Influence of Digestion Model, Product Type, and Enrichment Level on \textit{in vitro} Bioavailability of Lutein from High Lutein Functional Bakery Products

by
Andrew M. Read

A Thesis
Presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Human Health and Nutritional Science

Guelph, Ontario, Canada
© Andrew M. Read, October, 2011
ABSTRACT

INFLUENCE OF DIGESTION MODEL, PRODUCT TYPE, AND ENRICHMENT LEVEL ON IN VITRO BIOAVAILABILITY OF LUTEIN FROM HIGH LUTEIN FUNCTIONAL BAKERY PRODUCTS

Andrew Read
University of Guelph, 2011

Advisors:
Amanda Wright
Elsayed Abdel-Aal

Lutein is a lipid soluble plant pigment with recognized health benefits, although intake levels by the general population and bioavailability are generally low. These factors have led to interest in producing high lutein functional foods, including baked products. Cookies, muffins, and flatbreads, were produced at three enrichment levels (equivalent to 0.5, 1.0, and 2.0 mg per serving) and then subjected to an in vitro simulation of human gastric and duodenal digestion coupled with Caco-2 monolayers. Lutein transfer to the aqueous phase during digestion (i.e. bioaccessibility) and monolayer absorption were determined as estimates of potential bioavailability. The higher fat products (muffins and cookies) resulted in higher overall bioaccessibility (p<0.05) and absorption at most levels of enrichment. Digestive conditions representative of the fed and fasted state were compared, with the fed model resulting in much higher estimates of bioavailability. Lutein concentration in the aqueous was the most important factor in determining subsequent monolayer absorption.

Overall, the cookie was the most effective product for bioaccessibility, and enriching them to the highest level would result in the greatest delivery of bioavailable lutein to the body.
ACKNOWLEDGEMENTS

I am first grateful to my advisors Amanda Wright and Elsayed Abdelaal, as well as Alison Duncan for their insight, support, and motivation during this project. Further thanks are due to all those who provided technical support: Iwona Rabalski, Amir Malaki Nik, Christine Carey, Magdalena Kostrzynska, and others. Finally, I have to thank all the family, friends, and others who have been helpful and supportive during these years!
# TABLE OF CONTENTS

Abstract ........................................................................................................................................... ii

Acknowledgements ...................................................................................................................... iii

Table of Contents ........................................................................................................................ iv

List of Tables .................................................................................................................................. vii

List of Figures ............................................................................................................................... viii

List of Abbreviations .................................................................................................................... ix

1. Introduction .............................................................................................................................. 1

2. Literature Review .................................................................................................................... 4

   2.1 Introduction to Lutein ........................................................................................................ 4

   2.2 Motivation for Creation of High Lutein Functional Foods ............................................ 5

       2.2.1 Roles for Lutein in Human Health ........................................................................... 5

       2.2.2 Dietary Intake of Lutein .......................................................................................... 7

       2.2.3 Rise of Functional Foods ......................................................................................... 7

   2.3 Existing High Lutein Functional Foods and Prototypes .................................................. 8

   2.4 Lutein Functional Food Production ............................................................................... 10

   2.5 Stability of Lutein .............................................................................................................. 12

   2.6 Analytical Methods for Lutein ......................................................................................... 13

   2.7 Bioavailability of Lutein ................................................................................................. 14
2.7.1 Bioavailability ................................................................. 14
2.7.2 In Vitro Estimation of Bioavailability ................................. 15
2.7.3 Results of Bioavailability and Bioaccessibility Studies ........... 20
2.8 Considerations for Functional Food Optimization .................... 25
3.1 Product Preparation ................................................................ 27
  3.1.1 Flatbread Procedure: ...................................................... 28
  3.1.2 Muffin Procedure ............................................................. 29
  3.1.3 Cookie Procedure: Modified AACC Method 10-50D (92) ....... 30
3.2 In Vitro Digestion .................................................................. 31
  3.2.1 Fed Model ..................................................................... 31
  3.2.2 Fasted Model .................................................................. 33
3.3 Caco-2 Absorption Study ...................................................... 33
  3.3 Lutein Quantification ........................................................... 35
  3.4 Statistical Analysis ............................................................. 38
4. Results and Discussion ........................................................... 40
  4.1 Lutein Content of Products .................................................. 40
  4.2 Expression of Lutein Micellization and Absorption Data ........... 41
  4.3 Influence of Lutein Concentration on Bioaccessibility .......... 43
  4.4 Digestion Model Comparison ............................................... 50
4.5  Comparison of Product Types ................................................................. 52

4.5.1 Bioaccessibility (%)................................................................. 52

4.5.2 Quantity of Bioaccessible Lutein ........................................... 54

4.6 Cell Culture Data ........................................................................... 56

4.6.1 Influence of Lutein Concentration in the Products ............... 61

4.6.2 Comparison of Product Types .................................................. 61

5.  Conclusions ...................................................................................... 65

References Cited .................................................................................. 68
LIST OF TABLES

Table 1: Composition of Digestive Solutions................................................................. 32

Table 2: Lutein content of lutein-enriched bakery products prepared for in vitro
digestions................................................................................................................... 39
LIST OF FIGURES

Figure 1: Chemical structure of lutein.................................................................4

Figure 2: HPLC Chromatograms of dry products..................................................36

Figure 3: HPLC Chromatograms of wet extractions.............................................37

Figure 4: Proportion of lutein present in the products which was transferred to the aqueous phase during in vitro digestion.................................................................43

Figure 5: Absolute micellization for studies using fasted and fed conditions..........46

Figure 6: Proportion of lutein present in the digesta which was absorbed by the cells....57

Figure 7: Lutein present in the digesta introduced to the Caco-2 monolayer which was absorbed by the cells...............................................................58

Figure 8: Percentage of lutein initially present in the products which was absorbed by the Caco-2 monolayer following in vitro digestion and incubation..........................59
LIST OF ABBREVIATIONS

AMD – Age-related Macular Degeneration

ANCOVA – Analysis of Covariance

ANOVA – Analysis of Variance

BHT – Butylhydroxytoluene

DMEM – Dulbecco’s Modified Eagle Medium

DSHEA – Dietary Supplement Health and Education Act

DWB – Dry Weight Basis

FDA – Food and Drug Act (Canada); Food and Drug Administration (United States)

FFA – Free Fatty Acids

FOSHU – Foods for Specified Health Use

HBSS – Hank’s Buffered Salt Solution

HPLC – High Performance Liquid Chromatography

MAG – Monoacylglycerol (monoglycerides)

NHPD – Natural Health Product Directorate

RACC – Reference Amount Customarily Consumed
1. *INTRODUCTION*

Lutein is a member of the carotenoid family of lipid soluble pigments. It has gained recognition as being potentially beneficial to human health, particularly to prevent the development of certain chronic eye diseases, but also more generally as an antioxidant. However, the intake of lutein by the general population is low. Combined, these factors have led to interest in producing high lutein functional foods.

Lutein bioavailability from supplement and functional food matrices is an important consideration. As fat soluble molecules, the carotenoids must be transferred to the aqueous phase during digestion by incorporating into the mixed bile salt micelles. With lipophilic bioactives and micronutrients this transfer process is a limiting step in bioavailability. Direct evaluation of carotenoid bioavailability from foods is difficult as human studies are time consuming and expensive. They also may not permit a mechanistic understanding of the processes occurring during digestion. Therefore, *in vitro* methods for simulating human digestion which have become useful for estimating bioavailability. Specifically, lutein bioavailability can be estimated by determining the proportion of ‘bioaccessible’ lutein – i.e. the proportion of the compound which is transferred to the aqueous phase during an *in vitro* experiment. A number of food products and various lipophilic molecules have been studied using this approach. In some cases, the simulated digestive conditions are coupled with an absorption assay using Caco-2 cells which serve to mimic the intestinal epithelium, providing a way of estimating uptake of a micellized compound.

Recently, there has been interest in bakery products as lutein enriched functional food products due to their popularity and widespread consumption. To this end, previous
work has been done confirming that bakery products can be enriched relatively easily and to confirm the stability of lutein within bakery products. However, little research has been conducted to determine lutein bioavailability from these products. In particular, a comparison of specific product types is also required since it has not been established how food composition and matrix will impact lutein digestion and absorption. The purpose of this research was to evaluate three different bakery products as potential high-lutein functional food matrices using in vitro digestion and Caco-2 cells experiments to estimate lutein’s bioaccessibility and absorptive potential. To this end, flatbreads, muffins, and cookies were prepared with target lutein concentrations of 0.0, 0.5, 1.0, and 2.0 mg per 30 g serving in hopes of achieving a meaningful improvement to individuals’ lutein status according to the results of previous work in the field of lutein health benefits. In vitro digestion procedures representative of the fasted and fed conditions in the upper gastrointestinal tract during human digestion were employed. The aqueous digestate was evaluated for lutein to determine bioavailability. To evaluate the absorption characteristics of the different product types and enrichment levels, samples of the digestate were then incubated with Caco-2 cell monolayers and lutein absorption by the monolayer determined. According to previous work on carotenoid bioavailability and bioaccessibility, it was expected that the higher fat products – muffins and especially cookies – would demonstrate greater lutein bioaccessibility than the flatbreads, which do not contain any baking fat. Additionally, it was expected that the fed conditions would result in greater lipid digestion and as a result greater lutein bioaccessibility than when products were evaluated using the fasted model, as the influence of digestion model parameters has been previously investigated. This research is directed at achieving a
better understanding of the factors influencing lutein bioavailability and identifying preferred product types for high lutein bakery products.
2. LITERATURE REVIEW

2.1 Introduction to Lutein

Figure 1 Chemical structure of lutein

Phytochemicals are compounds obtained from plants which are not necessarily nutrients, but have been highly researched as they have varying and important benefits to human health. Among the most widespread phytochemicals are the carotenoids – 40-carbon pigments with many conjugated double bonds – of which at least 600 have been identified. Carotenoids contain a long conjugated double bond structure in the central portion of the molecule, which confers light absorbing properties. The absorbance of carotenoids peaks at roughly 450 nm, giving them their characteristic yellow and red colours. This also gives them free radical quenching properties, i.e. they can absorb energetic electrons and dissipate the energy by resonance and vibration. In the plant, this activity protects the chromoplast and vital chlorophyll from damage by radicals created during photosynthesis. Carotenoids are separated into two categories: carotenes, which are hydrocarbons (important examples are β-carotene and lycopene), and xanthophylls, which have some hydroxyl substitution (eg. zeaxanthin, lutein) and because of this can be found in pure form or esterified to fatty acids (1). In addition to this variation, many carotenoids including lutein can have one or more cis-bonds within the conjugated
structure, although the all-trans- form usually predominates. β-carotene has gained attention as a nutritional precursor to vitamin A which can be used to improve vitamin A status without the risk of toxicity associated with enrichment of the diet with vitamin A itself (2). The chemical properties of the carotenoids vary slightly by their structure, but all are lipophilic and must be incorporated into mixed micelles in order to be absorbed by the gastrointestinal tract (3). Animals do not have metabolic pathways to synthesize carotenoids; they are obtained exclusively from the diet.

2.2 Motivation for Creation of High Lutein Functional Foods

2.2.1 Roles for Lutein in Human Health

Carotenoids have long been thought to have positive effects in many areas of human health and there has been extensive research in order to determine potential health benefits associated with their consumption. Lutein and zeaxanthin are non-provitamin A carotenoids which may have effects on human health. A review evaluating the evidence for the effects of lutein and zeaxanthin intake on chronic disease development and progression identified key areas where evidence supports a beneficial effect of lutein. These are, specifically, eye disease (particularly age-related macular degeneration, or AMD, and cataracts), immune function, cardiovascular disease, and cancer.

