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Influence of simulated upper intestinal parameters on the efficiency of beta carotene micellarisation using an *in vitro* model of digestion

Amanda J. Wright *, Christina Pietrangelo, Amanda MacNaughton

Department of Human Health and Nutritional Sciences, College of Biological Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Abstract

This study was undertaken to better understand how variations in the parameters used to simulate the upper intestinal environment influence the transfer of a lipophilic molecule, beta carotene (BC), from the oily to the aqueous phase of digestate using a static model of digestion. Bile, pancreatin and pH were important and inter-related factors in determining the efficiency of BC transfer ($P < 0.05$). Less than 4% and 8% of the BC was transferred to the aqueous phase in the absence of bile and pancreatin, respectively. Generally, the proportion of BC transferred increased with bile and pancreatin concentrations and with pH. Under conditions which simulated the fed versus fasted states of digestion, significantly more BC was incorporated in the aqueous fraction (46.5% versus 18.8%). These results underscore the need to carefully consider and define the experimental parameters used for *in vitro* assays to study the digestion of carotenoids and other lipophilic molecules.

Keywords: Beta carotene; *In vitro* digestion; Intestinal; Micellarisation; Fasted; Fed

1. Introduction

The carotenoids are a group of lipophilic pigment molecules with important consequences for human health because of their pro-vitamin A activity and nutraceutical potential. Despite their prevalence in fruits and vegetables, however, the bioavailability of carotenoids from foods, and even some supplements, may be low and highly variable (Faulks & Southon, 2001). In fact, food matrix structure and interactions with dietary constituents, including between carotenoids, critically influence carotenoid digestion and absorption (Borel, 2003; Tyssandier et al., 2003).

During gastric digestion, carotenoids are released from foods and dissolve in the oily phase (Rich, Fillery-Travis, & Parker, 1998), which gets emulsified due to the shearing forces present (Furr & Clark, 1997; Parker, 1996). Digestive enzymes then hydrolyse the emulsified lipids into free fatty acids (FFA) and partial acylglycerols (Charman, Porter, Mithani, & Dressman, 1997). Pancreatic lipase activity, specifically, accounts for most of the lipid hydrolysis during digestion (Pafumi et al., 2002). The products of lipid digestion (i.e., primarily 2-monoacylglycerols and free fatty acids) play an important role in helping to solubilise lipophilic molecules into the bile salt micelles which form in the small intestine. These species intercalate into the bile salt micelles, causing them to swell and increasing their solubilisation capacity (Porter & Charman, 2001) for lipophilic molecules like carotenoids. Due to their lipophilic nature, carotenoids need to be incorporated into the mixed micelles in order to pass through the unstirred water layer and to approach the enterocyte membrane for absorption (Charman et al., 1997). Therefore, the efficiency of micellarisation and solubilisation of carotenoids within the micelles that form during digestion are important determinants of carotenoid bioavailability (Borel, 2003; Tyssandier, Lyan, & Borel, 2001; Tyssandier et al., 2003). Despite this, the processes by which lipophilic molecules are transferred

* Corresponding author. Tel.: +1 519 824 4120x54697; fax: +1 519 763 5902.
E-mail address: ajwright@uoguelph.ca (A.J. Wright).

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from the food matrix to the gastric emulsion and ultimately to the aqueous, micellar phase are not well understood.

In vitro assays have been adapted in order to study the processes of carotenoid digestion and absorption (Rich, Bailey, Faulks, Parker, Wickham, & Fillery-Travis, 2003a; Rich, Faulks, Wickham, & Travis, 2003b). This often involves quantifying how much of a carotenoid is transferred from a food matrix to the aqueous, micellar phase of the digestate after mimicking the conditions of the gastrointestinal tract. It is assumed that those carotenoids which are associated with the aqueous phase would be the most readily absorbable by the body (Porter & Charman, 2001).

Support for the use of *in vitro* digestion assays to study the pre-absorptive events which affect carotenoid digestion, is provided by the correlations which have been observed between *in vitro* and *in vivo* studies (Failla & Chitchumroonchokchai, 2005; Reboul et al., 2006). Still, there is much to learn about carotenoid digestion and about the relationships between the complex and interconnected factors which govern carotenoid digestion and absorption *in vivo* as well as during *in vitro* digestion assays. For example, carotenoid bioavailability is influenced by the presence and nature of food in the gastrointestinal tract (Borel, 2003). Despite this, most *in vitro* studies are conducted using conditions intended to simulate the fasted state of digestion. For example, pH values in the range of 6.5–7.5 are often used to simulate the intestinal environment (Garrett, Failla, & Sarama, 1999; Hedren, Diaz, & Svanberg, 2002; Liu, Glahn, & Liu, 2004; Veda, Kamath, Platel, Begum, & Srinivasan, 2006). While this may be representative of the duodenal fasted environment, the intestinal pH is influenced by the presence of food and is significantly lower in the fed state (Kalantzi, Goumas, Kalioras, Abrahamsson, Dressman, & Reppas, 2006). Depending on where measurements are made, fed pH values as low as 2 have been observed (Charman et al., 1997). Since carotenoids are often consumed as part of a mixed meal, there is strong rationale for studying the influence of the fed state on the efficiency of carotenoid transfer and micellarisation. The objective of this study was to determine the influence of simulated upper intestinal parameters on the efficiency of BC transfer from the oily to the aqueous fraction of digestate. Specifically, the influence of bile extract and pancreatin concentrations as well as pH are reported, in addition to the influence of conditions which are broadly representative of the fasted and fed states of digestion.

