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Use of Tween 40 and Tween 80 to deliver a mixture of phytochemicals to human colonic adenocarcinoma cell (CaCo-2) monolayers

Sinead M. O'Sullivan[1], Julie A. Woods[2] and Nora M. O'Brien[1]*

[1]*Department of Food and Nutritional Sciences, University College, Cork, Republic of Ireland*
[2]*Photobiology Unit, University of Dundee, Ninewells Hospital, Dundee DD1 9SY, Scotland, UK*

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Epidemiological evidence suggests that dietary intake of carotenoids and tocopherols may influence the risk of certain chronic diseases, such as cancer and CVD. *In vitro* studies investigating the synergistic effects of mixtures of carotenoids and tocopherols have been hindered due to the difficulty of solubilising these lipophilic compounds. The objective of the present study was to develop a system for delivering tocopherols and carotenoids simultaneously to cells in culture. Differentiated human colonic adenocarcinoma cells (CaCo-2) were incubated with a mixture of these phytochemicals for 24 h. The phytochemical mixture included carotenoids (astaxanthin, canthaxanthin, lutein, lycopene, α-carotene, β-carotene) and tocopherols (α-tocopherol and γ-tocopherol). The emulsifiers polyoxyethylene sorbitan monopalmitate (Tween 40) and polyoxyethylene sorbitan monooleate (Tween 80) were employed as the delivery vehicles, and were compared with tetrahydrofuran (THF). Each vehicle was added at a maximum concentration of 1 ml/l. No toxic effects to the CaCo-2 cells were noted when Tween 40 or Tween 80 were used. Both Tween 40 and Tween 80 resulted in greater solubility of the mixture and delivered substantially more carotenoids and tocopherols to the cells than THF. In particular, lycopene was detected within the cells when Tween 40 and Tween 80 were employed, whereas it was below the limits of detection by HPLC when THF was used as the delivery vehicle. The phytochemicals were retained within the cells for 24 h after supplementation. Tween 40 and Tween 80 have potential as simple, rapid and non-toxic methods for delivering mixtures of carotenoids and tocopherols to cells in culture.

Carotenoids: Polyoxyethylene sorbitan esters: Tween: Tetrahydrofuran: CaCo-2 cells

Epidemiological evidence suggests that the consumption of diets rich in fruits and vegetables decreases the occurrence of certain chronic diseases such as cancer and CVD (Steinmetz & Potter, 1996; van't Veer *et al.* 2000). Carotenoids and tocopherols are fat-soluble phytochemicals present in fruits and vegetables. These phytochemicals are known to have antioxidant properties (Brigelius-Flohe & Traber, 1999; Paiva & Russell, 1999) and may be responsible, in part, for the protective effects of fruits and vegetables. *In vitro* studies have reported on the potential protective effects of individual carotenoids or tocopherols; however, investigation of the potential synergistic effects of these phytochemicals has been hampered by the difficulty in delivering these hydrophobic compounds to cells in culture.

In vivo, carotenoids and tocopherols are incorporated into micelles after digestion; this facilitates absorption into the enterocyte of the intestinal mucosa. Within the enterocytes carotenoids and tocopherols are packaged into chylomicrons for transport to the liver, where they are transferred to VLDL for delivery to extra-hepathic tissue (Parker, 1996; Furr & Clark, 1997). Therefore, carotenoids are maintained in a bioavailable form throughout the body. Tocopherols have been successfully delivered to cultured cells using ethanol as the delivery vehicle (Martin-Nizard *et al.* 1998; Lyons *et al.* 2001), while carotenoids have been delivered to cells in culture incorporated in water-miscible beadlets, liposomes or mixed micelles (Grolier *et al.* 1992; Martin *et al.* 1996; Stivala *et al.* 1996; Garrett *et al.* 1999; Williams *et al.* 2000). The organic solvents tetrahydrofuran (THF), dimethylsulfoxide, ethanol and *n*-hexane have also been frequently employed as delivery vehicles for carotenoids (Chakraborty *et al.* 1987; Cooney *et al.* 1993; Stivala *et al.* 1996; van Vliet *et al.* 1996; Gross *et al.* 1997; O'Connor & O'Brien, 1998; Kozuki *et al.* 2000; Zhang & Omaye, 2001). However, a number of difficulties have been associated with the methods used for delivery of carotenoids to cells including instability and insolubility of the carotenoids, while cytotoxicity has been related to the use of solvents. The difficulty associated with solubilising carotenoids has also limited the number of these compounds that may be

Abbreviations: CAT, catalase; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; NRUA, Neutral Red uptake assay; SOD, superoxide dismutase; THF, tetrahydrofuran.

* **Corresponding author:** Dr Nora M. O'Brien, fax + 353 21 4270244, email nob@ucc.ie

simultaneously delivered to cells in culture. Pfitzner *et al.* (2000) developed a water-soluble complex of carotenoids with methyl-β-cyclodextrin to supplement cultured cells. However, carotenoids complexed to methyl-β-cyclodextrin were added individually to the cell cultures.

