Encapsulated Carotenoid Preparations from High-Carotenoid Canola Oil and Cyclodextrins and Their Stability[1]

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ABSTRACT: Cyclodextrin complexes were prepared using 1:1 and 1:0.5 molar ratios of cyclodextrins and high-carotenoid canola oil. β-Cyclodextrin formed powdered complexes with a molar ratio of 1:0.5, cyclodextrin/high-carotenoid canola oil. With a 1:1 molar ratio, the complex was clumpy. In the case of α-cyclodextrin, powdery complexes were formed with either 1:1 or 1:0.5 molar ratio. The triglyceride oil present in the complexes varied between 28.87 and 48.2%, and there was no segregation of the triglyceride oil during complex formation. The stability of carotenoids and tocopherols was also the same in brown bottles whether the complexes were kept under nitrogen or under oxygen. In clear glass vials, the amounts of α-and β-carotene went down, but there was very little change in tocopherols. With respect to sterols, more than 90% of the sterols present in the degummed oil were present in the α-cyclodextrin complexes, thereby indicating a higher affinity of the sterols in the cyclodextrin cavity.

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KEY WORDS: Complexes in brown bottles, cyclodextrin complex, fluorescent light, high-carotenoid canola oil, sterols.

Cyclodextrins, which are cyclic oligosaccharides, are produced by the enzymatic reaction of cyclodextrin glycosyl transferase enzyme on liquefied starch at appropriate conditions for the enzyme. The β-cyclodextrin which has seven glucopyranose units, is produced by adding toluene to the conversion mixture, forming an insoluble toluene complex. The β-cyclodextrin is recovered from the complex by removing toluene either by distillation or by solvent extraction. The material is suspended in water, filtered using activated carbon, and recrystallized. The solubility of β-cyclodextrin is 1.85 g per 100 mL water at room temperature. The β-cyclodextrin forms inclusion compounds in its hydrophobic cavities. Materials having hydrophobic characteristics are enclosed in those cavities, which have a volume of 0.14 mL/g of β-cyclodextrin. α-Cyclodextrin, which has six glucopyranose units, is produced by adding 1-decanol to the reaction mixture and is recovered by following the exact procedure as used for β-cyclodextrin except that the complex-forming agent is 1-decanol. The solubility of α-cyclodextrin is 14.5 g/100 mL water, and the cavity volume is 0.10 mL/g of α-cyclodextrin. The molecular weights of β- and α-cyclodextrins are 1135 and 972, respectively (1). The authors took advantage of this property of cyclodextrin to supply provitamin A and other carotenoids and vitamin E in a water-soluble form, which may be used in foods and liquid drinks to supply the essential vitamins. It had been shown in a recent study on the bioavailablity of β-carotene in volunteers that the rate of utilization of β-carotene from cyclodextrin tablets was 2.2 times less than that from the oily paste (2). The β-carotene absorbed from oily drugs was retained in the bloodstream for longer periods than that from cyclocar (cyclodextrin, β-carotene) tablets. There were reports on the hypolipidemic effects of β-cyclodextrin in the hamster and in the genetically hypercholesterolemic Rico rat (3). There was a report that fermentable carbohydrates exert a more potent cholesterol-lowering effect than cholestyramine (4). Cyclodextrins are known for their use as sustained-release drug carriers (5). They have been used as a novel substitute for bovine albumin in serum-free culture of mammalian cells (6). There are reports on the preparation of decolorized-cyclodextrin complexes (7) and fat substitutes containing water-soluble β-carotene (8). There are also patents on the method for the production of complexes of long-chain polyunsaturated fatty acids and their derivatives with cyclodextrin (9). Schlenk *et al*. (10) in a patent on the stability of organic compounds first described the stability of cyclodextrin and linoleic acid complexes. Reichenbach and Min (11) studied the oxidative stability and nuclear magnetic resonance analysis of linoleic acid encapsulated in cyclodextrins. Ishiguro *et al*. (12) also studied the autooxidation of cyclodextrin–fatty acid complexes. Cyclodextrins have also been used for removal of cholesterol from eggs, butter fat, lard, and tallow (13) and also for removal of free fatty acids from frying fat (14,15).

