Effects of Fats and Oils on the Bioaccessibility of Carotenoids and Vitamin E in Vegetables

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The low bioavailability of lipophilic micronutrients is mainly caused by their limited solubilization to an aqueous micelle, which hinders their ability to be taken up by the intestines. Bioaccessibility is the ratio of the solubilized portion to the whole amount ingested. We evaluated in this study the effects of individual fats and oils and their constituents on the bioaccessibility of carotenoids and vitamin E in vegetables by simulated digestion. Various fats and oils and long-chain triacylglycerols enhanced the bioaccessibility of β-carotene present in spinach, but not of lutein and α-tocopherol, which are less hydrophobic than β-carotene. Free fatty acid, monoaicylglycerol, and diacylglycerol also enhanced the bioaccessibility of β-carotene present in spinach. In addition to the long-chain triacylglycerols, their hydrolyzates formed during digestion would facilitate the dispersion and solubilization of β-carotene into mixed micelles. Dietary fats and oils would therefore enhance the bioaccessibility of hydrophobic carotenoids present in vegetables.

Key words: bioaccessibility; β-carotene; carotenoid; lutein; α-tocopherol

Dietary carotenoids have been shown to have the potential to prevent degenerative diseases, owing to their antioxidant activity, particularly their powerful singlet oxygen-quenching activity, as well as such other biological activities as those of immune enhancement, anti-inflammation, and anti-obesity. However, the bioavailability of dietary carotenoids is limited, principally because they are too hydrophobic to disperse in the aqueous milieu of the digestive tract. For example, the intestinal absorption from the oral administration of β-carotene dissolved in oil has been estimated to be 8.7–16.8%.¹²) β-Carotene bound to vegetables has been reported to have much less bioavailability than β-carotene dissolved in oils.³) Such other lipophilic micronutrients as vitamin E and vitamin K also have low bioavailability.⁴–⁶)

Several complex processes common to carotenoids and other lipophilic micronutrients are required before they can be absorbed by the intestines.⁷) Carotenoids must first be released from the food matrix. Although green leafy vegetables are rich in carotenoids the release of carotenoids from vegetables is hindered by the rigidity of the cell walls. Destroying the cell walls by heating and processing enhances the release of carotenoids. The carotenoids released from the food matrix are then dispersed in the digestive tract. Dietary fats and oils can promote the dispersion of carotenoids by dissolving them and by forming an emulsion in the digestive tract. The bile acids and phosphatidylcholine secreted as bile also facilitate the dispersion of dietary lipids and carotenoids. Mixed micelles are formed from the emulsified lipid particles as the lipolysis by pancreatic enzymes proceeds. These mixed micelles are composed of bile acids, phospholipids, cholesterol, fatty acids and monoaicylglycerols. Carotenoids are solubilized in the mixed micelles and can be taken up by intestinal epithelia.

Only a portion of the carotenoids ingested from foods becomes accessible to intestinal epithelia by being solubilized in the mixed micelles, making this solubilization one of the critical steps for intestinal absorption. The ratio of solubilized carotenoids to the whole amount ingested is referred to as the bioaccessibility, which can be evaluated by simulated digestion in vitro. Bioaccessibility is a useful index for elucidating the effects of the food matrix, cooking and food processing on the bioavailability of dietary carotenoids.⁸,⁹)

A portion of the carotenoids solubilized in the mixed micelles is taken up by the epithelial cells of the jejunum and assembled into chylomicrons, which are rich in triacylglycerol resynthesized from dietary lipids. The carotenoids in chylomicrons are delivered through lymph and blood to the tissues. This also makes the absorption of micellar carotenoids and secretion with chylomicrons by intestinal epithelia important processes for their bioavailability.

The intake of fats and oils is well known to enhance the bioavailability of lipophilic vitamins. Several studies have clearly demonstrated that dietary fats and oils enhanced the bioavailability of carotenoids present in vegetables in human subjects.¹⁰,¹¹) Dietary fats and oils are thought to enhance the bioaccessibility of dietary carotenoids by dispersing carotenoids in the digestive tract and indirectly by promoting the secretion of pancreatic juice. Moreover, chylomicron secretion promoted by dietary fats and oils would facilitate the transfer of micellar carotenoids to lymph.