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). This included all-*trans*-beta carotene (BC, Type 1 synthetic, >95% purity, C9750), porcine bile extract (B8631) and pancreatin (from porcine pancreas, P1750, 4 × USP). High purity nitrogen was supplied by BOC Gases (Guelph, ON).

2.2. Sample preparation

Refined, bleached and deodorised canola oil (CO) was generously provided by Bunge Canada (Toronto, Canada). The oil was re-bleached to ensure the removal of coloured impurities by the addition of 5 wt% bleaching clay (Engelhard F105, also provided by Bunge Canada) to the oil which was then heated to 90 °C, under vacuum in a rotary evaporator for 20 min prior to vacuum filtration.

A stock solution of BC in bleached canola oil (i.e., BC–CO) was initially prepared at a concentration of 0.1 mg BC per 100 mg CO. This concentration was selected based on reports that BC solubility in bulk triacylglycerols ranges from 0.11–0.14 wt% (Borel et al., 1996). To remove any potentially crystalline BC, the BC–CO was heated to room temperature and filtered (0.22 μm MAGNA, nylon, Fisher Scientific Inc.). The BC–CO was placed in an amber glass jar, flushed with nitrogen and stored at -20 °C until use within 2 weeks. To minimise BC degradation, all experiments were conducted under reduced lighting and at temperatures not exceeding 37 °C.

For each digestion experiment, the BC–CO was heated to 37 °C for 30 min and the desired amount was weighed into amber glass jars (typically). The concentration of the stock BC–CO was determined for each experiment (as described below) and found to be within the range of 0.035 mg BC/100 mg CO. Therefore, roughly 4.1×10^{-5} mg BC was present in a typical digestion sample. Samples were blanketed with nitrogen and stored at 4 °C overnight.

2.3. In vitro digestion assay

An *in vitro* digestion procedure which mimicked the upper intestinal stage of digestion was adapted from those described in the literature (Garrett et al., 1999; Hedren et al., 2002). During the gastric phase of digestion, carotenoids are transferred to the oily phase (Rich et al., 1998). In this study, samples consisted of BC already dissolved in the oily phase, and therefore only the intestinal phase of digestion was considered. In previous experiments, elimination of the gastric step did not significantly change the proportion of BC transferred to the aqueous phase (Garrett et al., 1999), while eliminating the intestinal phase of digestion resulted in a 90% reduction in transfer (Hedren et al., 2002).

At the start of each digestion, the desired amounts of bile and pancreatin were dissolved in 40 ml of saline (0.9 wt% NaCl) and 10 ml of 100 mM sodium bicarbonate solution, respectively, by vigorous mixing. Twenty millilitres of the bile solution and 10 ml of the pancreatin solution was then added to an amber glass jar, containing BC–CO and 1.3 mg butylated hydroxytoluene as an antiox-

idant. Samples were adjusted to the desired pH through the dropwise addition of either 1 N HCl or 1 N NaOH. Samples were then emulsified using a homogeniser (Ultra-Turrax, IKA T18 Basic) at 10,000 rpm for 30 s. This was done to roughly simulate the emulsified state at which the digestate enters the duodenum after the gastric phase (Armand et al., 1996). In preliminary studies, samples were not emulsified prior to the digestions, resulting in minimal to no BC being transferred to the aqueous phase (data not shown).

To minimise BC losses, 10 ml of the saline was used to rinse each of the pH electrode and emulsifier probe, resulting in a total sample volume of ~50 ml per jar. Samples were blanketed with nitrogen to minimise potential oxidative degradation, covered with Parafilm and sealed with lids. The jars were incubated in a 37 °C shaking water bath (New Brunswick Scientific Co., Inc., NJ) at 250 rpm for 2 h.

After 2 h, samples of the digestate were ultracentrifuged (Beckman L8-M) in order to isolate the aqueous micellar fraction. Duplicate ~6.5 ml aliquots of each digestate were spun at 144,800 g at 7 °C for 45 min. When small amounts of oil remained following the digestion, this adhered to the edges of the ultracentrifuge tubes and the aqueous fraction was collected with a fine tip disposable pipette, quantified in a graduated cylinder, and stored in glass vials under nitrogen at −20 °C until analysis (i.e., within 1 week). In preliminary experiments, the samples were filtered (nylon, 0.22 μm pore size) prior to storage. However, there was no significant effect of aqueous sample filtration on the determination of BC concentration ($P > 0.05$, data not shown) and no BC crystals were detected in the samples, by polarised light microscopy. Therefore, this step was subsequently eliminated. Garrett et al. (1999) similarly reported that filtration did not significantly affect the determination of carotene concentration in the aqueous fraction following an *in vitro* digestion.