The strongest evidence appears to be for a role in eye disease. Lutein and zeaxanthin are highly concentrated in the macula of humans and primates; concentrations of these compounds are around 1 mM (4). Such an extraordinary concentration of a compound not synthesized locally by the body suggests an important biological function. It is currently thought that these xanthophylls exert a protective role on macular
photoreceptors through the absorption of blue light (the absorption spectrum of these compounds is strong from 400-500 nm) or by a free-radical scavenging antioxidant effect, made possible by their extremely high concentrations (5). The strong absorbance of blue light by xanthophylls could act as a filter, protecting the macula from high energy visible light rays in the same way that external blue light filters protect against AMD (6). AMD is the leading cause of blindness in those 55 and older and is characterized by lesions of the macular retinal pigment epithelium (7). Among the leading risk factors for AMD is exposure to high intensity light, particularly short-wavelength light (7), and xanthophylls such as lutein may protect against this exposure.

The potential protective effects of lutein and zeaxanthin against AMD have been supported in clinical trials (8) and in vitro studies (9). This effect has not been observed for general antioxidant compounds not concentrated in the macula, such as β-carotene and anthocyanins. Negative correlations between lutein intake and nuclear cataract risk have been observed in the literature as well (10). Therefore, the primary role of lutein in human health is probably in ocular protection from high energy light damage. In addition, there is some evidence that increases in dietary lutein by 10-40 mg/d, whether through consumption of foods naturally high in lutein (11) or supplements (12) can improve visual acuity in patients already suffering from ocular diseases such as retinitis pigmentosa and scotomas metamorphosia. Tissue concentrations of lutein, even at high dietary intakes, are probably not high enough for its whole-body antioxidant properties to be significant in comparison to other compounds such as vitamins C, E, or glutathione, which are present at far greater levels in general tissues (13).
2.2.2 Dietary Intake of Lutein

The main sources of carotenoids in the diet are fruits and vegetables – these provide between 70 and 90% of total carotenoid intake. Specifically, yellow squashes, corn, and spinach are rich sources of lutein and zeaxanthin (14), as well as egg yolks. Current intake of lutein is low at 1-2 mg/d for the average person in the North American population (10,15). Prospective studies where the upper quintile intake of the subjects was 4-5 mg/d (16) have failed to find significant effects, or effects were significant only in this top quintile. Clinical supplementation found significant benefits in terms of AMD risk at 10 mg/d lutein in addition to normal dietary intakes (8). There is much more evidence linking carotenoid intake to improved macular pigment density (17-18), but additional research is needed in order to more conclusively link this to improved clinical outcomes. However, it is generally accepted that improved daily lutein intake would improve macular pigment status (17). Research suggests no toxic effects of lutein to major organs in rats at dosages up to 400 mg/kg (19), so enrichment of the diet with high lutein functional foods is considered to be a safe choice for those wishing to manage AMD risk. However, the effects of breakdown products of lutein are unknown; care is thus required to avoid potentially damaging effects of high lutein supplementation as occurred with a study of β-carotene and lung cancer in 1994 (20).

2.2.3 Rise of Functional Foods

Functional foods are an attractive way to improve the status of a given compound in the diet because they can be formulated to contain much higher concentrations than are achieveable when a ‘normal’ diet is consumed. In addition, functional foods are attractive to consumers and may be preferred to purified bioactive compounds or drugs,
as evidenced by the incredible growth of the functional foods market in North America in recent years (21). A recent survey found that Canadian dietitians support the development and consumption of functional foods, as well as allowing manufacturers to place health claims on the labels of such products (22). However, they also recommended that restrictions be in place to ensure the efficacy and safety of such products. As functional foods have risen to prominence in the market, legislation has followed to ensure that such products are properly regulated to protect consumers. These include Foods for Specified Health Use (FOSHU) in Japan, Dietary Supplement Health and Education Act (DSHEA) in the United States, and the Natural Health Product Directorate (NHPD) or more commonly the Food and Drug Act (FDA) in Canada.

Bakery functional food products are a particularly attractive route for delivering bioactive compounds like lutein. They could be made readily available and easily substituted in the diet for their non-functional counterparts since bakery products are a staple in the North American diet (23), and a wide range of product types are available to meet the demands of consumers. Functionalizing baked products also represents an opportunity to increase profits and marketability in the grains and cereals sector (22). Therefore, research towards a series of high-lutein functional bakery food products which could improve the lutein status of the population, and ultimately contribute to health in this manner is underway (24).

2.3 Existing High Lutein Functional Foods and Prototypes

High lutein functional foods in the market are currently relatively scarce. This is likely related to the fact that the US Food and Drug Administration (FDA) has not
currently approved any health claims which may be made for these foods. In 2005, the FDA denied a request by Xangold for a qualified health claim for their lutein supplement product (25). The claim was for risk reduction of cataracts and AMD and was denied due to insufficient scientific evidence (25). Similarly, Health Canada has not approved any health claims for lutein supplementation (26). It is difficult to market functional foods for which no health claims can be made. Despite this, Kemin Industries based in the United States has created a supplemental lutein product called FloraGLO intended to be incorporated by manufacturers into various products. FloraGLO is produced by purification of marigold flower oleoresin extract, resulting in a purified crystalline lutein supplement which is provided in capsules, as a suspension in oil, and in powder form. This lutein is supplied as pure free lutein, rather than esterified lutein. FloraGLO has received GRAS (generally recognized as safe, which indicates that it is permitted for use in food products) status for use at up to 10 mg of lutein per reference amount customarily consumed (RACC) (27). To date, most products containing FloraGLO have been supplements or multivitamins, but Jamieson Laboratories in Canada sells energy and meal replacement bars containing FloraGLO. In the United States, products by Ecco Bella (Health by Chocolate) are lutein enriched using FloraGLO as well. Marketing information for these products contain information regarding the functions of lutein in the body, including macular protection and antioxidant effects, although no specific or general health claims are used. According to the Lutein Information Bureau, products with FloraGLO contain the same crystalline lutein supplement used in the Veterans’ LAST study (8), where benefits of lutein supplementation at 10 mg/d were found.
Therefore, while specific health claim rights have been denied, high lutein products are being produced with some suggestions as to their potential benefits.

2.4 Lutein Functional Food Production

The two routes for producing high lutein foods are by using base ingredients which are high in lutein, or by enriching the foods using a lutein supplement. An important step towards the development of lutein-enriched products was the identification of grain species with high endogenous lutein content. Common wheat has a lutein content of about 2 µg/g dry matter (28). Durum wheat, which is used in pasta for its rich yellow hue, contains 5-6 µg lutein per gram dry matter (28). Einkorn is an ancient wheat variety very high in lutein as well as other phytochemicals and protein (29) and has satisfactory baking properties (29,30). The lutein content varies depending on the region the strain is obtained from, and ranges from 7-12 µg/g dry matter (28,31,32). This has led to suggestions that Einkorn wheat could be used in the production of high lutein functional foods (29,31).

When considering the production of functional foods using pure lutein as a supplement, it is important to consider the best method for physically incorporating the enriching supplement into the product matrix. This is important because it can affect the sensory properties of the product (33) and homogeneous nutrient distribution. The ideal enrichment strategy would result in a product with high consistency of nutrient content, even distribution, and no negative impact on sensory qualities of the product. An example is the enrichment of eggs with lutein, whereby eggs can be enriched up to 2.0 mg lutein per large size egg by enrichment of the chickens’ feed with up to 500 ppm of
lutein (34). Researchers in this case found no impact on egg quality from enrichment, other than that egg yolks were more strongly coloured. As with all eggs, lutein was concentrated almost exclusively in the yolks, with potential implications for bioavailability in the human diet described below (34,35).

In terms of baked products, there are a few strategies described in the literature for enrichment with lutein. One is the use of crystalline lutein in powdered form mixed with the dry material of the product. This is attractive because dry products are easy to store and dispense, but raises concerns as to the distribution of lutein in the products since the lutein is supplied as discrete grains, rather than a continuous medium. Regardless, this method has been used in products which already exist on the market, such as meal replacement bars from Jamieson Laboratories (Canada), and, as such, is presumably acceptable in practice. At any rate, it is an easy method to incorporate into baking procedures. Another method for lutein enrichment is by dispersing lutein in the aqueous or fat phases which represents a more continuous medium in which the lutein can be dispersed before mixing with the product’s dry ingredients, potentially resulting in more even lutein distribution. Distribution in fat may be particularly effective since lutein is fat soluble and thus should be easily dispersed in the liquid fats typically used in commercial baking applications. It would be relatively simple to replace the fat normally used in baking with fat containing lutein supplement. Distribution in water is more difficult to achieve since lutein is a fat-soluble nutrient, but lutein in oil suspensions are available (such as Lyc-O-Lutein, by LycoRed Ltd.) for use, and a suitable emulsifying agent could aid distribution in an aqueous solution. The benefit of this method is that
products with little or no fat can be produced without having to use powdered crystalline lutein supplements, since the lutein-oil suspension is dispersed in water rather than oil.

2.5 Stability of Lutein

It is important when creating functional foods to consider the realities of commercial food production, storage, and sale. Factory-packaged baked products are rarely consumed on the day of production, as fresh breads are, so it is important that enriched nutrients are stable within the food matrix during the time between production and consumption. In addition, baked products are subjected to the intense heat of the baking process, and thermal degradation may be an issue.

All-trans free lutein present in supplements is relatively unstable to heat, and temperatures as low as 60 °C will rapidly induce considerable loss in a benzene solvent system (36); half-life in this system was found to be roughly 30-40 hours. In addition, UV light induces rapid loss of lutein. Myristate esters of lutein are much less vulnerable to degradation (36), although protection with ascorbic acid also seems to be effective for slowing lutein degradation in solution (37). Lutein is degraded by isomerization to cis-isomers upon contact with acids as well as heat and light, and exposure to oxidizing environments causes the formation of epoxy- and apo-carotenoids. The instability of lutein is due, in large part, to the high degree of unsaturation. However, at room temperature, lutein is quite stable in common solvents over a 10 day period (38). The effects of isomerization to other carotenoids (particularly other forms of lutein, such as cis-isomers) on bioactivity and bioavailability are currently unclear.
In food matrices, lutein is similarly stable under normal product storage conditions. This has been studied in a number of different food types, including cheddar cheese (39), yogurt (40), and within enriched bakery products (24). During shelf storage conditions, degradation was negligible in the bakery products for a period of 8 weeks. These products were produced without preservatives, and the incorporation of protective agents such as the fat-soluble antioxidant BHT into commercial products could improve lutein stability even further.

2.6 Analytical Methods for Lutein

Lutein has a maximum wavelength of absorption of 450 nm and can therefore be quantified using spectrophotometry or HPLC with photodetection, following extraction into a solvent solution. As a hydrophobic molecule, lutein readily transfers to organic solvents in preference to the aqueous phase. Lutein is quite stable and soluble in most organic solvents (38), so solvents are chosen based on convenience and miscibility with water. In the literature solvent extractions using hexane, acetone, ethanol, and diethyl ether are commonplace (24,41,42). For reconstitution of dried extracts after extraction of liquid samples, it is best to use a less volatile solvent, such as 1-butanol, to minimize volume drift which can be a problem with volatile solvents. Water saturated 1-butanol is commonly used for extraction of dry products such as milled grain (28,32), although it is interesting to note that this method has been criticized for potentially failing to extract some of the lutein content of durum wheat species (43). Therefore, a new solvent extraction method involving soaking overnight was proposed and shown to improve lutein recovery by HPLC measurement from 42% of photometrically determined yellow
pigment content to 94% (43). These and other studies also highlight the complexity of lutein quantification.