Several studies have indicated that fats and oils enhanced the bioaccessibility of dietary carotenoids.⁹,¹²) However, the effects of the fatty acyl moiety of fats and oils, unsaponifiable matter, and other lipid classes on the bioaccessibility of carotenoids of diverse structures and...
other lipophilic micronutrients have not yet been fully revealed. We therefore evaluated in the present study the effects of various triacylglycerols, their hydrolyzates, and individual fats and oils on the bioaccessibility of carotenoids present in several vegetables in comparison with α-tocopherol.

Materials and Methods

Chemicals. β-Carotene (type II) and lutein were respectively purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Extrasyntese (Genay, France), and purified in a neutral alumina III column. δ-α-Tocopherol was obtained from Eisai Co. (Tokyo, Japan). Pepsin, pancreatin, and the bile extract from a porcine source were from Sigma-Aldrich. All other chemicals and solvents were of reagent grade. Fresh vegetables were purchased from a local market in Tsukuba, Japan.

Simulated digestion of the vegetables. The digestion of vegetables was by a method simulating the human digestive system. The leaves of washed spinach (Spinacia oleracea), qing gin cai (Brassica rapa var. chinensis), and komatsuna (Brassica campestris) were blanched for 2 min and then quickly cooled. Carrot (Daucus carota) and pumpkin (Cucurbita maxima) were sliced to a thickness of 5 mm and then branched in a similar manner. The branched samples were cut into small pieces and subsequently homogenized in two parts of deionized water with a Polytron homogenizer. The homogenates were divided into aliquots and stored at ~80°C until needed.

The homogenate (1.5 g) was put into a glass tube with a screw cap and mixed with 3 mL of a 0.5% pepsin solution (pH 2.0, adjusted with HCl) containing 3.6 mmol/L of CaCl2, 1.5 mmol/L of MgCl2, 49 mmol/L of NaCl, 12 mmol/L of KCl, and 6.4 mmol/L of KH2PO4. After the pH of the mixture had been adjusted to 2.0 with 1 mol/L of HCl, the mixture was shaken at 90 strokes per minute in the dark at 37°C for 1 h. The pH was then raised to 5 with 1 mol/L of NaHCO3 and then a mixture (3 mL) of pancreatin (8 g/L) and the bile extract (50 g/L) dissolved in 0.1% butylated hydroxytoluene. The mixture was centrifuged at 1500 g for 10 min at 4°C.

Carotenoids and α-tocopherol in the vegetables. Carotenoids and α-tocopherol present in each vegetable homogenate, which had been subjected to simulated digestion, were determined by solvent extraction and a subsequent HPLC analysis. The sample (5 g) was homogenized with a Polytron homogenizer in 15 mL of acetone containing 0.03% butylated hydroxytoluene. The mixture was centrifuged at 340 g for 10 min at 4°C. The resulting supernatant was collected, and the precipitate was subjected to an HPLC analysis of the carotenoids and α-tocopherol. Bioaccessibility is defined as the ratio of their amounts in the supernatant to those in the vegetable homogenate before simulated digestion.

Carotenoids and α-tocopherol present in each vegetable homogenate, which had been subjected to simulated digestion, were determined by solvent extraction and a subsequent HPLC analysis. The sample (5 g) was homogenized with a Polytron homogenizer in 15 mL of acetone containing 0.03% butylated hydroxytoluene. The mixture was centrifuged at 340 g for 10 min at 4°C. The resulting supernatant was collected, and the precipitate was subjected to extraction. After the combined supernatant had been dried, the extract was dissolved in 4 mL of dichloromethane:methanol (1:4, v/v) and subjected to an HPLC analysis. As lutein in pumpkin is present in an esterified form, the pumpkin extract and the filtered pumpkin digest by simulated digestion were further saponified by incubating overnight with 2.5% KOH in diethyl ether:90% ethanol (1:1, v/v) under argon gas at 4°C. The recovered unsaponifiable matter was subjected to an HPLC analysis of carotenoids.