The primary objective of the present study was to develop a system whereby a number of carotenoids and tocopherols could be delivered to cells in culture simultaneously, with the hope that this system could be used for future study of synergistic effects between phytochemicals. The phytochemical mixture employed in the present study consisted of the xanthophylls and carotenes (astaxanthin, canthaxanthin, lutein, lycopene, α-carotene and β-carotene), and tocopherols (γ-tocopherol and α-tocopherol). Two commercial emulsifiers, Tween 40 (polyoxyethylene sorbitan monopalmitate) and Tween 80 (polyoxyethylene sorbitan monooleate) were employed to solublise this mixture of phytochemicals. Tween 40 and Tween 80 are widely used in the pharmaceutical, cosmetic and food industries as vehicles for fat-soluble compounds (Lopez *et al.* 2000; Weiss & Liao, 2000; Malingre *et al.* 2001). They are found in foods such as soups, sauces, confectionary, desserts and dietary supplements. Tween 40 differs structurally from Tween 80 in that it has palmitic acid as its fatty acid side-chain while Tween 80 has oleic acid. In the present study, the delivery of the phytochemicals to human colonic adenocarcinoma cell (CaCo-2) monolayers using either Tween 40 or Tween 80 was compared with THF, a solvent that has been used successfully to deliver carotenoids to cell cultures, but is not without limitations.

Materials and methods

Materials

Astaxanthin and canthaxanthin were obtained from Quest International Ireland Ltd (Cork, Republic of Ireland). Fetal bovine serum was purchased from GIBCO BRL Technologies Ltd (Paisley, Scotland, UK). All other chemicals and reagents were purchased from Sigma-Aldrich Ireland Ltd (Dublin, Republic of Ireland) unless otherwise stated. Tissue culture plastics were supplied by Costar (Cambridge, MA, USA). All solvents employed were of HPLC grade.

Preparation of the compounds

The phytochemical mixture containing the carotenoids (astaxanthin, canthaxanthin, lutein, lycopene, α-carotene and β-carotene) and the tocopherols (α-tocopherol and γ-tocopherol) was prepared using Tween 40, Tween 80 or THF. Stock solutions of each compound were made up fresh for each experiment. The concentration of each compound was determined spectrophotometrically, using the published extinction coefficients, in either hexane or ethanol (astaxanthin, γ-tocopherol, lutein, retinol, astaxanthin acetate). Each compound (5 μM) was mixed together and dried under N_2. This residue was reconstituted in either Tween 40 (200 ml/l acetone), Tween 80 (200 ml/l acetone) or THF. These solutions were again dried under N_2 and reconstituted in growth media (Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (100 ml/l)) with vortexing, giving a final concentration in the growth media of 1 ml delivery vehicle/l. Undissolved compounds were removed and the growth media sterilised by passing it through a surfactant-free cellulose acetate filter (0·2 μm; Nalgene (190–2520), Biosciences, Dublin, Republic of Ireland).

Cell culture

CaCo-2 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, Wilts., UK). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (100 ml/l) and non-essential amino acids (10 ml/l). Cells were grown at 37°C and 5 % CO_2 in a humidified incubator, in the absence of antibiotics. Routine screening of cultures for the presence of mycoplasma was performed using the Hoechst staining method of Mowles (1990). For experiments, cells were seeded at a density of 4×10^4 cells/cm^2 in 2500 mm^2 flasks, and allowed to grow for 21–22 d until they had differentiated into enterocytes (Pinto *et al.* 1983; Hidalgo *et al.* 1989). Media were replaced every 2 d. Differentiation was confirmed by measuring the activity of the brush border enzymes alkaline phosphatase and amino peptidase, which have higher activities in enterocytes compared with undifferentiated CaCo-2 cells (results not shown).

Treatment of cells

Initially, when comparing the three delivery vehicles, differentiated cells were treated for 24 h with the phytochemical mixture dissolved in Tween 40, Tween 80 or THF. Following the 24 h period, cell and media samples were harvested, extracted and run on HPLC. In a second series of experiments, cellular accumulation of the carotenoids and tocopherols delivered to cells in Tween 40 or Tween 80 was assessed by treatment of cells with phytochemical mixture at *t* 0, removing flasks at designated time points post-treatment (2, 6, 12, 24 h), washing the cells three times in PBS and extracting the cells and media separately for HPLC analysis. In the final series of experiments the retention of the carotenoids and tocopherols by the cells after delivery by Tween 40 or Tween 80 was assessed by treatment of cells with the phytochemical mixture for 24 h. Following this 24 h period, the phytochemical-containing media was replaced with fresh phytochemical-free media. Samples were removed at the indicated time points (2, 6, 12, 24 h) following the media change-over, and both cells and media were analysed for their phytochemical content by HPLC.