For quantification of lutein, reverse-phase HPLC has an advantage over spectrophotometry in that it is possible to separate similar carotenoids with similar absorbance spectra. When separation of geometric isomers is desired (as with bioactive compounds such as lutein), a C30 carotenoid-specific column is used, with a gradient elution system using methanol and methyl-tert-butyl ether solvents (28,44). For wet extractions, internal recovery standards are used to correct for imperfect extraction efficiency. Researchers studying lutein bioaccessibility have used β-apo-8’-carotenal as a recovery standard to compensate for extraction efficiency (45,46).

2.7 Bioavailability of Lutein

2.7.1 Bioavailability

Bioavailability can refer to the proportion of a given compound, which after administration, is absorbed into the system, or the proportion which accumulates in the target tissue (47). For the purposes of this thesis, bioavailability will refer to the proportion of lutein absorbed by the intestinal epithelium. The determination of carotenoid bioavailability can be difficult, requiring human or animal experiments. Specifically, the ferret (48) and preruminant calf (49) have been suggested as good models for human carotenoid bioavailability studies. Although costly and time consuming, in vivo experiments are the gold standard for evaluation of nutrient bioavailability from foods. However, the approach of serving a meal to humans or animals and measuring the plasma response can be complicated due to wide variations in
metabolism (50) as well as relatively high risk and cost. Such studies also typically do not permit investigation of the digestive processes involved in the release and transfer of carotenoids and which impact bioavailability.

2.7.2 *In Vitro* Estimation of Bioavailability

*In vitro* estimation of bioavailability of lipid-soluble compounds such as carotenoids depends on an accurate model of human lipid digestion. The first significant digestion of lipids occurs in the stomach, where acid-stable lipases hydrolyze 10-30% of lipids. This leads to the generation of amphiphilic products which can contribute to the pool of interfacially active compounds which drive and stabilize the emulsion of fat droplets at the gastric phases and later in the small intestine (51). However, digestion of lipids occurs primarily in the small intestine when bile and pancreatin (composed of a number of different digestive enzymes) are released through the bile duct from the liver and pancreas, respectively (52). Bile salt micelles are created when bile adsorbs to and disrupts dietary fat droplets in the small intestinal lumen in order to improve the surface area and adsorption potential for lipases, which cleave triglycerides into primarily free fatty acids (FFA) and sn-2 monoacylglycerides (MAG) at the lipid-aqueous interface (52,53). The products of lipolysis are absorbed by simple diffusion by the enterocytes as well as by transport by the enterocytes’ membrane proteins (52). Therefore, lipophilic compounds, including the carotenoids, must be incorporated into the mixed bile salt micelles which form during digestion in order to be absorbed by the intestinal epithelium (50,54,55). Bioaccessibility is a term which describes the relative proportion of a compound which becomes available for absorption through this critical micellization step, which seems to be a major factor in determining the overall bioavailability of
lipophilic compounds (50). Therefore, measuring the proportion of carotenoids which are micellized during an *in vitro* simulation of digestion (i.e. bioaccessibility) is one approach to estimate the potential bioavailability of such compounds (56,57). Further, an *in vitro* simulation of digestion coupled with an assessment of absorption and chylomicron secretion characteristics using Caco-2 human intestinal epithelial cells enables determination of an estimate of the potential absorption by the intestinal wall (56,58,59). This approach has become a de facto standard for the estimation of bioavailability of carotenoids from meals (60-67). Due to variability in methodology between studies and differences between the *in vitro* and *in vivo* conditions, these studies are more useful for the comparison of different meal types or compositions in terms of potential bioavailability *in vivo* than determining absolute bioavailability. Despite this limitation, *in vitro* estimation of bioavailability remains a valuable tool because it can be performed relatively quickly and at lower cost and risk than human trials.

Two general methods for the simulation of digestion that are most frequently cited are those published by Garret (56) and Oomen (68). In 1999, Garrett et al (56) published a method for simulating human digestion that is coupled with an absorption assay using a human Caco-2 cell culture to mimic the absorption characteristics of the human intestinal epithelium. The method involves combining the ‘meal’ – a few grams of finely divided or pureed food – with a saline solution saturated with BHT to provide oxidant protection during the digestion procedure, as anoxic conditions are difficult to reproduce *in vitro*. To simulate gastric digestion, this mixture is acidified to pH 2 and porcine pepsin is added to achieve approximately 2 mg/mL enzyme concentration in the digestion mixture. The digestion vessel is an amber glass vessel in order to minimize photodegradation of
carotenoids. The gastric digestion occurs over 1 hour in a shaking water bath at 37 °C to simulate gastric churning. Following this, the digestion mixture is adjusted to pH 5.3 using 0.9 M sodium bicarbonate and a solution of porcine pancreatin and bile extract is added to achieve 0.4 mg/mL pancreatin and 2.4 mg/mL bile in the digestion mixture. Thereafter, the pH is adjusted to 7.5 with sodium hydroxide, the empty space in the jar is filled with argon, and the mixture sealed before returning to the shaking water bath for 2 hours. At this point, the mixture is considered fully digested and similar to the intestinal digesta available for absorption by the intestinal lining. The digesta is ultracentrifuged to isolate the aqueous fraction, since fat that is not micellized will sequester lutein and hinder the validity of the results if it remains. The second part of this method involves introduction of the aqueous fraction of digesta in a 1:3 ratio with cell culture media to the apical surface of a Caco-2 cell monolayer. The cells are incubated with the digesta-media mixture and washed with HBSS containing sodium taurocholate (used to remove any substances adhering to the apical surface of the cells that have not been absorbed). The cells are analyzed for lutein content by scraping them into an aqueous solution (HBSS) and extracted using organic solvents. The digesta is also independently analyzed for lutein content to provide an overview of how lutein is partitioned at each step. Many researchers have adopted this method for estimating bioavailability of carotenoids from various food and meal types (69-71). Some of the results of these studies are described in the next section.

In 2003, Oomen et al (68) updated an earlier \textit{in vitro} method (72) in order to study the bioaccessibility of soil contaminants such as lead. Superficially, the method is similar to that of Garrett et al (56), but includes an oral phase before the gastric digestion,
including the addition of α-amylase. This is an especially important addition for starchy foods because for these products, the initial stages of starch digestion begin in the mouth (73). Another important difference is that the addition of liquid to the digestion occurs at the beginning of each phase of digestion in the form of simulated whole digestive solutions, as compared to the Garrett method in which fluid is primarily added at the beginning of digestion and concentrated enzyme solutions are added only to achieve the appropriate enzyme levels. The simulated digestive solutions consist of more complex salt solutions derived from Documenta Geigy which are representative of physiologic concentrations of important ions and organic constituents. The pH ranges of the oral, gastric, and intestinal phases were 6.5, 1.2, and >5.5, respectively, in an attempt to achieve physiologically relevant conditions for the fasted state (i.e. digestive conditions where the individual has not recently consumed a meal). The fasted state was selected as it represents the worst case scenario for the contaminants examined in that study, i.e. bioaccessibility of heavy metals should be higher under the gastrointestinal conditions (specifically gastric pH) associated with the fasted state (74). The result of these modifications is that the Oomen method is more physiologically accurate, although more complex, than the Garrett method.

Researchers have modified the Garrett and Oomen methods for their own purposes and with the aim of improving the physiological relevance. For example, the Garrett method did not originally include an oral phase so researchers have added the oral method published by Oomen in order to achieve better starch digestion for foods such as cassava (62). Another modification involves replacing porcine bile extract with three pure bile salts, i.e. glycodeoxycholate, taurodeoxycholate, and taurocholate, totaling 2.2
mmol/L. It was claimed that pilot studies showed a substantial and significant improvement in lutein micellization efficiency from 27% to 49% (60) compared with the 2.4 mg/mL (roughly 2.5 mmol/L) bile extract concentration used in the Garrett studies. Of note, such a dramatic difference in micellization efficiency from a seemingly small modification in method creates interest in achieving intestinal digestion conditions which are most representative of human in vivo conditions, in order to avoid over- or underestimation of micellization efficiency. The most physiologically accurate digestion procedure seems to be the Oomen method, modified by the addition of fed-state levels of bile extract and pancreatin. The importance of this was highlighted by research into the impact of different intestinal experimental parameters on β-carotene micellization efficiency in vitro (75). Performing in vitro digestions (intestinal phase only) on a solution of β-carotene in canola oil, highlighted the importance of bile salt concentration in the intestinal phase of digestion. When the amount of bile salt was representative of the fed state (10-20 mmol/L (76)), β-carotene micellization improved dramatically compared with the digestions with lower bile salt concentration. Also, when pancreatin levels in the intestinal digestion were elevated to physiological fed-state levels, micellization efficiency improved even further, although at the highest bile salt concentrations (20 mg/mL) micellization efficiency was consistently high and relatively insensitive to pancreatin concentration. Finally, the influence of pH was examined for digestions in both the fed (2.4 mg/mL pancreatin, 20 mg/mL bile extract) and fasted (0.4 mg/mL pancreatin, 5 mg/mL bile extract) states of digestion. For the fed state, pH values from 5.0-9.5 resulted in similar micellization efficiency, while in the fasted state, pH is much more critical and higher pH values (8.0-9.5) result in micellization efficiency
similar to the fed state. The procedure by Garrett uses a relatively low bile salt concentration (roughly 2.5 mmol/L) and low pancreatin concentration (0.4 mmol/L) representative of the fasted state. The pH for the Garrett intestinal digestion is 7.5; this is higher than the in vivo intestinal pH after a meal (77), but may compensate for the poorer lipid and carotenoid micellization resultant from the low enzyme and bile load. It is worth noting that in human digestion, lipids are normally well digested and absorbed (78), indicating that if sufficient lipids are present to absorb most of a given carotenoid, absorption of those lipid micelles should be relatively good. Therefore, it seems appropriate to pay extra attention to micellization during digestion as it is likely a more critical step to bioavailability (50,79,80). Of note, colonic fermentation and digestion are nearly always overlooked by researchers conducting in vitro bioavailability research on carotenoids. For example, a 2006 study (81) showed that xanthophylls such as lutein are much more efficiently micellized than carotenes in the upper intestinal phase, but during colonic fermentation, the remainder of carotenes which are not micellized may be absorbed.

2.7.3 Results of Bioavailability and Bioaccessibility Studies

There are a number of factors that determine carotenoid bioavailability. In foods examined thus far, carotenoids are quite stable during in vitro digestion procedures; at least 75% of food carotenoids are present in samples of the digested food (46,82-84). The characteristics of the food being tested appear to be the most important factor in determining lutein bioavailability both in vivo and in vitro. For example, pure lutein dissolved in vegetable oil is generally micellized efficiently, i.e. at least 80% of lutein from oil is incorporated into the micelles during an in vitro digestion (60,85). Lutein
contained within egg yolks demonstrates similarly high bioavailability when evaluated in vivo. Eggs are a relatively good source of lutein, and the lutein content of egg yolks can be increased five-fold by feeding the laying hens a high-lutein diet (34,35). More importantly, the lutein contained within egg yolk is more bioavailable in vivo than lutein contained within spinach or a supplement, even when the accompanying meals are otherwise identical in terms of macronutrient content (35). As egg yolks are composed of a complex matrix of lipids and proteins, this demonstrates that lutein dissolved in a lipid matrix is much more easily incorporated into micelles during digestion than lutein in crystalline form or that contained within chromoplasts.