2.4. Beta carotene determination

The concentration of BC in the aqueous samples was determined following solvent extraction. Samples were thawed in a water bath at 37 °C. To each 0.5 ml aliquot of sample, 0.5, 3.0 and 1.0 ml of ethanol, acetone and deionised water, respectively, was added, with vortexing for 5 s after the addition of each liquid. Two ml of hexane was added, the vials inverted 10 times and the organic layer removed after 5 min. The hexane extraction was performed in triplicate for each sample and the 6 ml of hexane pooled and evaporated under nitrogen at 35 °C (N-Evap, Organomation, Berlin, MA). Samples were evaporated just to the point of dryness at which point 1 ml of fresh hexane was added. Samples were then vortexed for 5 s and placed in a sonicating water bath (Branson Ultrasonics Corporation, Danbury, CT) for 10 s to ensure re-dissolution of BC. This step was verified not to influence BC concentration ($P > 0.05$, data not shown). The contents of each vial were then transferred to a 1 ml microcuvette and the absorbance, at 450 nm (i.e., the maximum wavelength of absorption for all-*trans*-BC), was determined using a Hewlett Packard 8451A Diode Array Spectrophotometer. The concentration of BC in each sample was then calculated using Beer's law and a molar extinction coefficient of 137,400 ml mol^{-1} cm^{-1} (Rich et al., 1998). The proportion of BC transferred to the aqueous phase during digestion was calculated based on the initial amount of BC present in each sample jar. The extraction efficiency, determined by performing 10 replicate extractions on the stock BC–CO solution, was found to be 93.8 ± 1.8%.

Several sets of empty sample jars or those containing only CO (instead of BC–CO) were run as controls throughout the experiments. These were done across the ranges of oil, pancreatin and bile concentration studied (see below). In all cases, upon extraction into hexane, the $A_{450\ nm}$ values were within baseline for hexane, indicating that BC was solely responsible for the absorbance observed in the sample extracts.

2.5. Experimental parameters

The influences of bile and pancreatin concentration, pH and sample size on the transfer of BC to the aqueous fraction of the *in vitro* digestate were determined. Bile extract concentrations of 0.0, 1.25, 2.5, 5.0, 10.0 and 20.0 mg/ml were studied at low (0.4 mg/ml) and high (2.4 mg/ml) concentrations of pancreatin at pH 6.5. Similarly, the effect of pancreatin concentration (0.0, 0.1, 0.2, 0.4, 1.2, 2.4 and 4.8 mg/ml) was determined at pH 6.5 for both 5.0 and 20.0 mg bile/ml digestate. These ranges of bile and pancreatin were selected to span the expected physiological ranges (see below). The efficiency of BC micellarisation was also determined under conditions which approximated the lumen of the upper intestine in the fed state (i.e., after the ingestion of a lipid-containing meal). The fasted state was simulated using bile and pancreatin concentrations of 5.0 mg/ml and 0.4 mg/ml, respectively, and a pH of 6.5. The fed state was simulated using bile and pancreatin concentrations of 20.0 mg/ml and 2.4 mg/ml, respectively, and a pH of 5.0. Within each of the described fed and fasted scenarios, BC transfer was also compared at pH values ranging from 3.5 to 9.0. Lastly, the influences of BC–CO sample size and the presence of additional CO under both the fed and fasted conditions were studied.

2.6. Statistical analysis

At least six replicate digestion jars, conducted across at least two different days, were performed for each experimental treatment. From each jar, two samples were ultracentrifuged and subsequently two extractions from each aqueous sample were conducted. Therefore, results are reported as mean ± SEM (standard error of the mean) for each treatment. All statistical analysis was conducted using GraphPad Prism 4 and a significance level of $P < 0.05$. The effects of treatments on BC transfer to the aqueous phase were determined using one- and two-way

ANOVA (analysis of variance) testing, where appropriate. Differences between treatments were identified using Tukey's multiple comparison testing ($P < 0.05$).

3. Results and discussion

3.1. Influence of bile concentration on the efficiency of BC micellarisation

Typical bile salt concentrations in the lumen of the small intestine in the fasted and fed states are in the range of 4–6 and 10–20 mM, respectively (Porter & Charman, 2001; Rich et al., 2003b). Since bile salts constitute roughly half the weight of the bile extract used in this study, and assuming an average bile salt molecular weight of 500 g/mol (Nielsen, Mullertz, Norling, & Kristensen, 2001), the concentrations of bile extract examined in Fig. 1 (i.e., 0.0, 1.0, 1.3, 5.0, 10.0 and 20.0 mg/ml) correspond to bile salt concentrations of approximately 0.0, 1.2, 2.3, 4.9, 9.8 and 19.6 mmol/l. Therefore, Fig. 1 includes a range of bile salt concentrations with potential physiological relevance. Fig. 1 shows the influence of bile concentration on BC micellarisation at both low (Fig. 1A, 0.4 mg/ml) and high (Fig. 1B, 2.4 mg/ml) levels of pancreatin.