No previous attempt has been made to prepare the cyclodextrin complexes using high-carotenoid canola oil (HCCO) and cyclodextrin in a water solution. The objective of this work was to prepare complexes of HCCO with cyclodextrins so that some amounts of triglycerides, carotenoids, and other unsaponifiables are kept in the hydrophobic cavity of cyclodextrins. Thus the objective was to provide the antioxidants present in vegetable oil in a complex of cyclodextrin so as to increase their bioavailability when supplied in foods and drinks. The other objective was to protect

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the carotenoids from heat, light, and oxygen by inclusion in cyclodextrins. Thus the primary objective of this work was to provide a method for supplying carotenoids and vitamin E in triglyceride oil to make the carotenoids bioavailable in a water-soluble form.

EXPERIMENTAL PROCEDURES:

Materials. The β- and α-cyclodextrins were obtained from Cerestor USA Inc. (Hammond, IN). HCCO is a Calgene, Monsanto product (Davis, CA). The analytical composition of the oil with respect to carotenoids and tocopherols was as follows (values in ppm): α-carotene (including both *cis*- and *trans*), 604; β-carotene (including both *cis*- and *trans*-), 1214; lutein, 197; lycopene, 33; phytoene, 948. The percentage fatty acid composition was as follows: 16:0, 4.64; 16:1, 0.32; 18:0, 3.03; 18:1, 62.73; 18:2, 15.39; 18:3, 9.19; 20:0, 1.05; 20:1, 1.27; others, 2.33. All the solvents used for high-performance liquid chromatography (HPLC) analysis were of HPLC-grade and were from Aldrich (St. Louis, MO).

Methods. The following procedure was used for the extraction and HPLC determination of carotenoids from the samples. The solid sample (200 mg) was transferred to a mortar, and 3 mL of extraction solvent (hexane/acetone/ethanol, 50:25:25, vol/vol/vol) and 0.10 mL of internal standard solution were added, and the whole mass was ground with care to avoid spilling. The internal standard was made up in the following way: 5.0 mg of 8′-apo-β-carotene-8′al was taken in 100 mL hexane. Then 0.1 mL of this solution was added to 1.9 mL of acetonitrile/methylene chloride/methanol (50:40:10, vol/vol/vol) to make a 5 μg/mL internal standard solution. The extraction solution from the first grinding was then transferred to a screw-cap glass tube (10 mL). Then 3-mL fresh solution extraction was added to the residue in the mortar and grinding continued. The extraction solution was then transferred to the same glass tube as the first extraction solution. The process was repeated a few more times until there was no color in the extraction solution. The combined solution was then vortexed and spun for 3 to 5 min in a centrifuge. The solution was then concentrated under nitrogen to about 1 mL. To this solution was then added 4.0 mL hexane and 1 mL methanol, and the mixture was vortexed. Saturated sodium chloride solution (1 mL) was added, mixed, and centrifuged for 2–3 min. The upper hexane layer was then transferred to a screw-cap test tube. Hexane (3 mL) was added to the aqueous layer, vortexed, and then centrifuged. The hexane layers were combined. The extraction procedure was repeated until there was no color in the aqueous layer. The solvent was evaporated from the hexane extract under nitrogen. The residual material was then dissolved in 2 mL of acetonitrile/methylene chloride/methanol (50:40:10, vol/vol/vol). The sample was then filtered through a 0.45 μm filter in a brown autosampler vial for HPLC analysis. A Hewlett-Packard 1100 high-performance liquid chromatograph (Palo Alto, CA) was used for isoprenoid separation. A photodiode array detector was used for carotenoid determination. Isocratic separation was achieved on a Spherisorb ODS 2 reversed-phase C-18 (5 μm) column (4.6 mm × 25 cm) (Phenomenex, Corranee, CA) at 30°C. The mobile phase was 82% acetonitrile, 10% dioxane, 8% methanol containing 150 mM ammonium acetate and 0.1% triethylamine. Flow rate was 1.0 mL/min, sample injection volume was 20 μL, and running time was 48 min.