At some point during product creation or digestion it is necessary for lutein to localize to the fat phase in order to be incorporated into mixed micelles. Because lutein dissolved in oil or a complex lipid matrix is highly bioavailable, lutein supplements would be most effective if the carrier were an oil mixture or capsule containing lutein in oil (60,85). Therefore, for the creation of functional foods, it would be beneficial in terms of bioavailability to dissolve lutein in the fat used for baking, or create the product in such a way that the lutein supplement comes into close contact with fat so that it may localize to the fat phase.

The relatively low bioaccessibility of carotenoids from plants is probably the result of their sequestration within chromoplasts and blockage of digestive enzymes by cell walls. Disruption of plant structural features by microfluidization can improve lutein micellization efficiency (67). Cooking and processing operations generally have a positive effect on bioaccessibility and bioavailability of carotenoids from plant sources. Cooking has been shown to improve micellization of carotenes from carrots from 29% to
52% (63). Steaming and microwaving, specifically, generally improve micellization of lycopene and carotenes from vegetables (63,70). However, the effects of cooking on lutein bioavailability are uncertain. Cooking by boiling has been shown to improve micellization efficiency of lutein from spinach, but microwaving led to decreases (45). Furthermore, both cooking methods were associated with much lower cellular transport of lutein to the basolateral compartment of the Caco-2 monolayer (representative of bioavailability) compared with uncooked spinach, regardless of the spinach source (fresh, canned, or frozen) (45). It is unclear why this occurred, although it could be due to the release of surfactant molecules from spinach during cooking, which have been observed to inhibit lipid digestion and adsorption to the aqueous interface by competitive inhibition at the surface of micelles (86,87). This result demonstrates the importance of independently evaluating micellization and absorption steps in order to determine the efficiency of each step.

The lipid content of a food or supplement may also play an important role in determining carotenoid bioavailability. When examined in a pure carotenoid-oil system, increasing the concentration of oil lowered the micellization of lycopene (85), a very hydrophobic molecule. Lutein, which is more hydrophilic due to hydroxyl substitution, is more efficiently micellized with increasing amounts of both oil and cholesterol (85). A suggested explanation for this effect is that lycopene is competitively inhibited from entering the highly hydrophobic core of micelles by other hydrophobic molecules, such as the triglycerides from oil. This effect has only been observed in experiments using oil and pure carotenoid mixtures, rather than experiments involving a whole food matrix as described below.
Whole food and meal studies present a different trend. When bioaccessibility of carrot carotenes (which are not hydroxyl substituted, and thus are of similar hydrophobicity to lycopene) was measured \textit{in vitro}, the addition of oil to the test meal dramatically improved micellization (63), in contrast to the effect seen with pure carotenoids and oil. Another study found that fat composition (in terms of fatty acyl chain length) and quantity had no effect on xanthophyll bioaccessibility from a salad, in contrast to large improvements in bioaccessibility for lycopene and \( \alpha \)-carotene with long-chain fatty acids (triolein vs. trioctanoin) and larger amounts of lipid (64). Micellization of lutein and zeaxanthin from the salad in this study was 50-75\%. Therefore, for optimum bioavailability, it is desirable to incorporate a sufficient amount of lipid into a high lutein functional food, since this will give ample opportunity for lutein to come into contact with lipids as they begin to be digested in the gastric lumen and to stimulate the release of the bio-surfactants, as well as for the generation of lipolysis products which contribute to digestion.

There are relatively few studies reporting on the direct measurement or estimation of bioavailability of lutein from food matrices similar to the baked wheat-based functional foods which are the focus of this thesis. Grain-based food products are high in starch, which makes the oral phase of digestion more relevant than for vegetable or pure lutein systems. Therefore, in 2 recent studies examining carotenoid bioavailability and bioaccessibility from starchy foods (46,62), an oral phase of digestion was included. This consisted of a 10-minute exposure, with no stirring, at pH 6.5, according to Oomen et al (68), with 3000 units of \( \alpha \)-amylase per gram of food being digested. Kean et al (46) examined the bioaccessibility of lutein from maize-based products using this oral
digestion phase in order to determine their potential as a lutein source in the diet.

Micellization efficiency for lutein was high from these products – ranging from 50-80%. This suggests that grain-based functional foods high in lutein have potential as a dietary lutein source which can be digested and absorbed efficiently. Zeaxanthin (a xanthophyll similar to lutein) is also bioavailable from genetically modified zeaxanthin enriched potatoes when examined in human experiments (88). A significant plasma response was observed for zeaxanthin after consumption of the enriched potatoes. It is problematic in the best situations to compare in vivo results to the in vitro studies which predominate in this field, but this result provides evidence that lutein contained within starchy foods can be highly bioavailable, and supports the link between the in vitro and in vivo methods for evaluating bioavailability previously established by initial research in this area (56).

Finally, any property of a food which affects fat absorption can be expected to influence carotenoid bioavailability. For example, it has been suggested that fibre present in fruits and vegetables may limit the bioavailability of lipophilic molecules since the bulk and indigestibility of these substances tends to carry fat through the GI tract without digestion or absorption (3). This would account for the limited bioavailability of carotenoids from these sources, and is supported by research which demonstrates that bioaccessibility of most carotenoids from fruits and vegetables improves when they are subjected to mechanical and thermal processing steps, as mentioned above (63,69,70). Interestingly, in human studies, mechanical processing has been shown to have no effect on the bioavailability of lutein from spinach (89). Similarly, if products contained or were consumed as part of a meal containing artificial indigestible fats such as Olestra, these could sequester lutein and be excreted, with a severe impact on bioavailability. Human
studies have demonstrated that Olestra consumption can greatly decrease carotenoid serum concentration, implying a negative impact on bioavailability since the diet of these subjects was not otherwise altered (90).

2.8 Considerations for Functional Food Optimization

The impacts of different functional food matrices and compositions on lutein bioavailability are important considerations. Based on current research, bakery products appear to be ideal for enrichment with lutein. Starchy products including the maize-based baked products described above have been determined to be acceptable in terms of lutein bioavailability, and tend to be at least as good as fruits and non-tuberous vegetables in this respect (46). Furthermore, most bakery products contain an appreciable amount of fat, which will aid bioavailability. However, there are many different product types from which to choose with different levels of fat, sugar, leavening, not to mention consumer preferences.

Previous work has been completed demonstrating that bakery products (specifically cookies, muffins, and unleavened flatbreads) can be prepared by emulsifying a lutein in oil supplement in water with whey protein, and that lutein is stable in these products enriched to 1.0 mg per serving (24). These products have different composition in ways that could affect bioavailability. The cookies are high fat with moderate sugar content and leavening, muffins are moderate fat with high sugar content and leavening, and the flatbreads have very low sugar and no added fat, with no leavening ingredients. Differences in the ingredients induce differences in the microstructure of the final products (91). In particular, the quantity of lipids which are integrated in the insoluble
gluten phase, as opposed to available as free lipid droplets may have implications for digestibility of those lipids and any lutein which has migrated to the lipid phase during production. Specifically, cookies could be expected to have a greater quantity of lipids present as free droplets due to the higher quantity of lipids present in this product. The degree of leavening may also influence the ability of digestive fluids to saturate the foods in the \textit{in vitro} method due to the open porous structure of baked products. Research is thus required to determine the relative bioavailability of lutein from these different bakery products. While \textit{in vivo} studies are ideal for this purpose, a bioaccessibility study screening these three product types for micellization efficiency and absorption by Caco-2 cells is a reasonable first step towards determining how the different product characteristics might influence lutein bioavailability. Therefore, the present study investigated these properties of the three products selected for testing: flatbreads, muffins, and cookies.
3. METHODS

3.1 Product Preparation

Flour was milled from dehulled Einkorn wheat (chosen for its very high lutein content) on an Udy Cyclone sample mill (Udy Corp, Fort Collins, CO) using a 0.5mm screen, mixed thoroughly, and stored at 4°C.

Enrichment was achieved by addition of a 20% pure lutein in corn oil suspension (Lyc-O-Lutein, LycoRed Corp., Orange, NJ). For all preparations, lutein was mixed with whey protein extract and water mixture in order to achieve uniform dispersion throughout the product. Based on preliminary evaluation of lutein stability during baking, the quantity of lutein supplement was selected to achieve the target enrichment levels of 0.5, 1.0, and 2.0 mg/serving in the finished products. This corresponds respectively to approximately 17, 33, and 66 µg of lutein per gram of product respectively, since Health Canada’s Food Guide to Healthy Eating recommends grain product servings of 30–40 g (23). The three product recipes are below.
3.1.1 Flatbread Procedure:

The flatbread procedure was based on methods used previously (24). One point five grams of salt (Lifestream Sea Salt) and 0.1 g sugar (Redpath) were dissolved in 10 ml water and then combined with 35 ml water and 1 g whey protein isolate (Product #1580, Bulk Barn, Guelph, ON). Lutein supplement (Lyc-O-Lutein, 20% lutein in corn oil) was blended with this mixture using a hand mixer until homogeneous. This mixture was added to flour equivalent to 100 g at 14% moisture. Dough was mixed in 100 g Micro Mixer pin type mixer (National Manufacturing Co., Lincoln NE) for 3 minutes, and water added (up to 10 ml) to achieve an appropriate dough consistency for rolling. Dough was rolled into a ball and transferred to a plastic container with the lid placed on but not sealed. Container was placed in a proofing cabinet (37 °C, 90% humidity) for 1 hour.

Dough was divided into 40 g samples and rolled into rectangular flatbreads of 1.5 mm thickness. Loaves were placed on parchment paper on steel baking sheets and baked in a preheated rotary type oven (National Manufacturing Co., Lincoln NB) at 200 °C for 8 minutes. Loaves were removed from the oven and allowed to cool to room temperature before transfer to sealed plastic bags. All products were used within 24 hours.
3.1.2 Muffin Procedure

The muffin procedure was taken from previous work in this field (24). Flour (100 g, based on 14% moisture), sugar (62.5 g, Redpath), salt (1.25 g, Lifestream Sea Salt), and baking powder (2.5 g, Magic Baking Powder) were mixed together separately for 2 minutes (100 g Micro Mixer, National Manufacturing Co., Lincoln NE). Margarine (23 g, Becel Salt-Free Margarine) was added and mixing continued for 1 minute. Water (85 ml) was mixed with whey protein (2 g, Product #1580, Bulk Barn, Guelph, ON) using a hand mixer, Lyc-O-Lutein supplement was added, and mixing continued until homogeneous. This lutein solution was added to dry ingredients and margarine and this batter was mixed for 2 minutes in Micro Mixer until even and consistent. 30 g of batter was added to miniature muffin paper cups in muffin baking tray. Muffins were baked for 28 minutes in preheated 175 °C rotary oven (National Manufacturing Co., Lincoln, NB) and allowed to cool to room temperature before placing in sealed plastic bags. Muffins were used within 24 hours.
3.1.3 Cookie Procedure: Modified AACC Method 10-50D (92)

Margarine (40 g, Becel Salt-Free Margarine), sugar (40 g, Redpath), salt (1 g, Lifestream Sea Salt), and baking powder (1 g, Magic Baking Powder) were creamed together for 3 minutes in 100 g Micro Mixer (National Manufacturing Co., Lincoln, NB). Water (10 ml) was combined with whey protein isolate (1 g, Product #1580, Bulk Barn, Guelph, ON) and mixed with a hand mixer until combined. Lyc-O-Lutein supplement was added to this mixture and mixed thoroughly until homogeneous. This solution was added to the creamed ingredients in the micro mixer and mixed 1 minute. Flour (100 g based on 14% moisture) was added and batter was mixed for 2 minutes to achieve consistent dough. Dough was rolled to 6 mm thickness and cut into 60 mm diameter cookies. Cookies were placed on parchment paper on a steel baking sheet and baked at 175 °C for 13 minutes, allowed to cool to room temperature, and stored in sealed plastic bags. Cookies were used within 24 hours.
3.2 In Vitro Digestion

All digestion runs were performed in sets of 6 replicates. All enzymes and compounds used for digestions were obtained from Sigma-Aldrich.