According to Fig. 1, bile concentration had a significant influence on the proportion of BC transferred to the aqueous phase at both concentrations of pancreatin ($P < 0.05$).

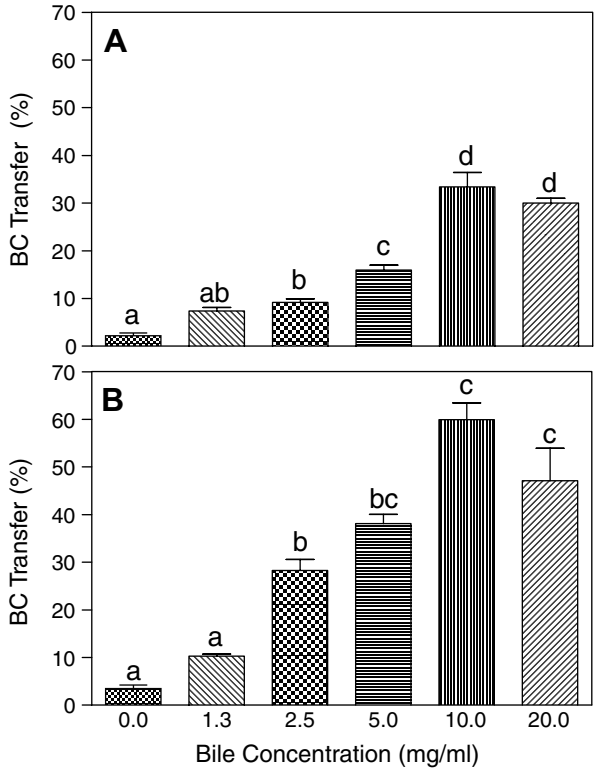

Fig. 1. Influence of bile extract concentration on BC transfer from the oily to the aqueous phase at (A) 0.4 and (B) 2.4 mg pancreatin/ml digestate. Error bars represent standard error of the mean. Different letters (a–d) indicate significant differences ($P < 0.05$) between treatment means within 1A or 1B.

In the absence of bile, BC transfer was minimal (i.e., 2.1 ± 1.4 and $3.5 \pm 2.8\%$ at 0.4 and 2.4 mg/ml pancreatin, respectively). Similarly, Hedren et al. (2002) reported an 80% reduction in the transfer of BC to the aqueous phase when bile extract was eliminated from their *in vitro* digestion assay. In contrast, Garrett et al. (1999) detected no BC in the aqueous fraction and less than 1% of lutein was transferred from the oily phase in the absence of bile extract.

Some of the decrease in BC transfer in the absence of bile is likely related to the influence of bile salts on pancreatic lipase activity (Shiau, 1981). For example, the presence of bile salts shifts the pH optimum of pancreatic lipase from 8.0 to 6.0 (Shiau, 1981). Furthermore, because they are highly surface active, bile salts tend to preferentially adsorb at the oil–water interface, displacing other surface active molecules that may be present, thereby allowing lipase to act. For example, while FFA were liberated from lecithin-stabilised emulsion droplets even in the absence of bile, the presence of bile promoted lipolysis (Mun, Decker, Park, Weiss, & McClements, 2006). Although bile salts themselves can inhibit lipolytic activity, this action is generally reversed by the presence of co-lipase (Gargouri, Julien, Bois, Verger, & Sarda, 1983), a small molecular weight cofactor which combines with lipase and which is present in pancreatic secretions (Porter & Charman, 2001).

The concept of critical micelle concentration (CMC) has been used to explain the influence of bile concentration on carotenoid transfer and absorption (El-Gorab & Underwood, 1973; Rich et al., 2003b; Tyssandier et al., 2001). The CMCs for pure bile salts are in the range of 0.5–4.2 mM (Borgstrom & Erlanson, 1973). In the duodenum, the CMC is around 1 mM (Hofmann, 1963), owing to the mixture of bile salts and various amphiphilic molecules such as phospholipids and the products of lipid digestion which are present (Charman et al., 1997). This is similar to the CMC reported for the bile extract used in this study (i.e., 0.07 ± 0.04 mM) (Mun et al., 2006). Therefore, except in the absence of bile extract, the CMC is expected to have been exceeded for each treatment in Fig. 1.