Cell viability

The potential cytotoxicity of the delivery vehicles was assessed by the 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the Neutral Red uptake assay (NRUA), and the lactate dehydrogenase (LDH) release assay. Cultures were incubated with Tween 40 or Tween 80 in the absence of the phytochemical mix at a concentration of 1 ml/l for a period of 24 h.

THF (1 ml/l) has previously been shown to be non-toxic as assessed by these endpoints (Woods *et al.* 1997). The MTT assay was carried out by the method of Edmonson *et al.* (1988). Briefly, following incubation with MTT all liquid was removed from the wells and the plates were frozen at −20°C overnight. After thawing, 200 μl isopropanol was added to each well to solubilise the formazan dye and the absorbance immediately read at 570 nm. Results were expressed as the percentage of surviving cells relative to the control samples. The NRUA was performed by modification of the method of Hunt *et al.* (1987). Following incubation with Tween 40 or Tween 80, Neutral Red dye was added to the cells for 3 h at 37°C. After this time, the dye was removed and the cells quickly rinsed in a solution of formaldehyde (40 ml/l). The dye was solubilised using a solution of ethanol (500 ml/l) containing glacial acetic acid (10 ml/l). Absorbance was measured at 540 nm. Results were expressed as a percentage of viable cells relative to the controls. The LDH release assay was performed using a Sigma LDH optimized kit (DG 1340-K; Dublin, Republic of Ireland). LDH release was expressed as a percentage of the total LDH concentration as per the manufacturer's instructions. A value of >5 % LDH released into the media was considered cytotoxic.

Antioxidant enzyme assays

For further toxicological assessment of Tween 40 and 80, their effects on the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were evaluated. In preparation for enzyme analysis, cells were incubated with emulsifier (1 ml/l) for 24 h. Cells were lysed by pulse sonication and sonicates were centrifuged at 100 000 ***g*** for 25 min at 4°C. CAT activity was determined on the day of harvesting, using the method of Baudhuin *et al.* (1964). CAT activity was estimated by measuring the formation of peroxytitanium sulfate at 465 nm. One unit of CAT activity was defined as the amount that will decompose 1 μmol H_2O_2/min (pH 7·5, 25°C). SOD activity was determined by the method of McCord & Fridovich (1969). The reduction of cytochrome *c* at 550 nm was used to monitor SOD activity. One unit of SOD activity was defined as the amount required to inhibit the maximum rate of cytochrome *c* reduction by 50 %. Enzyme activities were expressed as units/mg protein. Total protein was determined by the method of Lowry *et al.* (1951).

HPLC analysis

Uptake and retention of compounds in cells were analysed by reverse-phase HPLC. Cell pellets were sonicated (4°C) in 100 μl PBS. Ethanol–ethyl acetate (2:5, v/v) containing butylated hydroxytoluene and 500 μM-α-tocopherol acetate as a recovery standard was added to the sonicate. This solution was then extracted twice with ethyl acetate and once with hexane. The organic layers were pooled and dried under N_2. Media samples were extracted identically, but without the sonication. The residues were reconstituted in dichloromethane–acetonitrile–methanol (1:7:2, v/v) on the day of analysis, and analysed using the method of Craft & Wise (1992). The HPLC system (Shimadzu, Kyoto, Japan) consisted of a LC10-AD pump connected to a SIL-10A auto-injector and SPD-10AV UV-visible detector. Two 150·0 × 4·6 mm C_{18} columns were maintained at 25°C using a column oven. The injection volume was 50 μl; samples were eluted using an isocratic mobile phase composed of acetonitrile–methanol–dichloromethane (15:4:1, v/v) containing 10 mM-ammonium acetate, 4·5 mM-butylated hydroxytoluene and 3·6 mM-triethylamine at a flow rate of 1·5 ml/min. Carotenoids were detected at 450 nm and tocopherols at 325 nm. Results were collected and analysed using Millenium software (Waters Corporation, Milford, MA, USA).

Statistical analyses

Results are representative of two to three individual experiments with two to three flasks per data point. Errors are expressed as standard deviations or standard errors of the means. Where appropriate, data were analysed using ANOVA followed by Dunnett's test.

Results

Effects of Tween 40 and Tween 80 on CaCo-2 cells

The potential cytotoxicity of Tween 40 and Tween 80 on CaCo-2 cells in culture was evaluated. Cultures were incubated with the delivery vehicles at a concentration of 1 ml/l for 24 h. There were no cytotoxic effects observed for Tween 40 or Tween 80 in the differentiated CaCo-2 cells as assessed by the NRUA (lysosomal integrity), MTT (mitochondrial activity) assay and LDH release (membrane integrity) assay. Results for the MTT assay and the NRUA were expressed as a percentage of control cells. For the MTT assay, Tween 40 and Tween 80 had values of 99·5 (SD 1·7) and 98·3 (SD 2·6) % respectively. Results from the NRUA were 101·1 (SD 2·7) and 98·9 (SD 2·9) % for Tween 40 and Tween 80 respectively. With regard to the LDH release assay, values are expressed as % LDH released into the media. Control cells released 2·9 (SD 0·5) %. Cells treated with Tween 40 or Tween 80 released 2·9 (SD 1·1) and 2·6 (SD 0·5) % respectively.