3.2.1 Fed Model

The products were subjected to an in vitro digestion procedure adapted from those used by Garrett and Oomen (56,68). Samples of 3.00 g were cut radially from the products in order to achieve optimum uniformity across samples. The samples were broken into small pieces 2-4 mm in size and added to 70 ml amber glass jars with sealed lids. Digestion salt solutions for the entire series of experiments were prepared in one batch; active enzymes were added on the day of digestions and solutions were stirred for 5 minutes with a magnetic stirring bar before being warmed to 37°C in an incubator for use. Table 1 details the composition of the solutions. The oral phase of digestion was simulated by the addition of 10 ml of salivary solution. The pH was adjusted to 6.5 by dropwise addition of 1M HCl or 1M NaOH. The mixture was mixed using a homogenizer (Ultra-Turrax, Ika T18 Basic) for 45 seconds at 12000 rpm, and the homogenizer probe was rinsed with an additional 2 ml of salivary solution. Digestion jars were placed in a shaking water bath (New Brunswick Scientific Co., Inc., NJ) at 37°C for 5 minutes at 250 rpm.

The gastric phase was simulated by addition of 10 ml of gastric solution with adjustment to pH 3 and shaking for 1hr in a 37 degree water bath. Gastric pH varies with food intake (after a 1000 kcal meal it is close to 5.0, and returns exponentially to the fasting pH of 2.0 over the next 90-100 minutes) (93), so this is an approximation of
gastric conditions during digestion. Intestinal digestion was simulated by addition of 20 ml of intestinal solution. Final concentrations of pancreatin and lipase in the digestate of 2.4 and 0.4 mg/ml, respectively, are based on expected concentration of these intestinal enzymes following a meal, and are consistent with previous work in the area (75). Additionally, 10 ml of bile solution was added to achieve 12 mg/ml concentration of porcine bile extract in the digestion. This is also consistent with expected concentration in the fed state (75,94). Samples were adjusted to pH 6.0 and returned to the shaking water bath for 2 hours. Following the intestinal phase of digestion, separation of the aqueous phase containing the mixed bile salt micelles was achieved by ultracentrifugation at 144 000 g at 7°C for 1 hour. Samples of the aqueous phase were immediately filtered through a 0.22µm nylon syringe filter to remove crystalline carotenoids, and stored in glass vials at -80°C.

**Table 1 - Composition of Digestive Solutions**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Salivary</th>
<th>Gastric</th>
<th>Intestinal</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constituents</td>
<td>20mM KCl 4.0mM KSCN 15mM NaH₂PO₄ 10mM NaCl 3.6mM NaOH 6.7mM urea</td>
<td>88mM NaCl 4.5mM NaH₂PO₄ 18.4mM KCl 5.4mM CaCl₂·2 H₂O 11.4mM NH₄Cl 156mM HCl</td>
<td>224mM NaCl 81mM NaHCO₃ 1.2mM KH₂PO₄ 12.6mM KCl 0.95mM MgCl₂ 2.7mM CaCl₂·2 H₂O</td>
<td>168mM NaCl 138mM NaHCO₃ 28.4mM KCl 3.6mM HCl 3mM CaCl₂·2 H₂O</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.3 mg/ml porcine α-amylase</td>
<td>4 mg/ml porcine pepsin (Sigma) 12.6 mg/ml pyrogallol</td>
<td>6 mg/ml porcine pancreatin 1 mg/ml porcine lipase 5 mg/ml phosphatidylcholine</td>
<td>60 mg/ml porcine bile extract</td>
</tr>
</tbody>
</table>
3.2.2 Fasted Model

All saline solution used in the fasted model is 9 g/L NaCl, saturated with BHT. Digestions were carried out in 70 ml amber glass jars with sealed lids.

4 g of product was broken into small pieces (1-2 mm) and combined with 9 ml of saline solution containing 2.9 mg α-amylase and homogenized for 45 seconds at 12000 rpm (Ultra-Turrax, Ika T18 Basic). Jars were placed in a 37 °C shaking water bath for 5 minutes to complete the oral phase of digestion.

For the gastric phase of digestion, 12 ml of saline containing 40 mg of pepsin was added, and the mixture was acidified to pH 2 with 1 M HCl (approximately 1-1.5 ml). Jars were placed in the shaking water bath for 1 hour to complete the gastric phase. Concentration of pepsin during the gastric phase was 1.8 mg/ml.

The intestinal phase was simulated by the addition of 0.9 M NaHCO₃ to adjust the pH to 6.5 (approximately 1 ml), 20 ml of saline containing 20 mg porcine pancreatin, and 10 ml of saline containing 120 mg of porcine bile extract. Concentrations of pancreatin and bile extract during the intestinal phase of digestion were roughly 0.4 mg/ml and 2.4 mg/ml, respectively. The mixture was readjusted to pH 6.5 with 0.9 M NaHCO₃ or 1 M HCl before jars were sealed with parafilm and returned to the shaking water bath for 2 hours. After the intestinal phase, digesta was centrifuged and stored at -80 °C until further analyzed.

3.3 Caco-2 Absorption Study

*In vitro* absorption of micellized lutein was assayed using ATCC Caco-2 cells, HTB-37 (Cedarlane Laboratories Ltd., Burlington, ON) Cells were grown from frozen
aliquots. A vial containing HTB-37 cells was thawed in a 37 °C water bath for 5 minutes. Contents were suspended in 10 ml Caco-2 cell culture media (containing low-glucose DMEM plus 1% amino acids) and centrifuged at 2400 rpm for 3 minutes. The supernatent was discarded and the pellet was suspended with 5 ml of fresh media. The entire aliquot of media containing cells was dispensed into T-25 vented cap polycarbonate flask and 15 ml of additional media was added. Cells were then grown to a confluent monolayer at 37°C and 5 % CO₂. Upon reaching confluency cells were subcultured. Upon reaching confluency for the second time cells were considered ready for plating. Thereafter, T-25 flasks were used for maintenance of the cell line, and 12-well polycarbonate plates were used for absorption assay.

Cells were subcultured every 3-4 days by removal of cell culture media and pipetting 5 ml of 0.25% trypsin in DMEM, followed by incubation for 8 minutes. 5 ml of cell culture media was added and cells were centrifuged for 3 minutes at 2400 rpm. Cells were suspended with 5 ml of culture media and used to seed a new T-25 flask as well as 1 or 2 plates. Cell density was measured by haemocytometer and trypan blue staining was used to ensure cell viability. Density was adjusted to 1.25 x 10⁵ cells/ml. Flasks were seeded with 20 ml and plates were seeded with 1 ml per well. Plates were incubated for 25 days before use in absorption assay, with media changed (1 ml per well) every 3 days.

The assay was performed using 5 of the 6 replicates from the in vitro digestion. Each replicate was tested in 2 wells of a twelve-well plate of Caco-2 cells. To perform the absorption assay, the filtered aqueous fraction of the digestate was thawed in a 37°C water bath and combined 1:3 with culture media. This mixture was incubated at 37°C for 15 minutes. Media was aspirated from the Caco-2 cell monolayer were and the wells
rinsed with 1 ml per well with HBSS. 1 ml of the digestate-media mixture was gently
layered onto the Caco-2 monolayer and incubated for 4 hours. Each well was then rinsed
with 1 ml HBSS containing 5 mM sodium taurocholate, overlaid with 1 ml plain HBSS,
and frozen at -20 °C. Because of difficulty with cell destruction by enzymatic action of
the digestate, two wells from each plate were used to measure cell viability after the
incubation period. One was overlaid with 1 ml of plain cell culture media, and the other
with 1 ml of 1:3 digestate and culture media mixture. These wells were stained for 5
minutes with trypan blue after the incubation period, rinsed with HBSS, and number of
dead cells in 3 microscope fields (100x magnification) was recorded. The cell culture
wells treated with digesta and the control wells not treated with digesta were not
significantly different in terms of cell survivability (p=0.969). Qualitatively, there also
were no differences between the two treatment groups in terms of cell morphology.

For lutein analysis, tissue culture plates were thawed in a 20 °C shallow water bath.
Cells were removed from the bottom of the wells using a cell scraper, and the HBSS
containing Caco-2 cells was transferred to a 15 ml glass culture tube for extraction by the
same method as the digestate, except the first 2 ml aliquot of extraction solvent was used
to rinse the culture well before extracting the HBSS/cell mixture.

This Caco-2 assay was incompatible with the fed digestion model (see 4.6).

3.3 Lutein Quantification

All measurement of lutein was by HPLC. HPLC parameters and methods were as
established previously (24). Solvent extractions were as follows.

35
Chromatograms representative of dry samples are shown in Fig 2. For dry samples (flour and baked product), 0.5g samples were mixed with 10 ml of water saturated butanol and homogenized (Polytron PT 10-35) for 45 seconds. The material was allowed to rest for 1 hour to allow extraction of the lutein, and homogenized again for 45 seconds. The samples were filtered with 0.45µm nylon syringe-mounted filters before HPLC analysis.

**Figure 2** HPLC Chromatogram of dry product extract. Chromatograms are for unenriched (A) and enriched (B, 1.0 mg per serving) cookies. Peaks are labeled as follows: tL, all-trans lutein; cL, cis- isomers of lutein; Z, zeaxanthin.

Chromatograms representative of wet samples are shown in Fig 3. For wet samples (digestate and cells from absorption study), 0.5 ml samples were added to 0.1 ml of a 2.38 mg/L solution of β-apo-8’-carotenal in acetonitrile in a 15 ml glass culture tube, vortexed 10 seconds at a setting of 5 (Fisher Vortex Genie 2), and extracted three times.
with 2 ml of a 49/25/25/1 mixture of hexane/ethanol/acetone/1-butanol, with 10 seconds of vortexing per extraction. The organic fractions from each extraction were pooled by and evaporated under a stream of nitrogen (N-EVAP, Organomation Associates Inc., Berlin, MA) until just dry. The residue was reconstituted with 0.5 ml 1-butanol and vortexed 1 minute at setting 5 to ensure complete dissolution of lutein, filtered at 0.45μm with nylon syringe filters, and transferred to an HPLC vial for analysis.

**Figure 3** HPLC Chromatogram of wet extract. Chromatograms are for extracted digesta obtained from unenriched (A) and enriched (B, 1.0 mg per serving) cookies. Peaks are labeled as follows: tL, all-trans lutein; αC, β-apo-8'-carotenal (extraction standard).

HPLC analysis of lutein was carried out on an Agilent 1100 HPLC system with a quaternary pump and diode array detector (Hewlett-Packard Co., Palo Alto, CA). HPLC parameters were as follows: 4.6x100mm C30 reverse-phase column (Waters S-3 carotenoid column), 1.0 ml/min flow rate, solvents: A – 85% methanol, 11% methyl tert-butyl ether (MTBE), 4% nanopure water, B - 90% MTBE, 10% methanol. Solvent
profile begins with 100% solvent A, gradually changing to 90% solvent B over the first 12 minutes duration. This is followed by 1 minute at 100% solvent B, 1 minute of gradient from 100% B to 100% A, and 1 minute at 100% A. Detection of carotenoids was by photodiode array detector at 450 nm (bandwidth 8 nm). The elution time for lutein is approximately 5.2 minutes in this system. Data was recorded using Agilent ChemStation software. Software integration for lutein peak area was used for evaluation of total lutein content according to the standard curve established previously (24).