The dependence of BC transfer on bile concentration is evident from Fig. 1. The trend towards increasing carotenoid transfer with increasing bile concentration is consistent with previous reports (Garrett et al., 1999). Up to 10 mg/ml, an increase in bile extract likely resulted in the formation of more micelles, permitting more BC to be incorporated into the aqueous phase. However, according to Fig. 1, beyond a bile concentration of 10 mg/ml, there was no significant increase in the proportion of BC incorporated into the aqueous phase ($P > 0.05$) at either pancreatin concentration studied. Tyssandier et al. (2001) observed a similar leveling off of BC transfer with bile concentration, although this occurred at a lower bile concentration (i.e., 2 mM, corresponding to roughly 1.3 mg/ml in Fig. 1). Rich et al. (2003b) also observed that at concentrations above the CMC, initial increases in the proportion of BC and lutein solubilised in pure bile salt micelles were

observed, but that there was a subsequent attenuation of this effect and even decreases in the proportion of BC transferred at the highest concentrations studied. This was explained in the context of a possible change in micellar structure or possibly the solubilisation of compounds which compete with and prevent the solubilisation of BC (Rich et al., 2003b). There is evidence from *in vitro* studies that, at high concentrations, bile salts can form different polymolecular aggregate structures. The impact of these structures, on the solubilisation of lipophilic molecules and their physiological relevance, are not known (Shiau, 1981). It is also possible that solubility limits are exceeded at higher concentrations or that the presence of another molecule in the bile extract begins to impede and limit the capacity for BC micellarisation.

3.2. Influence of pancreatin concentration on the efficiency of BC micellarisation

According to Fig. 1, pancreatin concentration influenced the transfer of BC to the aqueous phase, i.e., comparing Fig. 1A and B the transfer was generally higher at 2.4 (1B) versus 0.4 mg/ml pancreatin (1A). Fig. 2 shows the influence of pancreatin at two bile extract concentrations (i.e., 5.0 and 20 mg/ml, A and B, respectively). These levels of bile were selected to approximate the bile salt concentrations in the small intestine during the fasted and fed states of digestion, respectively.

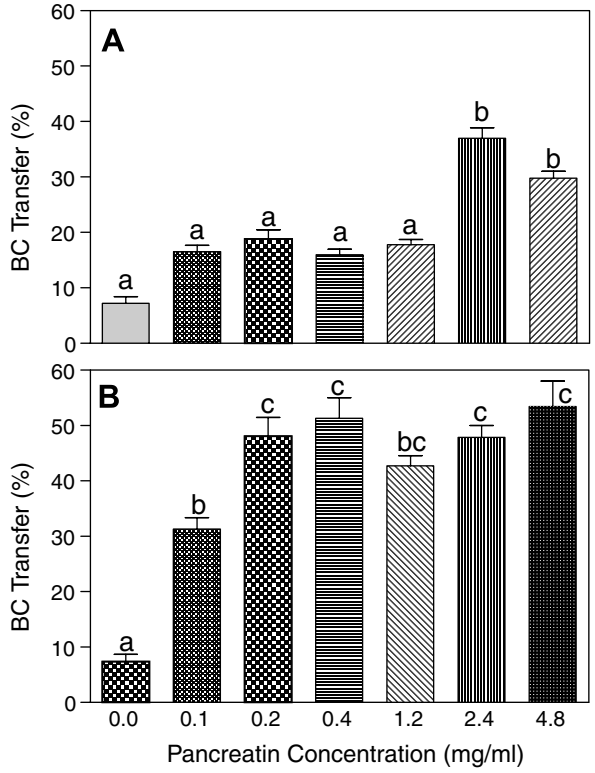

Fig. 2. Influence of pancreatin concentration on BC transfer from the oily to the aqueous phase at (A) 5.0 and (B) 20.0 mg bile extract/ml digestate. Error bars represent standard error of the mean. Different letters (a–c) indicate significant differences ($P < 0.05$) between treatment means within 2A or 2B.

Comparing Fig. 2A and B emphasizes the important role of bile extract in the transfer of BC to the aqueous phase. Significantly more BC was transferred at 20.0 (2B) versus 5.0 (2A) mg/ml bile extract ($P < 0.05$). In the absence of pancreatin, ~7.5% of the initial BC was transferred to the aqueous phase at both levels of bile. Although bile salt micelles form in the absence of pancreatin, there is no clear consensus as to how critical triacylglyerol lipolysis is for carotenoid micellarisation (Borel et al., 1996; Rich et al., 2003b; Tyssandier et al., 2001). In persons with exocrine pancreatic insufficiency, triacylglycerol hydrolysis is generally impaired and leads to a reduced capacity for the micellarisation of lipophilic molecules which is reflected in low plasma carotenoid levels (Yamada-Kusumi & Nakamura, 2000). When Garrett et al. (1999) eliminated pancreatin from their *in vitro* digestion assay, the amount of BC incorporated in the aqueous phase was reduced by 50%. However, in another study, only a 10% reduction in carotene transfer was observed (Hedren et al., 2002).