Activity of antioxidant enzymes

The activities of antioxidant enzymes CAT and SOD were measured in CaCo-2 cells following a 24 h incubation with Tween 40 or Tween 80 (1 ml/l). The CAT activity in the control cells was 77·5 (SE 12·2) units/mg protein and there were no significant alternations in the enzyme activities of cells treated with either Tween 40 (74·3 (SE 10·6) units/mg protein) or Tween 80 (69·9 (SE 7·0) units/mg protein). Similarly, there were no significant changes in SOD activity. The level of SOD activity in untreated CaCo-2 cells was found to be 3·08 (SE 0·51) units/mg protein. In cells treated with Tween 40 and Tween 80, SOD activity was 3·44 (SE 0·48) and 2·77 (SE 0·39) units/mg protein respectively.

Comparison of the effectiveness of Tween 40, Tween 80 and tetrahydrofuran for delivering phytochemicals to differentiated CaCo-2 cells

In order to determine which of the three delivery vehicles was most appropriate for delivering the compounds to the CaCo-2 cells, cells were incubated for 24 h, with the phytochemical mixture delivered by either Tween 40, Tween 80 or THF. The Tween vehicles delivered substantially more phytochemicals to the cells compared with THF (Fig. 1, note scales). In this figure control cells represent cells that were treated with normal growth media (Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (100 ml/l)) that were not supplemented with the phytochemicals. For all three vehicles the hydrocarbons α-carotene, β-carotene and lycopene were the most poorly absorbed compounds, whereas the oxygenated xanthophylls and tocopherols were readily taken up by cells. All eight phytochemicals were taken up by the CaCo-2 cells when delivered by either Tween vehicles. In contrast, lycopene was not detected when delivered by THF. In addition there was some evidence of retinol formation by the cells, as controls cells after the 24 h period had a retinol content of 0·025 (SD 0·010) ng/mg protein, whereas cells treated with carotenoids had a retinol content of 0·906 (SD 0·777) ng/mg protein. THF was the least efficient delivery vehicle for all phytochemicals analysed; therefore, the use of THF as a method to deliver a phytochemical mixture to CaCo-2 cells was discontinued in subsequent experiments.

Stability of phytochemicals in media

The stability of the phytochemical mixture in the media delivered in either Tween 40 or Tween 80 over the 24 h incubation period was analysed. Although 5 μM of each compound was initially prepared, the concentration of the phytochemicals in the media after filtration was lower and differed for each compound (Table 1). The filter used was designed for sterilising media (aqueous solution), but it also resulted in removal of some of the phytochemicals. This was carefully monitored over the course of the experiment. As can be seen from Fig. 2(a and b) there was little difference in phytochemical media content before and after the 24 h incubation period, and therefore the compounds remained stable within the media over the course of the experiment.