3.4 Statistical Analysis

Statistical analyses were performed using IBM SPSS 19 (for ANCOVA and bivariate regression) and GraphPad Prism 5 (univariate regression and ANOVA with Tukey’s post-hoc tests).

For single product type comparisons across enrichment levels, ANOVA analysis was performed, grouping by target enrichment level (i.e. 0.0, 0.5, 1.0, 2.0 mg per serving), and using post-hoc Tukey’s pairwise comparisons to identify significant differences in lutein micellization (by proportion of initial lutein as well as by absolute lutein micellization), as well as in lutein absorption by the Caco-2 monolayer.

For comparisons across product types, products were not subgrouped by enrichment level as these were not identical between different product types (i.e. the actual measured levels of lutein in the products were not exactly the same as the target levels, see Table 1). Therefore, analyses were performed taking into account lutein concentration as a continuous, rather than categorical variable. ANCOVA and bivariate linear regression analyses were chosen to meet this consideration. Bivariate regression models were generated using product fat content by dry weight and product lutein
concentration as independent variables. These analyses were performed for the fasted and fed model for micellization efficiency (proportional micellization as well as absolute micellization) as well as for the Caco-2 absorption assay (proportional absorption and absolute absorption).

To compare the two different digestion models in terms of micellization efficiency, separate ANCOVAs were performed for each product type, with the lutein content of the products used as a covariate.

The different product types were compared using a separate ANCOVA test for each digestion model, with product lutein content as a covariate. In order to more closely examine the relationship between product fat content and micellization efficiency, bivariate linear regression analysis was performed on the complete fed state digestion data set, with product fat and lutein contents as independent variables.
4. RESULTS AND DISCUSSION

4.1 Lutein Content of Products

The lutein content of the baked products tested are shown in Table 2.

<table>
<thead>
<tr>
<th>Target Enrichment</th>
<th>Non-enriched</th>
<th>Enriched to 0.5 mg per serving</th>
<th>Enriched to 1.0 mg per serving</th>
<th>Enriched to 2.0 mg per serving</th>
</tr>
</thead>
<tbody>
<tr>
<td>Products prepared for fasted state digestion</td>
<td>17 µg/g</td>
<td>33 µg/g</td>
<td>66 µg/g</td>
<td></td>
</tr>
<tr>
<td>Cookies</td>
<td>3.62 ± 0.0382a</td>
<td>23.29 ± 0.724b</td>
<td>22.03 ± 1.14b</td>
<td>81.48 ± 3.60c</td>
</tr>
<tr>
<td>Muffin</td>
<td>2.73 ± 0.0880a</td>
<td>18.75 ± 1.72a</td>
<td>18.18 ± 1.85a</td>
<td>65.70 ± 1.86a</td>
</tr>
<tr>
<td>Flatbread</td>
<td>4.41 ± 0.0747a</td>
<td>14.37 ± 0.539b</td>
<td>22.75 ± 1.18c</td>
<td>64.62 ± 4.00d</td>
</tr>
<tr>
<td>Products prepared for fed state digestion</td>
<td>N/D</td>
<td>35.18±2.52b</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>Cookies</td>
<td>2.18 ± 0.108a</td>
<td>N/D</td>
<td>36.47±1.37b</td>
<td></td>
</tr>
<tr>
<td>Muffin</td>
<td>1.82 ± 0.0548a</td>
<td>N/D</td>
<td>18.28±0.979b</td>
<td></td>
</tr>
<tr>
<td>Flatbread</td>
<td>6.36 ± 0.590a</td>
<td>N/D</td>
<td>18.28±0.979b</td>
<td></td>
</tr>
</tbody>
</table>

Values are µg of all-trans lutein per gram of product, ± SD; n = 4. Within each row, different superscript letters denote values significantly different by ANOVA followed by Tukey’s post-hoc analysis (p<0.05). 0.5 mg per serving and 2.0 mg per serving values not determined for fed state conditions. Target lutein content is based on 30 g servings.

The products ranged in lutein content from ~ 2 – 81 µg/g, corresponding to roughly 0.06-2.4 mg per serving, assuming 30 g servings. As expected, the non-enriched products contained minimal lutein. What was present was contributed by the Einkorn flour which is relatively high in lutein. The significant differences between the non-enriched products are attributable to differences in flour fraction, as flour is the main lutein source in these products. Of note, in many cases, the lutein content of the enriched products was less than what was intended. This can be related to heat-induced destruction of lutein (24,37), but possibly also because of inconsistencies in the lutein distribution from the supplements. It is important to note that some of the lutein loss during baking was due to isomerization to other carotenoids – primarily cis- isomers. These can be seen in the HPLC traces (Fig 2). During pilot studies, although loss of all-trans lutein during baking was 30-40%, the total carotenoid content of the products was quantified as well.
Overall carotenoid loss was roughly 15%, indicating that much of the all-trans lutein loss was due to isomerization, the biological effects of which are unclear. Products in this study were subjected to the digestion procedure within 2 hours of being produced in order to minimize losses due to degradation. This decision was also made in order to minimize any structural or other chemical changes within baked products which might occur during storage, including retrogradation of gelatinized starch granules (95). In future studies, it would be wise to evaluate and standardize products for lutein content prior to performing the digestions to simplify the statistical analyses and interpretation (see section 3.4 for a description of these). In this study it was not possible to do so because products were utilized for the digestion procedure within 2 hours of being produced in order to prevent any degradation of the products.

4.2 Expression of Lutein Micellization and Absorption Data

Analysis of both lutein micellization and Caco-2 cell absorption of lutein was done first proportionally to the lutein present in the foods prior to digestion (i.e. bioaccessibility is expressed as % of original lutein transferred to the aqueous phase of the digestate samples). This is consistent with how results from these types of studies are generally presented in the literature (56,63,66). It is an effective way to compare different products in terms of how bioaccessibility is expected to differ between products such as for comparisons between different cooking methods or different meal compositions.

This basis of comparison makes most sense when considering unenriched foods where lutein content is fixed. However, for enriched products, it is more meaningful to compare the products based on the absolute amount of lutein absorbed since the products may be
enriched to various levels intended to deliver enhanced amounts of bioavailable lutein. For the purposes of this discussion, both lutein transfer to the aqueous phase will be discussed both in terms of proportional and absolution amounts.
Figure 4 Proportion of lutein present in the products which was transferred to the aqueous phase during *in vitro* digestion. ANOVA and Tukey’s post-hoc test results displayed for fasted digestion data; different letters represent significantly (p<0.05) different results. Combined product type graph (A) represents fasted model data only, whereas B, C & D show fasted and fed model results for flatbread, muffin, and cookies respectively. Data are means ± SEM, n = 6.
Figure 4 shows the proportion of lutein which was transferred from the products to the aqueous phase during the *in vitro* digestion procedures (i.e. lutein bioaccessibility).

Bioaccessibility has become a common basis for studies of lipophilic bioactives, including the carotenoids. For all product types, micellization efficiency was negatively influenced by increased lutein concentration, both within the fed and fasted digestion models. When products were digested using fasted conditions, all the non-enriched products had significantly greater lutein bioaccessibility than the 1.0 and 2.0 mg per serving products (*p*<0.05). With fed conditions, all product types had significantly lower bioaccessibility (*p*<0.05) in the enriched (1.0 mg/serving) products in comparison to the non-enriched products. Values varied depending on product type and enrichment level, but agree reasonably well with similar studies in the literature. Kean et al (46) examined the bioaccessibility of lutein from unenriched (< 3.0 µg/g) maize based products with 10% fat content by weight, using a model of digestion similar to the fed model used here. Bioaccessibility ranged from 46% for extruded puffs to 85% for breads. Comparing with the unenriched cookies and muffins from this study, it appears that these wheat-based products are comparable in bioaccessibility, but bioaccessibility is lower when the products are enriched as discussed later. For reported studies where a fasted model was used, bioaccessibility results are variable but within the range of 20-50% for meals containing vegetables and towards the higher end of this range where fat was present as part of the meal (56,62,63,64,84). Note that all of these studies examined unenriched foods whose lutein concentrations were far lower than the enriched products evaluated here, i.e. lutein concentrations were in the range of 1-6 µg/g, compared with 2-81 µg/g
for this study. The results from the present study are slightly lower, as will be discussed below.
Figure 5 Absolute micellization for studies using fasted and fed conditions. Data represented as μg of lutein transferred to the aqueous phase of digestion per gram of product introduced to the in vitro digestion procedure. ANOVA and Tukey’s post-hoc test results displayed; different letters represent significantly (p<0.05) different results. Combined product type graph (A) represents fasted model data only, whereas B, C & D show fasted and fed model results for flatbread, muffin, and cookies respectively. Data are means ± SEM, n = 6.
Different trends were observed when product comparisons were made on the basis of absolute lutein transfer to the aqueous phase (Fig 5). In this case, a higher product lutein concentration was correlated with a higher total lutein being transferred to the aqueous phase of the digesta (p<0.05). Pairwise t-tests (with Tukey’s post-hoc correction) for the fasted conditions revealed that at 1.0 and 2.0 mg/serving, the quantity of lutein transferred was higher than for the non-enriched products. The fed model results were similar, i.e. for all products, the enriched products (1.0 mg/serving) had significantly more lutein transferred to the aqueous phase of digesta. However, the fed model resulted in much higher total lutein transfer at equivalent enrichment of 1.0 mg/serving (the result of higher micellization efficiency). This is expected given the presence of additional bile salts, phospholipids and lipid digestion products with the fed model, all of which contribute to the formation of mixed bile salt micelles and possibly other aqueous phase structures which solubilize lutein (15,51). Since foods are complex matrices and are typically consumed as part of mixed meals, utilization of conditions approximating fed state conditions is advisable. According to these results, although lutein bioaccessibility decreased with increasing enrichment, the total lutein available to the intestinal wall for absorption was increased in products enriched to a greater level. This is an important distinction which is not always made in the literature where discussions of bioaccessibility % (i.e. the proportional transfer) dominate. Also, the fact that more total lutein (g) was incorporated within the aqueous phase from products with higher enrichment levels also indicates that the solubilization capacity of the mixed micelles is not limiting in the model utilized, even in the case of the fasted state experiments. Pilot experiments were conducted using the same pancreatin concentration,
but much higher (i.e. 3x) lipase concentration. The results for lutein transfer were the same (data not shown). This supports that lipolysis and the presence of lipid digestion products were not the critical determinant of differences in lutein transfer between the fed and fasted models. In terms of the potential for the most highly enriched products, the most relevant observation is that the highest lutein content in the digesta was reached for products with the highest lutein enrichment levels (Fig 5).

Regression modeling was performed to explore the relationships between product lutein content and lutein transfer. Lutein was included as a continuous variable in the statistical analysis because product enrichment levels were not exactly the same, even within either the fed or fasted state analyses (Table 2). This was especially important when the influence of lutein concentration on micellization efficiency was studied, since variations in product lutein concentration affected bioaccessibility values. In total, five regression analyses were performed for the bioaccessibility data (i.e. Equations 1-4 below) and two for the cell culture studies (i.e. Equations 5 & 6 as shown in Section 4.6.2).

**Equation 1:** Lutein Bioaccessibility (fed, %) = 42.865 - 0.570 * LUT + 1.328 * FAT, 
\( R^2=0.62, \ p<0.01 \) for all terms

**Equation 2:** Absolute Lutein Transfer (fed, µg/g) = -1.980 + 0.424 * LUT + 0.184 * FAT, 
\( R^2=0.90, \ p<0.01 \) for all terms

**Equation 3:** Lutein Bioaccessibility (fasted, %) = 11.712 – 0.149 * LUT + 0.304 * FAT, 
\( R^2=0.37, \ p<0.01 \) for all terms
**Equation 4**: Absolute Lutein Transfer (fasted, µg/g) = -0.422 + 0.055 * LUT + 0.093 * FAT, $R^2$=0.69, LUT and FAT p<0.01, intercept p=0.107.

