing the same calories. In other words, a high intake of oil may be preferable, but a decrease in digestive enzyme secretion due to aging reduces carotenoid bioaccessibility. Thus, EMF consumption would be more favorable; since EMF has 30% fewer calories than vegetable oils of the same weight, there is room to add another calorie as a constituent oil of EMF in a calorie-based comparison. If this could be done, it would increase the potential of EMF in the bioaccessibility.

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5 Conclusions

Bile extract and pancreatin concentrations in our in vitro digestion assay significantly affected vegetable carotenoid bioaccessibility. In our previous report, neutral lipids did not promote lutein bioaccessibility. This was because the bile concentration was too high at 5%. Here various lipids showed effects on the bioaccessibility. EMF, comprising smaller amounts of oil also had similar effects on lutein as rapeseed oil, whereas rapeseed oil had a higher effect on β-carotene. Rapeseed oil and EMF also showed the same effects in the aging model, with reduced bile extract and pancreatin concentrations, suggesting that these molecules exert compensatory effects on the decrease in bile/pancreatic juice secretions due to aging and disease. EMF that can serve calories is may be applied to nursing care and post-sickness meals.

Author Contributions

H. K. and E. K.-N. designed the study, wrote the manuscript. R. H., M. H. 2, and H. K. designed and prepared the EMF. M. H. 1 and E. K.-N. performed the experiments of in vitro digestion assay. E. K.-N. analyzed the data. All authors contributed to the critical revision of the article.

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Conflict of Interest

The authors declare no conflicts of interest.

References

1) Loskutova, E.; Nolan, J.; Howard, A.; Beatty, S. Macular pigment and its contribution to vision. *Nutrients* 5, 1962-1969 (2013).

2) Berrow, E.J.; Bartlett, H.E.; Eperjesi, F. The effect of nutritional supplementation on the multifocal electroretinogram in healthy eyes. *Doc. Ophthalmol.* 132, 123-135 (2016).

3) Nagao, A.; Kotake-Nara, E.; Hase, M. Effects of fats and oils on the bioaccessibility of carotenoids and vitamin E in vegetables. *Biosci. Biotechnol. Biochem.* 77, 1055-1060 (2013).

4) Dawilai, S.; Muangnoi, C.; Praengamthanachoti, P.; Tuntipopipat, S. Anti-inflammatory activity of bioaccessible fraction from Eryngium foetidum leaves.
5) Rodrigues, D.B.; Mariutti, L.R.; Mercadante, A.Z. An in vitro digestion method adapted for carotenoids and carotenoid esters: moving forward towards standardization. Food Funct. 7, 4992-5001 (2016).

6) Garrett, D.A.; Failla, M.L.; Sarama, R.J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. J. Agric. Food Chem. 47, 4301-4309 (1999).

7) Werner, S.; Böhm, V. Bioaccessibility of carotenoids and vitamin E from pasta: Evaluation of an in vitro digestion model. J. Agric. Food Chem. 59, 1163-1170 (2011).

8) Spinelli, S.; Lecce, L.; Likyova, D.; Del Nobile, M.A.; Conte, A. Bioactive compounds from orange epicarp to enrich fish burgers. J. Sci. Food Agric. 98, 2582-2586 (2018).

9) Phan, M.A.T.; Bucknall, M.P.; Arcot, J. Co-ingestion of red cabbage with cherry tomato enhances digestive bioaccessibility of anthocyanins but decreases carotenoid bioavailability after simulated in vitro gastrointestinal digestion. Food Chem. 298, 125040 (2019).

10) Oliveira, A.; Pittard, M. Stability of polyphenols and carotenoids in strawberry and peach yoghurt throughout in vitro gastrointestinal digestion. Food Funct. 6, 1611-1619 (2015).

11) Corte-Real, J.; Bertucci, M.; Soukoulis, C.; Desmarchelier, C.; Borel, P. et al. Negative effects of divalent mineral cations on the bioaccessibility of carotenoids from plant food matrices and related physical properties of gastro-intestinal fluids. Food Funct. 8, 1008-1019 (2017).

12) Ishibashi, T.; Matsumoto, S.; Harada, H.; Ochi, K.; Tanaka, J. et al. Aging and exocrine pancreatic function evaluated by the recently standardized secretin test. Nihon Ronen Igakkai Zasshi 28, 599-605 (1991) (in Japanese).

13) Laugier, R.; Bernard, J.P.; Berthezene, P.; Dupuy, P. Changes in pancreatic exocrine secretion with age: pancreatic exocrine secretion does decrease in the elderly. Digestion 50, 202-211 (1991).

14) Kinugasa, K.; Morinaga, O.; Horii, Y.; Inada, Y.; Kataoka, K. et al. Bile acid and biliary lipid metabolism estimated in pancreozymin. Nihon Shokakibyo Gakkai Zasshi 79, 1452-1459 (1982) (in Japanese).

15) Miyashita, K.; Hirao, M.; Nara, E.; Ota, T. Oxidative stability of triglycerides from orbital fat of tuna and soybean oil in an emulsion. Fisher. Sci. 61, 273-275 (1995).

16) Kotake-Nara, E.; Yonekura, L.; Nagao, A. Glyceroglycerolipids affect uptake of carotenoids solubilized in mixed micelles by human intestinal Caco-2 cells. Lipids 50, 847-860 (2015).

17) Ishikawa, H. Lipid composition of yolk oil. Bull. Liv - ing Sci. 17, 57-63 (1995) (in Japanese).

18) Watanabe, K. Nutritional management in the elderly. J. Nihon Univ. Med. Ass. 78, 215-221 (2019) (in Japanese).

19) Abe, S.; Ezaki, O.; Suzuki, M. Medium-chain triglycerides in combination with leucine and vitamin D increase muscle strength and function in frail elderly adults in a randomized controlled trial. J. Nutr. 146, 1017-1026 (2016).

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