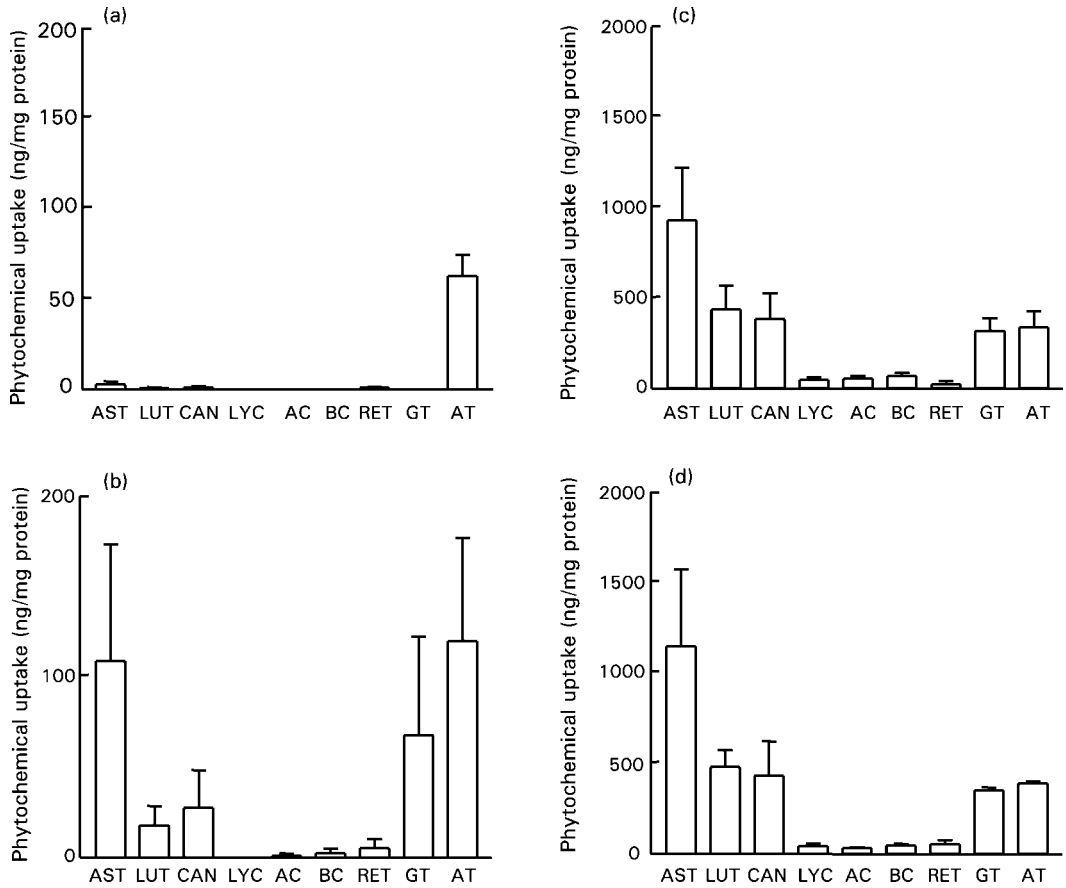

Fig. 1. Cellular uptake of phytochemicals following a 24 h incubation. The differentiated CaCo-2 cells were supplemented with the phytochemical mixture delivered to media in either control (a), tetrahydrofuran (b), Tween 40 (c) or Tween 80 (d) at a concentration of 1 ml/l. Control cells represent cells that were treated with normal growth media (Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (100 ml/l)) and that were not supplemented with the phytochemicals. Following a 24 h incubation period, cells were analysed for phytochemicals by HPLC. For details of procedures, see p. 758. Phytochemicals analysed were astaxanthin (AST), lutein (LUT) canthaxanthin (CAN), lycopene (LYC), α-carotene (AC), β-carotene (BC), retinol (RET), γ-tocopherol (GT) and α-tocopherol (AT). Values are means with standard deviations shown by vertical bars (four observations from two independent experiments).

Table 1. The initial concentration of the phytochemicals in the media after filtration*
(Mean values and standard deviations for four observations from two independent experiments)

	Tween 40 (μM)		Tween 80 (μM)		Tween 40 ng/ml		Tween 80 ng/ml	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Astaxanthin	0·700	0·025	0·680	0·002	418	0·001	406	0·92
Lutein	1·175	0·232	1·216	0·238	666	131	689	135
Canthaxanthin	0·249	0·035	0·223	0·035	140	19·5	126	19·5
Lycopene	0·345	0·075	0·343	0·114	185	40·1	184	60·9
α-Carotene	0·922	0·079	1·043	0·077	494	42·1	560	41·4
β-Carotene	0·667	0·007	0·760	0·002	358	3·77	408	0·85
γ-Tocopherol	1·647	0·379	1·431	0·038	685	157	595	16·0
α-Tocopherol	2·221	0·333	1·887	0·089	955	143	811	382

* A concentration of 5 μM of each phytochemical was initially prepared. These were mixed together and dried under N_2. The residue was reconstituted in TWEEN 40 or TWEEN 80 (200 ml/l acetone). These solutions were dried under N_2 and reconstituted in growth media with vortexing. Undissolved compounds were removed and the growth media sterilized by passing through a 0.2 μM filter. After filtration, the phytochemicals contents within the media were analysed by HPLC. For details of procedures, see p. 758. Media phytochemical contents are expressed in μM concentrations and also as ng phytochemical/ml media.

Cellular accumulation of phytochemicals

The cellular accumulation of the phytochemicals dissolved in Tween 40 or Tween 80 was monitored over a 24 h incubation period. Cells were treated with the phytochemical mixture (37°C, 5 % CO_2) delivered in Tween 40 or Tween 80, and samples were removed at 2, 6, 12 or 24 h post-treatment (Fig. 3(a and b)). Cellular concentrations of all compounds increased during the 24 h period, reaching an apparent saturation after 6–12 h incubation. All eight phytochemicals were detected in the cells after as little as 2 h incubation. As previously demonstrated, astaxanthin, canthaxanthin, lutein, α-tocopherol and γ-tocopherol were the most readily sequestered into the cells. Retinol was also detected in the cells at all time points and appeared to gradually increase over the 24 h. Little difference between the Tween vehicles was observed with regard to the oxygenated compounds; however, Tween 40 appeared slightly better at delivering the more hydrophobic compounds (α-carotene, β-carotene and lycopene).