HPLC analysis. Analytical HPLC was carried out with an HP-1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a photodiode array detector and a fluorescence detector. β-Carotene, lutein and α-tocopherol were separated in a TSK-gel ODS-80Ts column (2 × 150 mm, Tosoh, Tokyo) attached to an ODS-S1 guard column (2 × 10 mm, Tosoh) by gradient elution with solvents A and B: acetonitrile/methanol/water (75:15:10, v/v/v) containing 0.1% ammonium acetate, and methanol/ethyl acetate (70:30, v/v) containing 0.1% ammonium acetate, respectively. The gradient was programmed as follows: 0–10 min, a linear gradient from A/B (40:60, v/v) to 100% B; 10–25 min, 100% B at a flow rate of 0.2 mL/min. The carotenoids were monitored at 450 nm with a photodiode array detector, and α-tocopherol was monitored at 325 nm with an UV detector. Each was quantified from the peak area by using a calibration curve for each authentic standard.

Data analysis. Data are presented as the mean ± SD and were treated by one-way ANOVA and subsequent Student’s t test and the Tukey-Kramer test by using StatView for Windows (version 5.0, SAS Institute, Cary, NC, USA). Differences were considered significant at p < 0.05.

Results

Effects of acylglycerols and fatty acid on the bioaccessibility of β-carotene, lutein, and α-tocopherol

The bioaccessibility of β-carotene (9.8%) evaluated by simulated digestion of the spinach homogenate was extremely low compared to that of both lutein (65.7%) and α-tocopherol (58.6%) (Fig. 1). The addition of trioleoylglycerol to the spinach homogenate markedly enhanced the bioaccessibility of β-carotene to 14.6%. As dietary triacylglycerol is hydrolyzed to diacylglycerol, monoaoylglycerol, and free fatty acid during digestion, these hydrolyzates were evaluated for their ability to enhance bioaccessibility. Oleic acid and dioleoylglycerol markedly enhanced the bioaccessibility of spinach β-carotene, followed by monooleoylglycerol which had higher efficacy than trioleoylglycerol. In contrast, these acylglycerols and fatty acid did not enhance the bioaccessibility of lutein and α-tocopherol present in spinach (Fig. 1).

Effects of fatty acids on the bioaccessibility of β-carotene

The effects of a series of saturated fatty acids with different carbon-chain lengths on the bioaccessibility of β-carotene are not significantly different. The values for β-carotene and lutein not sharing a common letter are significantly different among the lipids by the Tukey-Kramer test (p < 0.05), and those of α-tocopherol are not significantly different.
the bioaccessibility of the same trend as that of lutein (data not shown). (Fig. 3B). The bioaccessibility of reduced the bioaccessibility compared with oleic acid lutein, while linoleic and linolenic acids significantly rated fatty acids had no effect on the bioaccessibility of than the monounsaturated fatty acids. The monounsaturated fatty acids showed significantly lower bioaccessibility/C12 chain fatty acids tested enhanced the bioaccessibility of or not enhance the bioaccessibility of lutein (65.8–78.8%) than oleic acid. On the other hand, these fatty acids did C10, and C12) and myristic acid significantly enhanced bioaccessibility. The medium-chain fatty acids (C8, C10, C12) were exceptions, significantly enhanced the bioaccessibility of other vegetables showed the same level of bioaccessibility. The latter two oils also tended to sesame oils as exceptions, significantly enhanced the bioaccessibility of lutein and \( \beta \)-carotene in spinach are shown in Fig. 2. The addition of \( n \)-hexanoic acid and palmitic acid did not affect the bioaccessibility. The medium-chain fatty acids (C8, C10, and C12) and myristic acid significantly enhanced the bioaccessibility, although they were less effective than oleic acid. On the other hand, these fatty acids did not enhance the bioaccessibility of lutein (65.8–78.8%) or \( \alpha \)-tocopherol (70.2–79.7%). All the unsaturated long-chain fatty acids tested enhanced the bioaccessibility of \( \beta \)-carotene in spinach (Fig. 3A). The polyunsaturated fatty acids showed significantly lower bioaccessibility than the monounsaturated fatty acids. The monounsaturated fatty acids had no effect on the bioaccessibility of lutein, while linoleic and linolenic acids significantly reduced the bioaccessibility compared with oleic acid (Fig. 3B). The bioaccessibility of \( \alpha \)-tocopherol exhibited the same trend as that of lutein (data not shown here).