Fatty acid compositions were determined by converting the glycerides into methyl esters using either sodium methoxide/methanol procedure or sulfuric acid/methanol procedure. Methyl ester analyses were determined using a Hewlett-Packard model 6890 gas chromatograph (GC) equipped with a split/splitless capillary inlet, auto sampler, flame-ionization detector (FID), and 3390 integrator. The column was 100 m × 0.25 mm i.d. SP-2560™ capillary column(Supelco Park, Bellefonte, PA). Carrier gas, helium; make-up gas, nitrogen; FID gas, hydrogen plus air; injection port temperature, 250°C; detector temperature, 270°C; initial temperature was 180°C, and final temperature was 240°C. Head pressure, 44 psi (constant pressure mode); split ratio, 60.

The extraction of oil from the complexes was performed following the procedure as described in this example: To the complex (2.1 g) was added 100 mL deionized water. The mixture was agitated with magnetic stirrer and with nitrogen sparging and heated to 58–60°C. At the end of 1 h, 50 mL hexane (Mallinckrot chrom AR®HPLC-grade, Paris, KY) was added, and the mixture was agitated for 5 min; then an additional 150 mL hexane was added. The mixture was transferred into a separatory funnel. After one-half hour, the hexane layer was taken out and the bottom layer containing the solids was taken out and again extracted with 50 mL hexane. This operation was performed one more time. The total hexane layer was then dried over anhydrous sodium sulfate. Hexane was removed under vacuum in a rotary evaporator. The material was then dried under vacuum at 70°C.

Processes for preparing the complexes. An outline of the processes for preparing the complexes is depicted in Figure 1.

Experiment 1. β-Cyclodextrin, 20.3 g (0.0179 mol), was dissolved in 500 g deionized water at 40–45°C with agitation. The solution was cooled to 29°C with agitation. Degummed HCCO 15.40 g (0.0175 mol) was slowly added to the agitated solution with nitrogen sparging. The reaction was carried out for 24 h, and the mixture was allowed to settle for 72 h, in the presence of nitrogen. The precipitate was filtered, and most of the water was removed under high vacuum (1 mm Hg). The material was dried under high vacuum (0.1 mm Hg) at a water-bath temperature of 60–70°C for 4 h. The material was cooled to room temperature and vacuum was released under nitrogen. The recovery was 25.0 g (70% of the combined weight of β-cyclodextrin and degummed HCCO).

Experiment 2. β-Cyclodextrin, 10.34 g (0.0091 mol), was dissolved in 176 g deionized water by stirring and heating to 45°C. After cooling to room temperature, degummed oil, 4.38 g (0.0049 mol), was added slowly with nitrogen sparging. The agitation was continued for 16 h, after which the reaction mixture was allowed to settle for one-half hour under nitrogen. The top layer containing the oil and some coloring