Equations are based on the proportional (for Equations 1-2) and absolute (for Equations 3-4) lutein transfer to the aqueous phase for the fed and fasted models separately. In each Equation, LUT represents the lutein content of the products (µg/g) and FAT represents the dry weight percentage of fat in the product recipe.

The effect of lutein concentration on bioaccessibility can be seen in the Equations 1 and 3. A negative influence of increasing product lutein concentration on percentage bioaccessibility is apparent. This suggests a saturation effect, whereby a limited quantity of the mixed bile salt micelles – or some other factor- is limiting the lutein transfer to the aqueous phase. The fasted digesta contained lower micelle forming compounds including free fatty acids and monoglycerides, in addition to the lower bile salt concentration. However the effect of lutein concentration was also significant (p<0.05) in the fed model (Equation 1), suggesting another factor may be at play.

In contrast to this result, Thakkar et al (62), using bile salt and pancreatin concentrations similar to those of the fasted model in this study found that β-carotene bioaccessibility was similar from cassava, regardless of carotenoid concentration. The β-carotene concentrations in the cultivars of cassava studied were relatively low at 0.0-6.9 µg/g, so the effect of higher carotenoid concentration on micellization may have been undetectable in this experiment. Also, β-carotene is relatively more hydrophobic than the xanthophyll lutein studied here and these molecules are known to partition differently within oil droplets (96) resulting in differences in bioaccessibility (56,97). β-carotene does not have the polar hydroxyl substitutions of lutein, which could increase its affinity
for the highly nonpolar cores of micelles rather than competing for space toward the periphery with other products of lipid digestion. Finally, bile and pancreatin concentrations in the Thakkar study (62) were similar to the fasted state, while the analysis discussed here are based on both fed and fasted data. These differences could account for the difference between β-carotene and lutein in terms of micellization efficiency, as their concentration increases relative to micelle quantity.

4.4 Digestion Model Comparison

To compare the fed versus fasted digestion models in terms of bioaccessibility, separate ANCOVAs were performed for each product type, with the lutein content of the products treated as a covariate. All three ANCOVAs revealed significant influences of digestion model and product lutein content (p<0.001). For each of muffins, cookies and flatbread (at the 1.0 mg per serving enrichment level), micellization efficiency and absolute lutein transfer were significantly higher (p<0.001) with the fed state conditions. The 0.5 and 2.0 ppm lutein enrichment levels could not be compared because these products were not subjected to the fed digestion procedure.

These results were as predicted and in agreement with previous findings for β-carotene where different intestinal digestion experimental parameters were evaluated (75). When the fasted intestinal digestion procedure was complete, including sample ultracentrifugation, undigested droplets of fat were evident for the muffins and cookies. The amount of fat remaining was estimated by visual comparison with equivalent amounts of water in identical centrifuge tubes, Accordingly, roughly 50-100 µL and 100-200 µL undigested lipid remained after 2 h intestinal phase digestion of the muffins and
cookies, respectively. Therefore, the low micellization efficiency observed is probably at least partly related to lutein being retained in the undigested fat when fasting conditions were utilized. As tested in pilot studies, no fat droplets were present after digestion using fed state conditions. This can be attributed to the higher concentration of bile salts aiding in fat emulsification and possibly lipolytic activity, as well as the higher lipase concentration in the fed model. Other researchers have found that using higher bile salt concentrations substantially increases measurements of carotenoid bioaccessibility (56,60,75). These two factors result in higher amounts of amphiphilic lipid digestion products such as free fatty acids and monoglycerides which contribute to the formation of aqueous phase micelles. These findings indicate that the differences between the fasted and fed models are considerable enough to question the relevance of using fasted conditions when relatively high fat products are being evaluated. Because the flatbreads contained a negligible amount of fat from the corn oil in which the lutein was suspended, this was not a concern.

Despite the results of this study, pure lutein in oil systems have been evaluated using similar methods as here, with bioaccessibility results being higher than measured here - even with fasted conditions. Bioaccessibility values in the range of 60-90% have been observed (66,85), in contrast to the values of 3-30% observed in this study. However, these studies did not mention that residual lipid droplets were present so the quantities of oil used may have been within the lipolytic capacity of the fasted digestion model.

The large difference in micellization between the fed and fasted conditions also indicates it is inappropriate to compare results obtained using the different conditions. It
highlights the challenges in comparing results from different studies. In recognition of this, there have been recent attempts to better understand the influence of in vitro digestive modeling conditions on the results obtained and to propose standard methods and approaches to publishing methods (65,75). Therefore, for physiologically relevant estimation of lutein bioavailability, and if the results are to be compared with those observed in other studies, it is important to use an appropriate digestion model for the products being tested and the circumstances under which the products are likely to be consumed. Since lipid digestion is generally complete in healthy individuals (78), it is most appropriate to select experimental conditions which allow for complete digestion of lipids. This also suggests that fed state conditions should be more physiologically relevant, although, pilot studies should also be conducted to ensure this condition is met.

4.5 Comparison of Product Types

4.5.1 Bioaccessibility (%)

The different product types were compared using a separate ANCOVA test for each digestion model (i.e. fed and fasted), with product lutein content as a covariate. Product lutein content had a significant (p<0.001) influence on bioaccessibility (%). In terms of absolute lutein transfer, lutein content had a significant positive influence (p<0.05). The three product types had significantly different (p<0.001) results in terms of lutein bioaccessibility. Under fed state conditions, micellization efficiency from the products followed a very clear trend, i.e. bioaccessibility was the lowest for the fat-free flatbread (mean 22.99% lutein transferred to aqueous phase), followed by the muffin (mean 37.93% lutein transfer, 12 % fat dwb), followed by the cookie (mean 55.95% lutein transfer, 22% fat dwb).
In order to more closely examine the relationship between product fat content and micellization efficiency, bivariate linear regression analysis was performed on the complete fed state digestion data set, with product fat and lutein contents as independent variables (see Equation 2). The strong significance of each term in the model (p<0.01 for all terms) indicates that lutein and fat content of the products were both strongly correlated with bioaccessibility. The R-squared value for this analysis was 0.62, indicating that not all of the variability can be explained by the lutein and fat contents of these products using a simple linear model. A more complex model may better explain the observed variation, but other differences in product composition (please see product recipes in methods section) could also be contributing to the remaining variability. For example, the mass fraction of flour was different among these products, and the protein and starch present in the flour may have had an influence on micellization efficiency. Changes in fruit pectin structure during ripening have been observed to affect micellization efficiency for β-carotene (71), and differences in baked product microstructure may similarly affect micellization in this case. However, fat content was statistically the strongest predictor of bioaccessibility among the product characteristics, and is supported by the literature (60,63,64,66,85) as a likely explanation for the different lutein micellization observed between the cookies, muffins and flatbread.

The bioaccessibility data for the fasted experimental conditions was more difficult to analyze in terms of product type. Variability was generally higher and trends were less easily recognized compared with the fed model data, as discussed below. ANCOVA analysis was performed as for the fed model, and revealed significant contributions (p<0.05) from lutein concentration in the products and from the product types.
4.5.2 Quantity of Bioaccessible Lutein

When absolute lutein transfer to the aqueous phase was modeled instead of bioaccessibility, the bivariate linear models obtained were quite different. The fed digestion conditions are modeled in Equation 2. Total lutein transferred in this case refers to µg of lutein micellized per gram of product introduced to the digestion model. When total micellized lutein was examined in the fed model, product lutein content stands out as the strongest predictor of lutein micellization. This is due to its large coefficient: 0.484 compared with 0.184 for FAT, as well as the numerically higher value which LUT has in the experiments performed presently. Lutein concentrations are as high as 81 µg/g, while fat content does not exceed 22%. Since total micellized lutein is the best indicator of how much lutein could be available by absorption by the enterocyte, a high product enrichment level does seem to be a reasonable approach. In other words, the somewhat lower bioaccessibility of the lower fat products (even with the fed model) can be partly offset by increasing the lutein content of these products (see Fig 5). This is particularly relevant in light of recent market developments. The lutein supplement FloraGLO has GRAS status and is permissible for use at up to 6 mg per reference amount customarily consumed (RACC) in cookies, i.e. roughly 30 g (27) and this could probably be extended to other baked products such as muffins and flatbreads. As the quantities of lutein involved are very small in comparison to the primary baking ingredients, there is little to suggest that enrichment would have an impact on the qualities of the products at this level – roughly three times the highest level examined here.

Regression analysis for the fasted model bioaccessibility data is modeled by Equation 3. Although the trends were the same as for the fed model, the coefficients are
much smaller than for the fed model, and the model fit is not very strong ($R^2 = 0.372$). As expected, bioaccessibility was generally lower with the fasted model, as described previously. However, even considering the overall bioaccessibility with this model, the contribution of the FAT coefficient is very low. The fact that fat contributes less in this model supports the hypothesis that lutein micellization is negatively influenced by sequestration of the carotenoid in undigested fat, more of which was present in the higher fat products. It is apparent that the high fat content of the cookies is impairing lutein bioaccessibility in our *in vitro* model, in comparison to the muffins. Bioaccessibility was significantly higher from the muffins versus cookies at the 0.0 and 2.0 mg/serving levels, (p<0.01). It is interesting that the trend was not maintained at 2.0 mg/serving lutein enrichment and that the relationship was stronger at high and low lutein levels. This could be an artefact of incomplete fat digestion in some samples, i.e. undigested fat (containing lutein) could have adhered to the pipette tip during removal of the aqueous phase, boosting the apparent lutein concentration of the aqueous phase.

At the 0.0 mg/serving level (i.e. non-enriched products), micellization was not significantly different between cookie and flatbread, although the differences were significant at higher enrichment levels. This is likely due to a saturation of lutein carrying capacity of the digesta from the fat-free flatbread. Products of lipid digestion (FFA and MAG, especially) are important in forming and increasing the solubilization capacity of mixed bile salt micelles. Because the flatbread fat content is so low, there is limited capacity for the formation of lipolysis products. Although the results were mixed, they generally support the premise that when lipolytic capacity is limited in a digestion procedure, fat becomes less important for lutein micellization efficiency. It has been
found previously that with a fasted digestion model, fat content was not associated with lutein or zeaxanthin micellization (64), supporting this premise. With the fasted model, the muffin appears to strike a balance between the positive influence of fat content and the negative influence of incomplete fat digestion in terms of lutein bioaccessibility.

For the fasted digestion conditions, the total lutein micellized is represented by Equation 5. As seen previously, lutein bioaccessibility under the fasted conditions was very low, and the influence of fat content was minimal compared with the fed model. Although the FAT coefficient was statistically significant, its value was very small, highlighting the poor correlation of fat content with micellization under the fasted conditions. Also of note is that the LUT coefficient was very small, indicating that very little (roughly 5.5%, according to the model) of additional lutein was micellized as enrichment level increases.