At a bile concentration of 5.0 mg/ml (2 A), there was no significant effect of pancreatin concentration ($P > 0.05$) on BC transfer until 2.4 mg/ml. Beyond this, increasing the pancreatin concentration to 4.8 mg/ml did not significantly change the amount of BC transferred ($P > 0.05$). At 20.0 mg/ml bile (2B), beyond 0.2 mg/ml pancreatin, no further increases in BC transfer observed ($P > 0.05$). Conceivably, at a bile concentration of 5.0 mg/ml, more lipase is required to reach a maximum transfer because there are fewer micelles present to solubilise the BC, and therefore, more lipolysis products are required to swell the existing micelles in order to increase the solubilisation capacity of the aqueous phase.

The presence of bile may also change the nature of the oil droplet interfacial area. Bile generally results in a promotion of pancreatic lipase activity because the highly surface active bile salts displace other surface active molecules from the interface, thereby allowing lipase and co-lipase to act (Gargouri et al., 1983). However, Fig. 2 suggests that, even in the presence of bile, the transfer of BC to the aqueous phase reaches a maximum once a certain level of lipolytic activity is achieved. Surface active molecules, including free fatty acids (FFA), can absorb at the oil–water interface and displace lipase (Pafumi et al., 2002). Therefore, lipid hydrolysis may lead to the formation of FFA which, beyond a certain level, displace lipase from the interface and subsequently limit further increases in activity. These results support the conclusion that bile, and to a lesser extent pancreatin concentration, are important and inter-related factors in the process of BC micellarisation

The observation that generally more BC is transferred to the aqueous phase with increasing pancreatin concentration is consistent with previous findings (Garrett et al., 1999) and the collective understanding of lipid digestion, i.e., products of lipid digestion increase the capacity of

micelles to solubilise lipophilic molecules by promoting swelling of the micelles (Porter & Charman, 2001). However, it is difficult to draw accurate comparisons between many *in vitro* digestion studies because enzyme sources and/or activities are not necessarily reported. In this study, the pancreatin had a lipase activity of 8 USP units/mg (USP 4×). Therefore, the treatments in Fig. 2 with pancreatin concentrations of 0.0, 0.1, 0.2, 0.4, 1.2, 2.4 and 4.8 mg/ml had lipase activities corresponding to approximately 0.0, 0.8, 1.6, 3.2, 9.6, 19.2 and 38.4 units/ml.

3.3. BC micellarisation under conditions which simulate the fasted versus fed states of digestion

The transfer of BC to the aqueous fraction under conditions which are broadly representative of the fasted and fed states of digestion was determined. The rationale for selecting the bile extract concentrations of 5.0 and 20.0 mg/ml was discussed previously. Lipase concentrations in the small intestine are reportedly 2.5–5 times higher in the fed versus the fasted state (Brunner, Northfield, Hofmann, Go, & Summerskill, 1974; Porter & Charman, 2001). However, this is variable and depends on factors such as the contents of a meal. Schwizer et al. (1997) analysed pancreaticobiliary secretions in response to the intragastric instillation of TAGS or FFA and observed a 4–9 times increase in lipase activity in the duodenum. Therefore, pancreatin concentrations of 0.4 and 2.4 mg/ml were chosen to approximate the range of concentrations expected in the absence and presence of a lipid-containing food and to parallel the experiment in Fig. 1.

Duodenal fasting pH values between 6 and 7 are often reported (Kalantzi et al., 2006; Kararli, 1995). In the presence of foods, duodenal pH is significantly lower and in the range of 5 (Kalantzi et al., 2006). Therefore, in this study, the fasted state in the upper small intestine was approximated using bile and pancreatin concentrations of 5.0 mg/ml and 0.4 mg/ml, respectively, and a pH of 6.5. To simulate the environment of the upper intestine following the ingestion of a meal (i.e., the fed state), concentrations of bile and pancreatin were 20.0 and 2.4 mg/ml, respectively, and the digestate pH was adjusted to 5.0.

Under the fasted conditions, 18.8 ± 0.7% of the BC in the sample was transferred to the aqueous, micellar phase. Significantly more (i.e., 46.5 ± 5.3%) BC was transferred when conditions which mimic the fed state were used ($P < 0.05$). Many *in vitro* digestion experiments are performed using conditions which simulate the fasted state. However, because carotenoids and other lipophilic micronutrients are generally consumed within the context of a snack or meal, the potential carotenoid transferred may be significantly underestimated. According to Figs. 1 and 2, the higher proportion of BC transferred under the fed versus fasted conditions is related to increases in both bile and pancreatin for the fed treatments. To examine the role of pH on BC transfer to the aqueous phase, the pH was varied between 3.5 and 9.5 for each of the fasted and fed treatments.

3.4. Influence of pH on BC micellarisation under conditions approximating the fasted and fed states

Fig. 3 shows the influence of pH on BC transfer to the aqueous fraction at bile and pancreatin concentrations which approximate the fasted and fed small intestinal environments. Depending on meal contents and where measurements are made, intestinal pH values vary and values as low as 2 have been reported (Charman et al., 1997; Kalantzi et al., 2006; Kararli, 1995; Tyssandier et al., 2001). pH 9.5 is outside the range of physiological relevance.