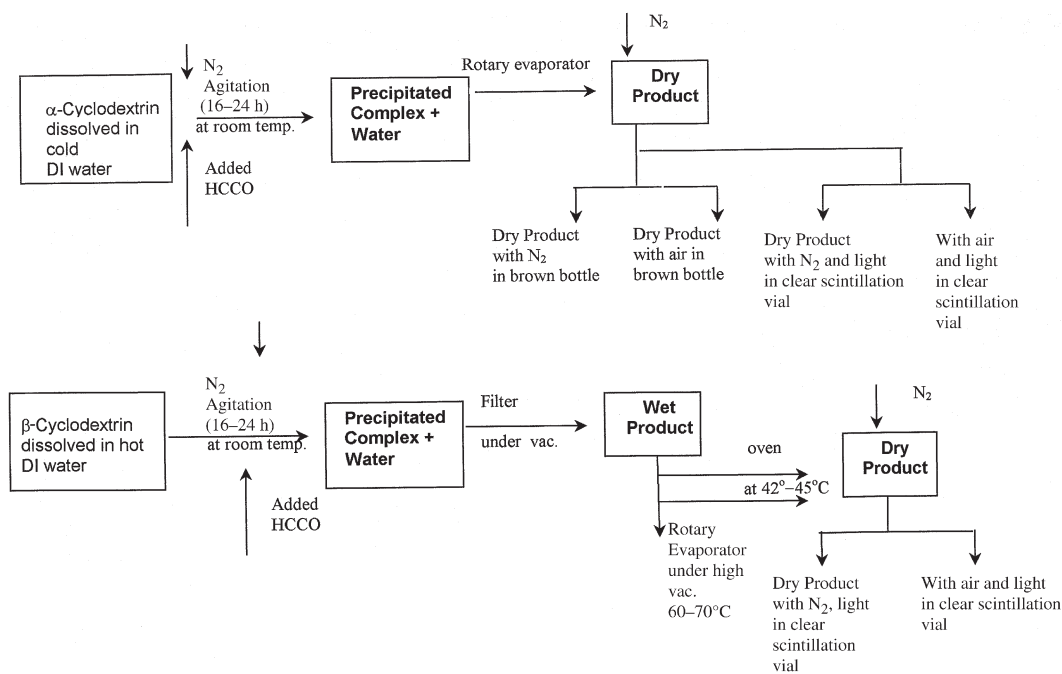

FIG. 1. Cyclodextrin high-carotenoid canola oil (HCCO) complexes, methods of preparation and evaluation of stability. DI water = deionized water.

matters was removed. The precipitate was filtered separately and washed with 100 mL deionized water. The precipitate was then transferred in a brown-colored rotary evaporator flask and dried under high vacuum as was done in Experiment 1. The recovered powder material weighed 7.33 g (49.8%).

Experiment 3. β-Cyclodextrin, 10.34 g (0.0091 mol), was dissolved in 167 g deionized water at 40–45°C with stirring. The solution was cooled to 29°C, and degummed oil, 8.33 g (0.0094 mol), was added slowly with stirring and under nitrogen atmosphere. The reaction was carried out for 16 h. After settling for 15 min, most of the water was removed by centrifuge. The precipitate with the rest of the water was transferred to a brown-colored rotary evaporator, and water was removed under a vacuum, 26″ of Hg. The whole material was dried under high vacuum and at a water-bath temperature of 55°C for 4 h. The amount of recovered material was 18.0 g (96.4%).

Experiment 4. α-Cyclodextrin, 20.0 g (0.206 mol), was dissolved in 205 g deionized water at 25°C with agitation. Degummed oil, 9.61 g (0.109 mol), was added slowly to the solution with nitrogen sparging. Agitation was continued for 24 h. The water was removed in a rotary evaporator under a vacuum of 26″ Hg and then under high vacuum at a water-bath temperature of 55°C. The whole operation took 4 h, after which the reaction mixture was cooled to room temperature and vacuum was released under nitrogen. The amount of solid powder recovered was 29.8 g (98.9%).

Experiment 5. α-Cyclodextrin, 9.80 g (0.010 mol), was dissolved in 64 g deionized water with agitation at room temperature. After 21 min, 9.13 g degummed oil (0.0104 mol) was added slowly with agitation and nitrogen sparging. The reaction was carried out for 24 h. The whole reaction mixture was then transferred to a brown-colored rotary evaporator flask, and water was removed under high vacuum as described before. The recovered material weighed 18.2 g (96.14%).

Experiments were conducted to determine the stability of carotenoids in the complexes. These were performed in the following manner.

Example 1. (i) In one experiment, 2.12 g of α-cyclodextrin complex (Expt. 4) was placed in a brown bottle, and nitrogen was passed through the sample for 5 min before the bottle was sealed. The bottle was kept at room temperature in the laboratory for 27 d before analysis. (ii) The same complex (2.13 g) was placed in a brown bottle, and air was passed through the sample for 5 min before being sealed. The bottle was kept at room temperature in the laboratory for 27 d before analysis. (iii) The same complex of α-cyclodextrin was taken in two clear scintillation tubes, and the tubes were separately filled with nitrogen and air for 5 min before being sealed. Then these tubes were kept under laboratory fluorescent light for 648 h before being analyzed.

Example 2. (i) In this experiment, 1.5 g of β-cyclodextrin complex (Expt. 2) was placed in a brown bottle, and nitrogen was passed through the sample for 5 min before the bottle was sealed and kept at laboratory temperature (24–25°C) for 27 d before being analyzed. (ii) The same complex, 1.5 g, was placed in a brown bottle, and air was passed through the sample for 5 min. After that the bottle was sealed and kept in the laboratory (24–25°C) for 27 d before analysis. (iii) The same complex was taken in two clear scintillation tubes and filled with nitrogen and

air separately, sealed, and the bottles were kept under laboratory fluorescent light for 648 h before analysis.