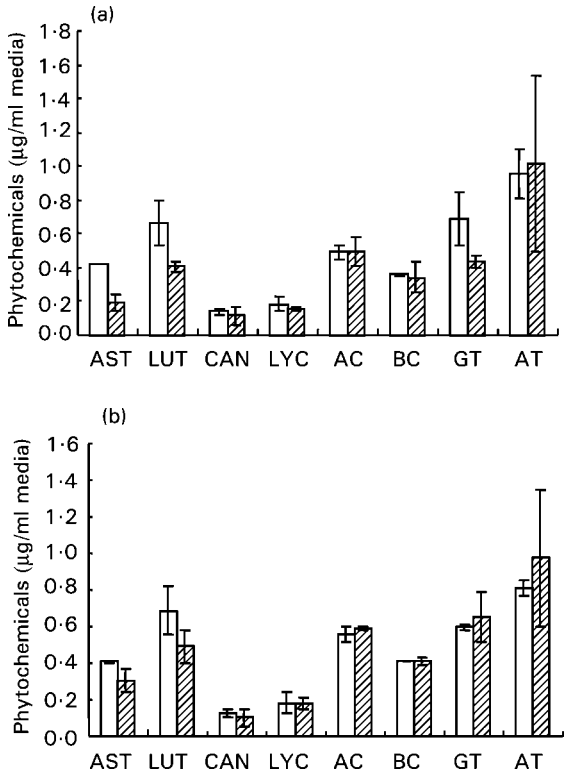

Fig. 2. The stability of the phytochemicals in the media over a 24 h incubation. The phytochemical mixture was delivered to media in either Tween 40 (a) or Tween 80 (b) at a concentration of 1 ml/l and incubated for a period of 24 h. Media samples before and after the 24 h incubation were analysed for their phytochemical content by HPLC. □, Initial concentration; ▨, concentration after 24 h incubation. For details of procedures, see p. 758. Phytochemicals analysed were astaxanthin (AST), lutein (LUT) canthaxanthin (CAN), lycopene (LYC), α-carotene (AC), β-carotene (BC), γ-tocopherol (GT) and α-tocopherol (AT). Values are means with standard deviations shown by vertical bars (four observations from two independent experiments).

Retention of phytochemicals within the cells

To assess retention of the phytochemicals, cell samples at 2, 6, 12 and 24 h post-phytochemical removal were analysed. Overall, retention of the compounds within the cells was stable, with no major loss of any of the compounds over the 24 h period. There was a trend for the oxygenated compounds to be lost from the cells, whereas β-carotene, α-carotene and lycopene levels remained fairly constant. This is best illustrated by the cells treated with Tween 40 (Fig. 4(a)). Again, the phytochemical concentration of the cells was similar when Tween 40 or Tween 80 was used as the delivery vehicle (Fig. 4(a and b)).

Discussion

Recent advances in drug manufacture have led to the production of drugs that are generally more lipophilic and less water-soluble. This has necessitated the development of techniques to enhance absorption and bioavailability of these drugs and often involves the use of surfactants. Polyoxyethylene sorbitan esters (Tweens) represent one type of non-toxic surfactant that has been used for the solubilisation of drugs for oral, topical and ocular administration (El-Sabbagh *et al.* 1978; Skaare *et al.* 1997; Zurowska-Pryczkowska *et al.* 1999; Lopez *et al.* 2000; Malingre *et al.* 2001). Tween 80 and Tween 60 have been used

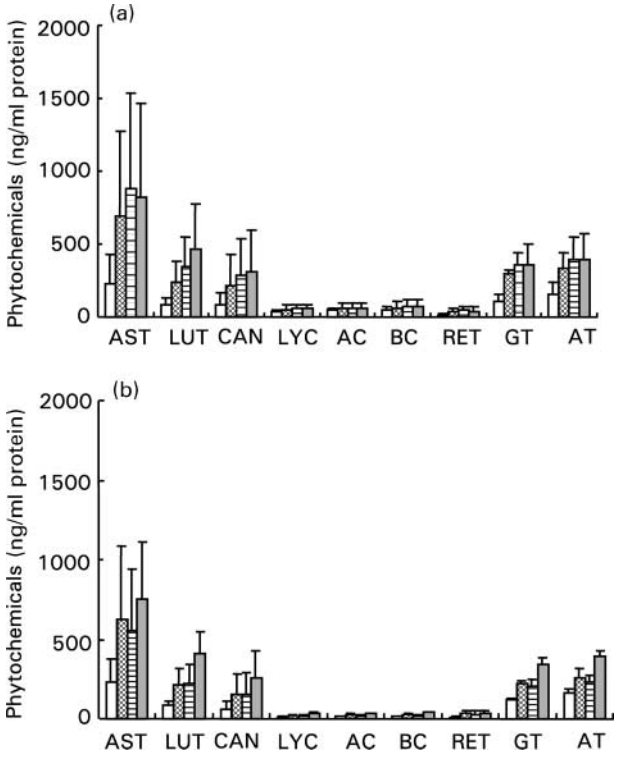

Fig. 3. Cellular accumulation of the phytochemicals dissolved in Tween 40 (a) or Tween 80 (b). Differentiated CaCo-2 cells were incubated with media containing the phytochemical mixture dissolved in Tween 40 or Tween 80 at concentrations of 1 ml/l for a period of 24 h. During the 24 h incubation, cells samples were removed at indicated time points and analysed by HPLC for their phytochemical accumulation. For details of procedures, see p. 758. □, 2 h; ▨, 6 h; ▤, 12 h; ■, 24 h. Phytochemicals analysed were astaxanthin (AST), lutein (LUT) canthaxanthin (CAN), lycopene (LYC), α-carotene (AC), β-carotene (BC), retinol (RET), γ-tocopherol (GT) and α-tocopherol (AT). Values are means with standard deviations shown by vertical bars (four observations from two independent experiments).