Effects of triacylglycerols and edible fats and oils on the bioaccessibility of \( \beta \)-carotene Triacylglycerols consisting of a single acyl moiety with different carbon-chain lengths (C8 to C18) were evaluated for their effect on the bioaccessibility of \( \beta \)-carotene in spinach (Fig. 4). Tridecanoxyglycerol (C10) and tridodecaneoylglycerol (C12) slightly enhanced the bioaccessibility, while trioctanoxyglycerol (C8), trineylsysteoylglycerol (C14) and tripalmitoylglycerol (C16) did not enhance it. Triacylglycerols consisting of such long-chain unsaturated fatty acids as oleic acid and linoleic acid markedly enhanced the bioaccessibility. None of the triacylglycerols tested affected the bioaccessibility of lutein and \( \alpha \)-tocopherol in spinach (data not shown).

The effects of various fats and oils on the bioaccessibility of \( \beta \)-carotene in spinach are shown in Fig. 5. Most of the fats and oils tested, with safflower and sesame oils as exceptions, significantly enhanced the bioaccessibility. The latter two oils also tended to enhance the bioaccessibility of \( \beta \)-carotene. Although most of the fats and oils tested did not affect the bioaccessibility of lutein and \( \alpha \)-tocopherol, sesame oil significantly reduced their bioaccessibility by 15%, compared with the control (data not shown here).

As with the case of spinach, the bioaccessibility of \( \beta \)-carotene present in such other vegetables as komatsuna, pumpkin, and carrot was enhanced by the addition of soybean oil. The bioaccessibility of lutein and \( \alpha \)-tocopherol in these vegetables was higher than that of \( \beta \)-carotene and was not enhanced by the addition of soybean oil (Fig. 6). Lutein in pumpkin showed half the bioaccessibility of \( \alpha \)-tocopherol, although lutein in the other vegetables showed the same level of bioaccessibility as \( \alpha \)-tocopherol.

Discussion

The poor solubilization of lipophilic micronutrients in the digestive tract has been thought to be a cause of their low bioavailability. The present study clearly demonstrated that the solubilization among the lipophilic micronutrients varied significantly. The bioaccessibility of \( \beta \)-carotene in vegetables was substantially lower than that of both lutein and \( \alpha \)-tocopherol. A similar difference
Effects of Soybean Oil on the Bioaccessibility of β-Carotene, Lutein and α-Tocopherol in Vegetables.

The vegetable homogenates supplemented with 15 mg of soybean oil (hatched bar) or without supplementation (unfilled bar) were digested in vitro. A, spinach; B, komatsuna; C, pumpkin; and D, carrot. β-Car, β-carotene; Lut, lutein; α-Toc, α-tocopherol. Bars represent the mean and SD of three independent experiments. Values with an asterisk were significantly different from those of the corresponding digestion without soybean oil by Student’s t test (p < 0.05).

Fig. 5. Effects of Fats and Oils on the Bioaccessibility of β-Carotene.

A spinach homogenate was digested in vitro with 15 mg of fats and oils. Rapeseed oil with a high icosenoic acid content (42%) was used. Bars represent the mean and SD of data from three independent experiments. Values not sharing a common letter are significantly different by the Tukey-Kramer test (p < 0.05).