4.6 Cell Culture Data

Note that data for this section is only for the fasted model. Samples following digestion with the fed conditions were incompatible with the cell culture assay (i.e. the cells were destroyed, leaving no identifiable cellular structures behind). Therefore, comparisons between digestion models for lutein absorption by Caco-2 cells could not be performed. From pilot tests, it appeared that the high concentrations of digestive enzymes (particularly pancreatin) and bile were responsible for destruction of the Caco-2 monolayer. The high ionic strength, pH, and antioxidants used in the digestion procedures (pyrogallol, BHT) were ruled out as being the cause of the incompatibility during preliminary testing. Also, DMEM dilution of digesta samples from the fed
experiments to levels with roughly equivalent enzyme concentrations as in the fasted model samples resulted in acceptable cell survivability. The approach of diluting fed digesta samples prior to was not utilized given the likelihood of this inducing changes in the aqueous phase structures in which lutein is solubilized. In previous studies, digestive conditions (specifically concentrations of these key enzymes) were typically similar to the fasted model used in the present experiments (45,46,56,62). This may have been by design to avoid issues with cell survivability, although this was not been reported. In other studies (62), conditions which mimic the fasted conditions but with higher lipase concentrations have also been used – perhaps to prevent the negative effects of pancreatin on the Caco-2 cells. Of note too, fasted conditions have been the default approach in drug and food studies to mimic the gastrointestinal environment. An appreciation of the importance of considering the fed state is still developing.
Figure 6 Proportion of lutein present in the digesta which was absorbed by the cells. Combined data (A) shown along with data for flatbread, muffin, and cookie (B, C, D, respectively). ANOVA and Tukey’s post-hoc test results shown; different letters represent significantly (p<0.05) different results. Data are means ± SEM, n = 5. Fasted model digestions only.
Figure 7 Lutein present in the digesta (measured in µg lutein per gram of product undergoing digestion) introduced to the Caco-2 monolayer which was absorbed by the cells. Data shown as combined (A) as well as individually for flatbread (B), muffins (C), and cookies (D). ANOVA and Tukey’s post-hoc test results displayed for fasted digestion data; different letters represent significantly (p<0.05) different results. Data are means ± SEM, n = 5. Fasted model digestions only. Linear regression slope is not
**Figure 8** Percentage of lutein initially present in the products which was absorbed by the Caco-2 monolayer following *in vitro* digestion and incubation. Data shown as combined (A) as well as individually for flatbread (B), muffins (C), and cookies (D). ANOVA and Tukey’s post-hoc test results displayed for fasted digestion data; different letters represent significantly (p<0.05) different results. Data are means ± SEM, n = 5. Fasted model digestions only.
4.6.1 Influence of Lutein Concentration in the Products

Trends for relative and absolute lutein absorption by the cell monolayers were similar to those observed for micellization. At higher lutein product concentration, absorption efficiency (the proportion of lutein in the digesta which is absorbed, Fig 6) decreased, although the absolute amount of lutein absorbed by the Caco-2 cells (Fig 7) increased. In other words, the total system efficiency of incorporation from lutein in the products to lutein absorbed by the cells decreased (see Fig 8), but the net lutein that would theoretically be absorbed by the enterocytes, according to this model, would be higher for products containing more lutein. This is discussed further below.

4.6.2 Comparison of Product Types

In addition to ANOVA analysis between enrichment levels for each product type, product types were compared using ANCOVA and linear regression analysis of the fasted model absorption data. Two analyses were performed, i.e. one based on the proportion of lutein present in the digesta which was absorbed, and one based on the absolute amount of lutein absorbed by the Caco-2 monolayers.

For the first analysis, the lutein content of the cell monolayer was evaluated as a proportion of the total lutein presented to the cell culture well in the digesta (i.e. absorption efficiency) in order to characterize the absorption characteristics of lutein present in the mixed micelles. This data is presented in Fig 6. As mentioned, an ANCOVA test was used to evaluate the lutein absorption characteristics of different product types with lutein content of the digesta as a covariate. Both product type (cookie, muffin and flatbread) and digesta lutein content were significantly correlated (p<0.0005) with absorption efficiency of the monolayer.
In the literature, data is generally presented as a proportion of carotenoids available in the digesta. As mentioned, monolayer absorption data is generally only available for studies where a fasted method has been used. Using similar methodology to estimate the bioavailability of β-carotene from cassava, Thakkar et al (62) found that 10.8-11.5% of β-carotene present in a digesta was accumulated by the Caco-2 cells. This is generally lower than the range of values observed in the present study, however β-carotene is less polar than lutein and may partition differently in the micelles. Interestingly, β-carotene absorption was directly linearly related to β-carotene content of the digesta ($R^2=0.99$), unlike the present study where absorption efficiency was dependent on lutein concentration of the product and of the digesta. The cassava products were not enriched, however, and lutein levels were relatively low at under 7 µg/g cassava. Garrett et al (55) found that approximately 22% of lutein in the digesta was absorbed by Caco-2 cells following digestion of a vegetable meal and 4 hour monolayer incubation.

Linear regression was performed in order to evaluate the influence of differing fat content, between product types, for both percentage lutein absorbed from the initial product and total lutein absorbed per gram of product. The results are as follows:

**Equation 5:** % lutein absorbed = 64.13 – 0.447 * LUT – 1.106 * FAT, $R^2=0.486$, p<0.01 for all terms

**Equation 6:** Lutein absorbed (µg/g of product) = 7.973 + 0.134 * FAT + 0.102 * LUT, $R^2=0.458$, p<0.01 for all terms

Note that equation 6 has influence from both bioaccessibility and absorption, since products with greater micellization results will have more lutein available for absorption by the Caco-2 cells.
R² for the % absorption regression was 0.609, indicating there is variability which cannot be explained by this simple linear model. All terms were significant according to the ANOVA analysis (p<0.01). The trend for fat content vs. % absorption was opposite to what was found at the level of bioaccessibility. A higher fat content appears to decrease absorption efficiency. This suggests there could be a saturation of micelle absorptive capacity by the Caco-2 monolayer. This is consistent with Caco-2 cell studies which have demonstrated that micelle absorption is indeed a saturable process (98). The digesta samples from the higher fat products are expected to contain more and possibly larger mixed micelles. A smaller quantity of micelles richer in lutein formed during the digestion of the lower fat products could be more efficiently absorbed if a larger proportion of these micelles are taken up by the limited capacity of the Caco-2 brush border. However, it is important to note that this effect may be an artefact of the absorption characteristics of the Caco-2 monolayers used and might not be observed in vivo. As noted above, lipid digestion and absorption in healthy individuals are generally quite complete (78), so it seems unlikely that a large fraction of lipid-soluble compounds would escape absorption.

It is also possible that differences between the products other than fat content contribute to the observed differences in absorption efficiency. Metal ions – particularly iron – have been observed to negatively impact lutein micellization and uptake by Caco-2 cells (99). These could be present in higher amounts in products which have higher flour fractions (cookies and especially flatbread), as einkorn wheat varieties have been observed to contain variable amounts of these micronutrients (100). In addition, the proofing stage of the flatbread production results in higher amounts of solubilized starch.
and release of dextrins from starch granules (101), which could also negatively impact lutein micellization and uptake by Caco-2 cells (102), probably by increasing the medium viscosity and thus decreasing diffusion rates.

Absolute lutein absorption was compared between product types using ANCOVA. This revealed a significant influence of product lutein content (p<0.0005) and of product type (p=0.026). These results were further analyzed using linear regression, using product fat and the lutein content of the product (in µg/g dry product weight) as independent variables. The regression result is described by Equation 6 (R²=0.458 for this regression). All coefficients were significant (p<0.007). Therefore, product lutein content was more important than product type in terms of lutein transfer through the entire system (i.e. micellization and monolayer absorption results combined). Additionally, the fat content of products did not exceed 25%, while the lutein content of the products tested was up to 81 µg/g. Therefore, lutein content has a greater total influence in the model due to its greater numeric value. Therefore, if the results of this digestion model and absorption assay are an accurate representation of the situation in vivo, a given target dose of lutein in terms of the actual amount which is delivered to the bloodstream can be provided by enriching products to the level which produces the required serum or tissue response.
5. CONCLUSIONS

The purpose of this study was to evaluate three different baked product matrices in terms of their ability to deliver lutein, by focusing on the stages of bioaccessibility and absorption using models of human digestion and intestinal absorption. Flatbreads containing zero fat, muffins containing 12% fat (dwb), and cookies containing 22% fat (dwb) were prepared with targeted lutein concentrations of 0.0, 0.5, 1.0, and 2.0 µg/g. These products were subjected to conditions representative of the fasted and fed states of human digestion in the upper gastrointestinal tract. The aqueous digestate was analyzed for lutein content to determine the bioaccessible fraction. To study the absorption characteristics of the micellar phase structures formed during digestion of the different product types and enrichment levels, samples of the digestate were incubated with Caco-2 cell monolayers. The proportional as well as absolute amounts of lutein absorbed by each monolayer were determined and compared.

The strongest conclusions relate to the digestion model comparison and the comparison of micellization efficiency between the three product types under the fed state. Under the most physiologically relevant conditions studied (i.e. the fed state) the differences between the product types in terms of lutein bioaccessibility were dramatic: the lutein within cookies was the most bioaccessible in both unenriched and enriched products, and the flatbread lutein was much less bioaccessible in the enriched products. This clearly demonstrates the importance of the presence of fat in products prepared for lutein delivery and is consistent with the literature. Previous work has shown the importance of fat during in vitro digestion of plant products such as spinach and mango (71,85) and human studies on the bioavailability of lutein supplements consumed in
combination with fat (103,104). While lipid is known to improve lutein bioaccessibility, the results of the present study support the conclusion that lipid digestion products are necessary for lutein to be efficiently micellized. When digestion conditions did not sufficiently promote lipid digestion, relatively little lutein was bioaccessible.

The Caco-2 cell absorption assay demonstrated that increasing the level of lutein enrichment in the products decreases the efficiency of the system (i.e. a smaller percentage of lutein is absorbed by the Caco-2 cells). However, while this might suggest theoretical decreases in bioavailability, the total quantity of lutein delivered to the Caco-2 monolayer was higher as lutein enrichment level increased. Differences between the products at the absorption stage were less dramatic than at the micellization stage, indicating a greater importance of the products on micelle formation, as opposed to absorption of the micelles formed.

One objective of the work was to identify an ideal baked product in which to deliver lutein. In that regard, the cookie and muffin were superior to the flatbread. Because the methods used in this study are not identical to those of other studies, it is difficult to assess exactly what the in vivo bioavailability would be in comparison to common sources (i.e. spinach, egg yolks). Without this frame of reference, it is difficult to make conclusions about how much product would be required to deliver measurable health benefits. The overall estimates of lutein bioavailability predicted from this study are quite low, i.e. 10-15% for the unenriched products and 1-2% for the products enriched at the highest level. However, given the demonstrated health benefits with lutein consumption of 10 mg/d (8) and dosages in the test products of up to 2 mg/d, two servings per day of the high-lutein functional food products could make a significant contribution towards
improving lutein status. In addition, even higher lutein concentrations should be achievable in the products studied.

The importance of digestion model parameters is also highlighted by the results of this study. Previous work (75) showed similar results, i.e. that digestion experiments with higher levels of bile and pancreatin increase measurements of carotenoid bioaccessibility. A number of different digestion models are currently in use for evaluation of lutein bioaccessibility. It is advisable to standardize these procedures further, since the methods. Lastly, care is needed in comparing the results of different studies. However, comparisons within studies where the methodology is constant remain useful, especially as a precursor in screening candidate products or food matrices for *in vivo* studies. While *in vivo* studies continue to be the gold standard for determination of product bioavailability, the present study supports the development of lutein rich functional food baked products and *in vitro* testing as a useful tool for understanding the impact of product matrix and composition on potential bioavailability.
REFERENCES CITED

76. Rich GT, Faulks RM, Wickham MS, Fillery-Travis A. Solubilization of carotenoids from carrot juice and spinach in lipid phases: II. modeling the duodenal environment. Lipids. 2003 Sep;38(9):947-56.