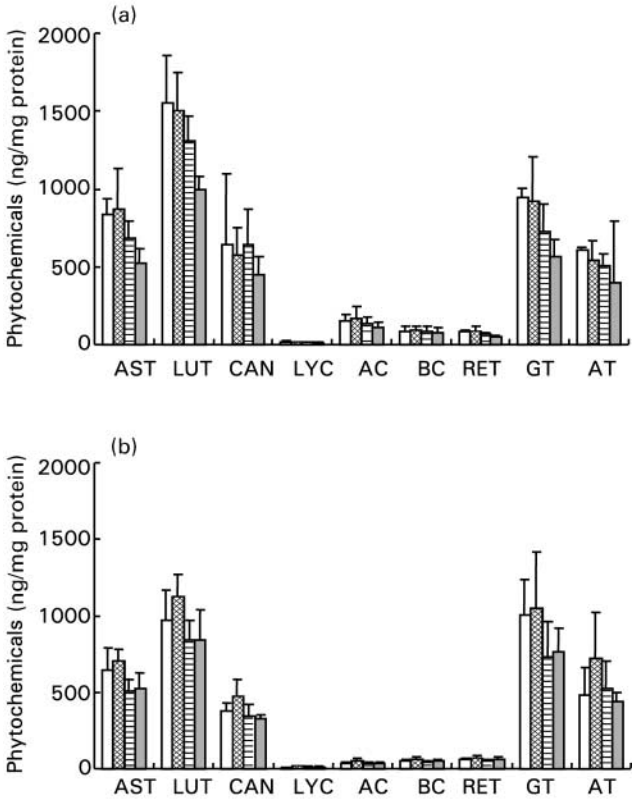

Fig. 4. Retention of the phytochemicals, delivered in Tween 40 or Tween 80, within the cells. Differentiated CaCo-2 cells were incubated with media containing the phytochemical mixture dissolved in Tween 40 (a) or Tween 80 (b) at a concentration of 1 ml/l for a period of 24 h. After this time the phytochemical-containing growth media were replaced with fresh phytochemical-free media and incubated at 37°C. Cell samples were removed at indicated times and analysed for their phytochemical content by HPLC. For details of procedures, see p. 758. □, 2 h; ▨, 6 h; ▤, 12 h; ■, 24 h. Phytochemicals analysed were astaxanthin (AST), lutein (LUT) canthaxanthin (CAN), lycopene (LYC), α-carotene (AC), β-carotene (BC), retinol (RET), γ-tocopherol (GT) and α-tocopherol (AT). Values are means with their standard errors shown by vertical bars (six to nine observations from three independent experiments).

previously to emulsify retinol and retinol esters in a drug preparation for ocular use (Radomska & Dobrucki, 2000). *In vitro*, Tween 20 has been used to solubilise retinol and β-carotene for delivery to rat everted-gut sacs (El-Gorab *et al.* 1975) and Tween 40 has solubilised β-carotene for delivery to CaCo-2 cells (During *et al.* 1998, 2002).

Carotenoids have undergone intense scrutiny as a result of their potential modulatory role in diseases such as cancer and CVD. *In vitro* evidence suggests that these diseases can be influenced by different carotenoids in isolation, especially β-carotene and lycopene. However, few studies have examined the possible synergistic or inhibitory effects of carotenoids, perhaps due to the difficulty involved in solubilising carotenoid mixtures for supplementation to cells. A previous study found that lipid peroxidation was reduced to a greater extent when lutein and lycopene were used together than when used on their own (Stahl *et al.* 1998). Bohm *et al.* (1998) observed a synergistic protection of β-carotene, α-tocopherol and ascorbic acid against damage to human lung fibroblasts due to UV light. Similarly, a combination of the compounds β-carotene, α-tocopherol and ascorbic acid showed greater protection to cellular damage induced by 2,2′-azobis (2-amidinopropane) dihydrochloride, a free radical initiator, than the individual compounds alone (Zhang & Omaye, 2001).