Fig. 6. Effects of Soybean Oil on the Bioaccessibility of β-Carotene, Lutein and α-Tocopherol in Vegetables.

between β-carotene and lutein has been found in several studies. Although there are few reports on the bioaccessibility of α-tocopherol, Borel et al. have reported that its bioaccessibility was highly variable between foods. They have found that lettuce and banana had high bioaccessibility, whereas nuts had low bioaccessibility. We found in the present study consistently high bioaccessibility of α-tocopherol in spinach and other vegetables. β-Carotene is more hydrophobic than lutein and α-tocopherol, as the respective values for the octanol-water partition coefficient (log P) of β-carotene, lutein and α-tocopherol were 17.6, 14.8, and 12.2. The dispersion of β-carotene in an aqueous milieu is more limited due to its higher hydrophobicity than that of both lutein and α-tocopherol. In fact, the transfer of carotenoids from emulsified oils to an aqueous micellar phase has been found to be inversely related to their hydrophobicity. Taken together, these results indicate that less lipophilic α-tocopherol and lutein in vegetables were more bioaccessible than highly lipophilic β-carotene.

These results for bioaccessibility found in the simulated digestion would explain the bioavailability of spinach carotenoids in a human study by West et al. They have reported that the relative bioavailability of β-carotene in several spinach products was extremely low compared to that of a β-carotene suspension in vegetable oil. On the other hand, the bioavailability of lutein in spinach products was the same as that of a lutein suspension in vegetable oil. These results observed in a human study indicate that the bioaccessibility of β-carotene present in vegetables was limited, whereas that of lutein was high irrespective of the food matrix.

It is thought that the intake of fats and oils enhances the bioavailability of lipophilic micronutrients mainly by facilitating the solubilization of lipophilic micronutrients into the aqueous phase of digesta in a physicochemical manner, and also indirectly by inducing the secretion of digestive juice. The direct effects of fats and oils on solubilization were found in the present study to be quite different among the lipophilic micronutrients. The addition of long-chain triacylglycerols to simulated digestion enhanced the bioaccessibility of β-carotene, but did not enhance that of lutein or α-tocopherol. Triacylglycerol would increase the solubilization of β-carotene by dissolving β-carotene in the bulk oil phase and by dispersing it as digestion of the triacylglycerols proceeded. On the other hand, a large portion of both lutein and α-tocopherol in vegetable was solubilized in the absence of the triacylglycerols. The effect of triacylglycerols would be negligible even if they could enhance solubilization. The lack of an effect of triacylglycerols on lutein might also have been due to the lower solubility of xanthophylls in fats and oils than that of β-carotene. This was not the case with α-tocopherol because of its miscibility with oils.

The bioavailability of β-carotene has been shown in human studies to be increased by the intake of fats and oils. This would have been partly caused by the increased bioaccessibility of β-carotene, as shown in the present study. The intake of Orlistat, a lipase inhibitor, as well as of Olestra, an indigestible fat substitute, has been reported to reduce the carotene level in human plasma. These chemicals were thought to suppress the solubilization of dietary carotenoids to mixed micelles by impairing fat digestion. On the other hand, they had little effect on the plasma levels of xanthophylls, suggesting that the bioavailability of xanthophylls was independent of the intake of fats and oils. The effects of fats and oils on the bioaccessibility of carotenoids found by simulated digestion would thus be relevant in vivo. However, Ferruzzi et al. have reported that the bioavailability of xanthophylls as well as that of carotenones in salad vegetables was enhanced by the intake of fats and oils. Although an intake of fats and oils would not directly enhance the bioaccessibility of xanthophylls, it would enhance the bioavailability of both xanthophylls and carotenones by inducing the secretion of bile and pancreatic juice via cholecystokinin, the secretion of which is stimulated by free fatty acids derived from dietary fats and oils.
Triacylglycerol is certainly an outstanding medium for the dispersion of hydrophobic \( \beta \)-carotene in the intestinal tract. If triacylglycerol hydrolysis is impaired, however, the bioaccessibility of \( \beta \)-carotene can be suppressed because of the retention of \( \beta \)-carotene in the bulk phase of triacylglycerol. The simulated digestion in the present study showed that the bioaccessibility of \( \beta \)-carotene was suppressed when an excess amount of oils relative to digestive juices was loaded (data not shown). The intake by human subjects of a lipase inhibitor decreased the \( \beta \)-carotene level in plasma, as already mentioned. Therefore, triacylglycerol hydrolysis would be essential for enhancing the \( \beta \)-carotene solubilization to the mixed micelles.