According to Fig. 3, pH had a significant influence on BC transfer to the aqueous phase at both low and high levels of bile and pancreatin ($P < 0.05$). Within the fasted treatments, the biggest difference was observed between pH 6.5 and 8.0. This is within the range of pH values used to simulate the upper intestinal environment in typical *in vitro* digestion assays. According to these results, large differences could be expected between studies which differ slightly in terms of experimental pH.

Within the fed treatments, pH had no influence on the proportion of BC transferred between pH 5.0 and 9.5 ($P > 0.05$). However, significantly less BC was transferred to the aqueous fraction of digestate at pH 3.5 ($P < 0.05$). This decrease in BC transfer below pH 5.0 in the fed treatments is consistent with previous observations of a biphasic response in carotenoid transfer above and below the range of pH 4–5 (Tyssandier et al., 2001). These observations are likely related to the precipitation of bile salts under the acidic conditions used. At low pH, bile salts, which would otherwise form micelles and solubilise BC, precipitate. Below pH 4, up to 60% of bile salts are precipitated (Yamada-Kusumi & Nakamura, 2000).

pH may also influence BC transfer because of its effect on the ionisation of species, such as FFA. Above their

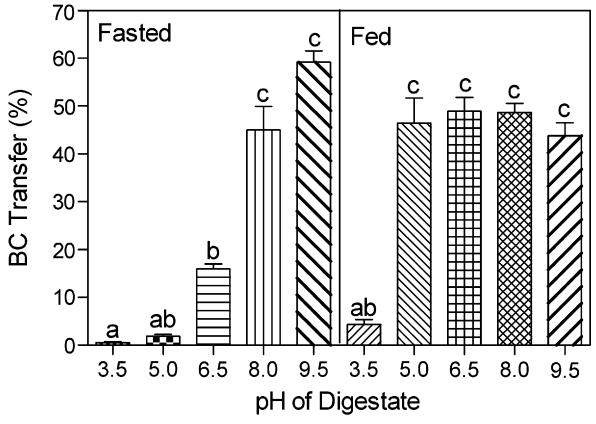

Fig. 3. Influence of digestate pH on BC transfer from the oily to the aqueous phase under conditions which approximate the upper intestinal lumenal environment during the fasted (pancreatin 0.4 mg/ml, bile extract 5.0 mg/ml) and fed (pancreatin 2.4 mg/ml, bile extract 20.0 mg/ml) states of digestion. Error bars represent standard error the mean. Different letters (a–c) indicate significant differences ($P < 0.05$) between treatment means.

pK_a, FFAs are ionised and can swell, leading to increased solubilisation capacity of the micelles present in the aqueous phase (Shiau, 1981). Below their pK_a, FFAs are protonated, and therefore predominate in the oily phase. Furthermore, pancreatic lipase is inactivated at low pH (Charman et al., 1997; Kalantzi et al., 2006; Kararli, 1995; Tyssandier et al., 2001). Porcine pancreatic lipase has an isoelectric point of about 5.7 (Evram & Telefoncu, 2005) and a pH optimum around 8.0, although this is shifted to roughly 6.0 in the presence of bile salts. Therefore, decreased lipase, bile salt precipitation and protonation of FFA all contribute to the decrease in BC transfer to the aqueous phase at pH 3.5 for both the fasted and fed conditions. Although bile and pancreatin concentration significantly influenced BC transfer at pH 6.5 (Figs. 1 and 2), increasing the pH to 8.0 eliminated any differences (Fig. 3) and a maximum BC transfer of ~50% was observed. Therefore, pH was a major determinant of BC transfer from the oily to the aqueous phase during the simulated digestion.

3.5. Influence of sample size on BC transfer under simulated fasted and fed intestinal conditions

Dietary fat has important implications for carotenoid bioavailability in terms of its presence, type and amount. Ingested lipids serve to increase biliary secretions from the gallbladder and as a sink to dissolve hydrophobic compounds. Their digestion products are also important in helping to solubilise carotenoids by increasing the size of bile salt micelles which form (Furr & Clark, 1997). Fig. 4 shows the amount (4A) and proportion (4B) of BC transferred for samples which consisted of 120 or 960 mg BC–CO, in addition to samples which contained 120 mg of BC–CO plus additional CO up to a total sample weight of 960 mg, under fed and fasted conditions.

According to Fig. 4A, under fasted conditions, the same amount of BC was incorporated into the aqueous phase with 120 mg BC–CO as with 120 mg BC–CO plus additional CO up to 960 mg. With 960 mg BC–CO, significantly more BC was transferred ($P < 0.05$), although this was not nearly equivalent to the increased amount of BC initially present. These results suggest there was a limit to the amount of BC which could be transferred to the micellar phase, under these experimental conditions (i.e., pH, bile, pancreatin, time, static conditions, etc.). Furthermore, the presence of CO above 120 mg does not seem to impede BC incorporation into the aqueous phase, i.e. there was no significant difference in the amount of BC transferred between the 120 and 120+ samples ($P > 0.05$).