To date, carotenoids have generally been delivered to cells in culture solubilised in organic solvents or incorporated into water dispersible beadlets, liposomes and/or mixed micelles. A number of disadvantages have been associated with these methods. Water miscible beadlets are only commercially available for β-carotene and canthaxanthin; in addition, as pointed out by Xu *et al.* (1999), the reagents required for the preparation of these beadlets are difficult to source. Absorption of β-carotene from liposomes was shown to be lower when compared with an emulsion preparation (Grolier *et al.* 1992) or with THF (Williams *et al.* 2000). In many studies micellarised β-carotene was shown to be unstable in tissue culture media. However, a micellarised formulation,

developed by Xu *et al.* (1999), was shown to stabilise and solubilise lycopene. Martin *et al.* (1997) also attempted to stabilise and solubilise carotenoids using human lipoproteins, but although these efforts proved successful, the procedure involved was difficult and expensive. Cooney *et al.* (1993) investigated a number of organic solvents for their abilities to solubilise carotenoids, but found that physiological concentrations could not be solubilised using either dimethylsulfoxide or acetone–dimethylsulfoxide mixtures. They also noted that chloroform was cytotoxic and that the bioavailability of carotenoids was low with hexane. Methyl-β-cyclodextrin was used successfully to deliver β-carotene, zeaxanthin, lutein and lycopene to human skin fibroblasts, but these were solubilised and delivered individually (Pfitzner *et al.* 2000). In another study β-carotene-enriched steer serum proved a more efficient delivery vehicle when compared with THF, beadlets and liposomes, but this procedure is not practical for many investigators (Williams *et al.* 2000). Numerous studies have successfully used THF as a solvent for carotenoid delivery to cells in culture (Stivala *et al.* 1996; Wei *et al.* 1998; Xu *et al.* 1999); however, as Cooney *et al.* (1993) observed, THF can be toxic to certain cell lines, possibly due to peroxide formation, and also it is difficult to solubilise high concentrations of the more hydrophobic carotenoids such as lycopene.

In the present study, the use of THF was compared with Tween 40 and Tween 80 as a vehicle for delivering a mixture of carotenoids to cells in culture. No toxic effects to the membranes or to the energy metabolism of the CaCo-2 cells were found under the conditions of the experiment when Tween 40 and Tween 80 were used as delivery vehicles at a concentration of 1 ml/l. Furthermore, the Tweens did not disrupt the antioxidant potential of the cells. THF was found to be least efficient at delivering the phytochemicals to the CaCo-2 cells. THF was incapable of delivering lycopene, the most hydrophobic phytochemical used in the mixture to the cells. In contrast, lycopene was detected when Tween 40 and Tween 80 were used as the vehicles. Of the remaining compounds, all three vehicles were more effective at delivering the more polar or oxygenated compounds, such as lutein, canthaxanthin, astaxanthin and α-tocopherol, compared with the non-polar compounds. In comparison with THF, the Tweens delivered substantially more phytochemicals to the cells than THF.

When Tween 40 and Tween 80 were used as the vehicles, the phytochemicals were shown to be stable within the media over the incubation period. The compounds were taken up by the CaCo-2 cells within 6–12 h (Fig. 3(a and b)). Although test media containing the phytochemicals were removed after 24 h and replaced with normal growth media that did not contain the phytochemicals, the compounds remained stable within the cells for up to 24 h afterwards, with only minor losses of the more oxygenated compounds (Fig. 4(a and b)). The hydrocabons remained stable within the cells. There was little difference observed between Tween 40 and Tween 80 as vehicles for the oxygenated compounds. However, Tween 40 appeared to be slightly better at delivering the hydrocarbons β-carotene, α-carotene and lycopene to the cells. Tween 80 has oleic acid as its fatty acid side-chain, whereas Tween 40 has palmitic acid. The slight difference in cellular uptake of the hydrocarbons may be attributed to the hydrophilic–lipophilic balance value of the Tweens. Tween 40 has a higher hydrophilic–lipophilic balance value (15·6) than Tween 80 (15·0).

Carotenoids that contain at least one unsubstituted β-ionone ring with a polyene side-chain attached exhibit pro-vitamin A activity. Of the carotenoids studied in this experiment, only β-carotene and α-carotene are capable of being converted to retinol. In the present study there was a greater retinol concentration in cells treated with the carotenoids than in control cells after 24 h incubation (Fig. 1). A previous study by Wei *et al.* (1998) has shown that treatment with β-carotene increased the production of retinol in several cell lines including rabbit corneal epithelial cells (SIRS) and humans skin fibroblasts (Hs27). The enzyme β-carotene 15,15′-dioxygenase has been shown to be present in certain subclones of CaCo-2 cells (During *et al.* 1998) and this enzyme is known to convert β-carotene to retinal (precursor of retinol and retinoic acid).

In conclusion, the Tween vehicles were found to be more effective delivery vehicles for carotenoids when compared with THF under these experimental conditions. Tween 40 and Tween 80 are rapid, inexpensive and non-toxic methods for delivering complex mixtures of carotenoids and tocopherols to cells in culture for the study of the biological interactions of these phytochemicals.

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