In addition to triacylglycerol, we found that such hydrolyzates as monoaclglycerol and free fatty acids had high potential to enhance the bioaccessibility of \( \beta \)-carotene. The bulk phase of these hydrolyzates could work as a medium for \( \beta \)-carotene as triacylglycerol. In addition, these hydrolyzates would facilitate the solubilization of \( \beta \)-carotene into the mixed micelles because they are amphiphilic constituents of the micelles.

The acyl chain length of free fatty acids and triacylglycerols added for simulated digestion significantly affected the bioaccessibility of \( \beta \)-carotene. The fatty acids with medium-chain lengths from C6 to C10 were less effective than oleic acid at enhancing the bioaccessibility of \( \beta \)-carotene, indicating that long-chain fatty acids with low hydrophil-lipophile balance were effective. The fatty acids from C12 to C16, whose melting points are higher than a digestion temperature of 37 °C, were less effective than C10, because of the difficulty in dispersing them. All of the unsaturated fatty acids tested had a melting point lower than 37 °C. Oleic acid and the two monoenoic acids had an enhancing effect at the same level, while the polyunsaturated fatty acids were less effective than the monoenoic acids. This would have been due to the oxidative degradation of \( \beta \)-carotene associated with the peroxidation of polyunsaturated fatty acids during digestion. This notion is supported by the result that lutein bioaccessibility, which neither saturated nor mono-unsaturated lipid supplementation affected, was significantly reduced in the presence of linoleinic acid.

The effects of the fatty acyl chain length of triacylglycerols added to simulated digestion on the bioaccessibility of \( \beta \)-carotene were similar to those of free fatty acids, although the medium-chain triacylglycerols appeared less effective than the corresponding free fatty acids. The long-chain triacylglycerols of major edible oils were superior to the medium-chain triacylglycerols in enhancing the bioaccessibility of \( \beta \)-carotene, although the solubility of carotenoids has been reported to be higher in triecoatnoglycerol (C8) than in such long-chain triacylglycerols (C18) as trioleoylglycerol and trilinoleoylglycerol.\(^{18}\) This was quite unexpected, considering that triacylglycerol enhances the bioaccessibility of \( \beta \)-carotene by dissolving carotenoid in its bulk phase. The bioaccessibility of \( \beta \)-carotene in the presence of triacylglycerol was therefore not associated with the solubility of the carotenoids in triacylglycerol, although it was well associated with the effects of the corresponding free fatty acids per se as already mentioned. These results indicate that hydrolyzates released from the triacylglycerols were more intensively involved than triacylglycerols in the solubilization of \( \beta \)-carotene. In other words, the amphiphilicity of fatty acids and monoacylglycerols would be more effective in dispersing \( \beta \)-carotene in digesta than the solubility of \( \beta \)-carotene in the bulk phase of triacylglycerol. Although the contents of free fatty acids and monoacylglycerols in foods are low, they would mediate the enhancement of \( \beta \)-carotene solubilization following the hydrolysis of triacylglycerols in the digestive tract.

Most edible fats and oils were as effective as trioleoylglycerol in enhancing the bioaccessibility of \( \beta \)-carotene. This enhancement by fats and oils would be relevant in any vegetable, as soybean oil enhanced the \( \beta \)-carotene bioaccessibility of several vegetables in addition to spinach. The bioaccessibility of lutein and \( \alpha \)-tocopherol in the vegetables evaluated was higher than that of \( \beta \)-carotene, and nearly at the same level, except for pumpkin which showed lower bioaccessibility of lutein than that of \( \alpha \)-tocopherol. Lutein in pumpkin is present as a fatty acid ester. The high lipophilicity of the esters would restrict the solubilization of lutein esters or lutein derived from the esters to the mixed micelles when compared with free lutein present in the other vegetables.

In conclusion, dietary fats and oils enhanced the bioaccessibility of \( \beta \)-carotene, but not that of lutein or \( \alpha \)-tocopherol, present in vegetables. The hydrolyzate of triacylglycerol formed by digestion would also be involved in the solubilization of \( \beta \)-carotene. This enhancement partly explains the increased bioavailability of dietary carotenes by fats and oils.

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