Example 3. (i) In this experiment, 2.04 g of β-cyclodextrin complex (Expt. 1) was placed in a brown glass bottle, and nitrogen was passed through the sample for 5 min before the bottle was sealed and kept at laboratory temperature (24–25°C) for 28 d. (ii) Of the same complex, 2.14 g was flushed with air for 5 min in a brown glass bottle, sealed, and kept at laboratory temperature for 28 d. (iii) The same complex was subjected to light experiments following the same procedure as described in Examples 1 and 2.

Example 4. (i) Of the β-cyclodextrin complex (Expt. 3) 2.12 g was treated in the same way as described in Experiments 1(i), 2(i), and 3(i). (ii) Of the same complex 2.23 g was used, and air was passed through the sample as described in Experiments 1(ii), 2(ii), and 3(ii). (iii) The same complex was subjected to the same light experiments as described in Experiments 1(iii), 2(iii), and 3(iii).

Example 5. (i) Of α-cyclodextrin complex (Expt. 5) 2.16 g was treated in the same way by passing nitrogen through the sample as described in 1(i), 2(i), 3(i), and 4(i). (ii) Of the complex from Experiment 5, 2.01 g was also treated in the same way as described previously in 1(ii), 2(ii), 3(ii), and 4(ii). (iii) The same complex was subjected to light experiments as described before.

RESULTS AND DISCUSSION

The analytical data on carotenoids and tocopherols from the experiments are shown in Tables 1 and 2. Carotenoids and tocopherols are in ppm (μg/g). From the experiments, it was clear that α-cyclodextrin formed powdered complexes with HCCO when present in an equimolar ratio and in a molar ratio of 1:0.5. The β-cyclodextrin formed powdered material when present in a molar ratio of 1:0.5. When the molar ratio of β-cyclodextrin and oil was 1:1, clumps formed.

The amount of oil present in the complexes varied between 23 and 48%. Thus, the amounts of oil present in the complexes from Experiments 1, 2, 4, and 5 were 48.18, 29.87, 28.97, and 47.5%, respectively. The fatty acid composition was the same in all the oil samples. The percentage fatty composition was as follows: 16:0, 4.89; 16:1, 0.32; 18:0, 2.97; 18:1, 64.04; 18:2, 15.9; 18:3, 8.83; 20:0, 0.98; 20:1, 1.29; others, 0.8. From these results it was clear that there was no segregation of the oil during complex formation.

From Tables 1 and 2 one can see that both carotenoids and tocopherols were present in the complexes. The α- and β-carotenes and tocopherols were present in lesser amounts in powder complexes when the molar ratio between cyclodextrins and degummed oil was 1:0.5. However, when the molar ratio between α-cyclodextrin and oil was 1:1, it formed a powdered complex as in Experiment 5. The analytical data on carotenoids and tocopherols from examples are shown in Tables 3 and 4. Carotenoids and tocopherols are expressed in ppm (μg/g). It was also clear from Tables 3 and 4 that in brown bottles the stability of the carotenoids and tocopherols was almost the same whether the materials were in the presence of nitrogen or in the presence of oxygen for the time pe-

TABLE 1
Carotenoids (μg/g)[a] Present in Cyclodextrin Complexes

Experiment	Form	α-Carotene	β-Carotene	Lutein	Lycopene	Total C
1	Clump	254	579	79	14	1301
2	Powder	175	402	53	9	895
3	Clump	240	549	85	14	1241
4	Powder	147	382	50	8	756
5	Powder	247	572	86	13	1274

[a]The α and β-carotenes contain both *cis*- and *trans*-isomers. Total C = total carotenoids including phytoene.

TABLE 2
Tocopherols (μg/g) Present in Cyclodextrin Complexes

Experiment	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherol
1	54	157	3	214
2	52	121	3	176
3	56	158	4	218
4	30	93	2	125
5	49	155	3	207

TABLE 3
Amount of Carotenoids (μg/g)[a] in the Complexes in Brown Bottles in Presence of Nitrogen and Air

Example	Sample	α-Carotene	β-Carotene	Lutein	Lycopene	Total C
1	With N_2	137	302	36	8	698
1	With air	140	312	36	8	721
2	With N_2	165	367	47	8	839
2	With air	162	364	45	7	829
3	With N_2	230	508	69	16	1209
3	With air	222	492	64	13	1170
4	With N_2	220	486	70	15	1142
4	With air	215	475	68	14	1120
5	With N_2	71	150	9	7	403
5	With air	213	473	70	13	1108

[a]The α- and β-carotenes contain both *cis*- and *trans*-isomers. Total C = total carotenoids including phytoene. Sample in brown bottle.