Under fed conditions (Fig. 4A), significantly less BC was transferred from 120 mg BC–CO in the presence of additional CO ($P < 0.05$). Fig. 4A also shows that significantly less BC was micellarised from 960 mg BC–CO than from 120 mg BC–CO ($P < 0.05$). This is a different trend than was observed for the fasted treatments. Observations in the fed treatments point to possible competition between

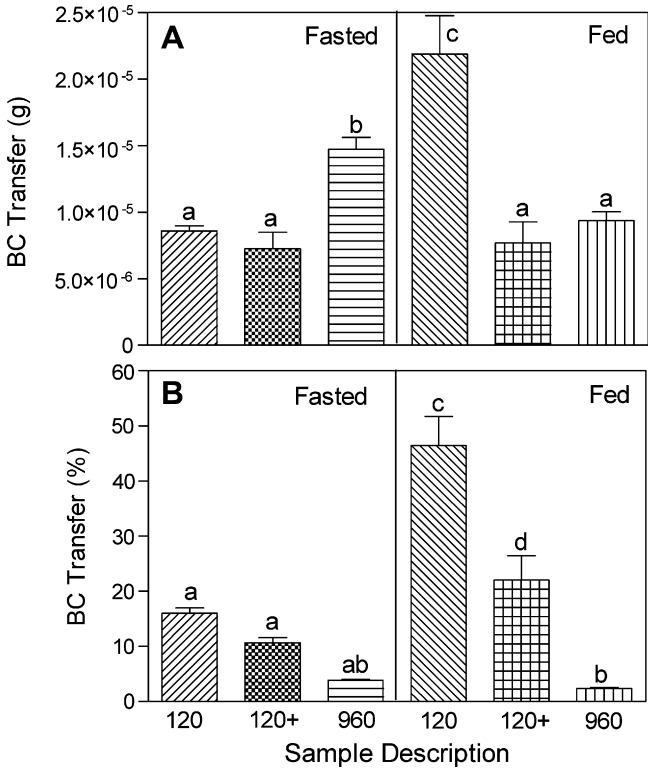

Fig. 4. Influence of sample (120 mg BC–CO *(120)*, 120 mg BC–CO plus CO for a total of 960 mg (*120+*), and 960 mg BC–CO (*960*)) on grams (A) and percentage (B) of BC transferred from the oily to the aqueous phase under conditions which approximate the upper intestinal lumenal environment during the fasted (pH 6.5, pancreatin 0.4 mg/ml, bile extract 5.0 mg/ml) and fed (pH 5.0, pancreatin 2.4 mg/ml, bile extract 20.0 mg/ml) states of digestion. Error bars represent standard error of the mean. Different letters (a–d) indicate significant differences ($P < 0.05$) between treatment means.

BC and CO or between BC and the products of CO digestion in terms of incorporation into the mixed micelles.

The efficiency of lipid digestion is determined both by factors which influence the ability of the lipase and colipase to act at the oil droplet surface and by the removal of lipid digestion products from the surface (Porter & Charman, 2001). Experimental conditions will be critically important in influencing these processes. For example, it is possible that BC transfer was limited within the 2 h digestion timeframe or by the ratio of lipid:pancreatic lipase in some of the samples. Furthermore, differences between the treatments in terms of lipid, bile and pancreatin concentration are expected to influence emulsion formation, droplet size and interfacial properties, all of which impact on lipid digestion (Armand et al., 1996) and could influence the transfer of lipophilic molecules to the aqueous, micellar phase.

In conclusion, the influence of varying the conditions used to simulate the upper intestinal digestive environment on the transfer of BC from CO to the aqueous, micellar fraction of digestate was studied. Bile, pancreatin and pH were found to be important and inter-related factors. In the absence of bile, BC transfer to the aqueous phase was minimal, although the presence of pancreatin was relatively less critical. Bile and pancreatin concentration, as well as a more basic pH, were

positively correlated with BC transfer. When experimental conditions were selected to mimic the fed (pH 5.0, pancreatin 0.4 mg/ml, bile 5.0 mg/ml) versus fasted (pH 6.5, pancreatin 2.4 mg/ml, bile 20.0 mg/ml) state of digestion, significantly more BC was transferred (46.5% versus 18.8%). However, within each level of bile and pancreatin studied, by pH 8.0, the same, maximum proportion (~50%) of BC was transferred to the aqueous phase. These findings further emphasizes the inter-related nature of the experimental parameters used in *in vitro* digestion assays and the need to carefully use defined experimental parameters when simulating gastrointestinal conditions for the study of lipophilic molecule transfer to the aqueous phase during digestion.

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