TABLE 4
Amount of Tocopherols (μg/g) in Complexes in Brown Bottles in Presence of Nitrogen and Air

Example	Sample[a]	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
1	With N_2	19	80	2	101
1	With air	20	82	2	104
2	With N_2	41	118	3	162
2	With air	30	106	2	138
3	With N_2	36	151	4	191
3	With air	28	147	4	179
4	With N_2	39	149	3	191
4	With air	38	148	4	190
5	With N_2	0	59	2	61
5	With air	27	135	3	165

[a]Sample in brown bottle.

TABLE 5
Amount of Carotenoids (μg/g)[a] in Complexes in Clear Glass Vial in Presence of Nitrogen and Air and Under Laboratory Fluorescent Light

Example	α-Carotene	β-Carotene	Lutein	Lycopene	Total C
1 (c) with N_2	98	213	25	8	521
1 (c) with air	100	213	24	7	504
2 (c) with N_2	109	233	24	5	541
2 (c) with air	110	234	25	5	544
3 (c) with N_2	170	367	50	11	898
3 (c) with air	153	347	45	11	858
4 (c) with N_2	155	336	44	12	809
4 (c) with air	153	327	45	11	798
5 (c) with N_2	153	326	48	11	783
5 (c) with air	187	406	60	12	977

[a]The α- and β-carotenes contain *cis*- and *trans*-isomers. Total C = total carotenoids including phytoene. (c) = sample in clear vial and laboratory fluorescent light.

riod tested in these examples. However, the results of Example 5 (with N_2) are very confusing. The analytical data on carotenoids and tocopherols from laboratory fluorescent light in clear-glass vial samples from examples item (iii) are shown in Tables 5 and 6. From these tables, it was evident that in clear-glass vials and in laboratory light, the amounts of α- and β-carotenes present in the complexes went down, but there was very little change in tocopherol contents whether the complexes were in the presence of nitrogen or in the presence of air. The amount of carotenoids and tocopherols present in the samples (complexes) was less than that of brown bottles. The analytical data on the sterol contents (ppm) of the extracted oils from complexes including the original oil are shown in Table 7. From Table 7, it was seen that most of the sterols present in degummed oil were present in the complexes, thereby indicating that they occupy most of the places in the hydrophobic cavity of the cyclodextrins. From the analytical results, it was evident that brassicasterol was absorbed to the extent of 67.68% in Experiment 1, whereas in Experiments 4 and 5 it was absorbed to 95.4 and 93.1%, respectively. It was also observed that higher percentages of

TABLE 6
Amount of Tocopherols (μg/g)[a] in Complexes in Clear Glass Vial Under Presence of Nitrogen and Air and in Laboratory Fluorescent Light

Example	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
1 (c) with N_2	19	71	2	92
1 (c) with air	18	72	3	93
2 (c) with N_2	22	93	4	119
2 (c) with air	20	93	3	116
3 (c) with N_2	20	121	4	145
3 (c) with air	0	114	3	117
4 (c) with N_2	20	116	3	139
4 (c) with air	0	117	4	121
5 (c) with N_2	19	111	5	135
5 with air	26	135	3	164

[a](c) = sample in clear vial and laboratory fluorescent light.

TABLE 7
Amount of Sterols (μg/g) in Original Oil and in Complexes

Oil	Brassicasterol	Campesterol	β-Sitosterol
Original oil	1020	2753	5685
Oil from Experiment 1	690	1940	4590
Oil from Experiment 4	970	2610	5310
Oil from Experiment 5	950	2620	5320

campesterol and β-sitosterol were present in the complexes of Experiments 4 and 5 compared to Experiment 1.

From these experiments, it was clear that provitamin A and vitamin E could be delivered in powder forms in foods and drinks. Since α-cyclodextrin is not cytotoxic to mammalian cells (6), the authors strongly believe that it would be a better way to deliver the vitamins in α-cyclodextrin to alleviate the vitamin A deficiency and night blindness in underdeveloped countries.

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