

Universitat de Lleida

Facultat de Medicina

Grau en Nutrició Humana i Dietètica

BIOAVAILABILITY OF CAROTENOIDS INCORPORATED INTO PROCESSED FOODS: BREAD AND MAYONNAISE

Mariona Fort Zaragoza

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Tutor: Robert Soliva Fortuny

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Abstract

This project involved the analysis of samples from the CAROTFood study, which is investigating the bioavailability of carotenoids that have been incorporated into processed foods (bread and mayonnaise). The volunteers in the study, judged to be healthy, returned for four stand-alone test sessions where they consumed one of four treatment breakfasts (treatment 1: plain bread with plain mayonnaise; treatment 2: plain bread with vegetable mayonnaise; treatment 3: vegetable bread with plain mayonnaise; treatment 4: vegetable bread). Following the breakfast, hourly blood samples are taken for the subsequent 8 hours and after 24 hours for measuring carotenoid concentration (lutein/zeaxanthin, lycopene, β -cryptoxanthin, β -carotene and α -carotene). The analysis of carotenoids in blood was carried out on two of its components: the amount of free carotenoids in plasma and the carotenoid concentrations in the chylomicron-rich fraction of plasma. The data obtained in this study clearly show that consuming carotenoids with mayonnaise increases their absorption. Furthermore incorporating the carotenoids in processed food could be an alternative to raise the consumption of this pigment in the UK. As the sample size analysed for the purposes of this thesis was small the carotenoid contents in the plasma and chylomicrons were not significantly different between the four treatments ($p>0.05$). Even so the study suggested that the presence of fat in an emulsified form enhances the absorption of carotenoids.

Resum

Aquest projecte recull l'anàlisi de les mostres de l'estudi CAROTFood, el qual està investigant la biodisponibilitat dels carotenoides que han estat incorporats en aliments processats (pa i maionesa). Els voluntaris de l'estudi, en bon estat de salut, van participar en quatre sessions independents en cadascuna de les quals van consumir un dels quatre tractaments (tractament 1: pa amb maionesa; tractament 2: pa amb maionesa vegetal; tractament 3: pa vegetal amb maionesa; tractament 4: pa vegetal). Després de l'esmorzar, es prenen mostres de sang cada hora durant les posteriors 8 hores i després de 24 hores, per mesurar la concentració de carotenoides (luteïna/zeaxantina, licopè, β -criptoxantina, β -carotè i α -carotè). L'anàlisi de carotenoides en sang es va dur a terme en dos dels seus components: la quantitat de carotenoides lliures en plasma i la concentració de carotenoides en la fracció rica en quilomicrons del plasma. Les dades obtingudes en aquest estudi mostren que el consum de carotenoides amb maionesa augmenta la seva absorció. A més, incorporar els carotenoides als aliments processats podria ser una alternativa per augmentar el consum d'aquest pigment al Regne Unit. Donat que la mostra analitzada per aquesta tesi era petita el contingut de carotenoides en el plasma i en els quilomicrons no van ser significativament diferent entre els quatre tractaments ($p > 0,05$). Tot i així, l'estudi suggereix que la presència de greix en una forma emulsionada millora l'absorció de carotenoides.

Resumen

Este proyecto recoge el análisis de las muestras del estudio CAROTFood, el cual está investigando la biodisponibilidad de los carotenoides que han estado incorporados en alimentos procesados (pan y mayonesa). Los voluntarios del estudio, en buen estado de salud, participaron en cuatro sesiones independientes en cada una de las cuales consumieron uno de los cuatro tratamientos (tratamiento 1: pan con mayonesa; tratamiento 2: pan con mayonesa vegetal; tratamiento 3: pan vegetal con mayonesa; tratamiento 4: pan vegetal). Después del desayuno, se tomaron muestras de sangre cada hora durante las posteriores 8 horas y después de 24 horas, para medir la concentración de carotenoides (luteína/zeaxantina, licopeno, β -criptoxantina, β -caroteno y α -caroteno). El análisis de carotenoides en sangre se llevó a cabo en dos de sus componentes: la cantidad de carotenoides libres en plasma y la concentración de carotenoides en la fracción rica en quilomicrones del plasma. Los datos obtenidos en este estudio muestran que el consumo de carotenoides con mayonesa aumenta su absorción. Además, incorporar los carotenoides en los alimentos procesados podría ser una alternativa para aumentar el consumo de este pigmento en el Reino Unido. Dado que la muestra analizada por esta tesis era pequeña el contenido de carotenoides en el plasma y en los quilomicrones no fueron significativamente diferentes entre los cuatro tratamientos ($p>0,05$). Sin embargo, el estudio sugiere que la presencia de grasa en una forma emulsionada mejora la absorción de carotenoides.

1. Introduction

1.1 Antioxidant compounds

There is scientific evidence that oxidative stress, induced by reactive oxygen species (ROS) that are generated by normal metabolic activity as well as lifestyle factors such as smoking, exercise, and diet, contribute to the progression of several chronic diseases such as cardiovascular diseases, cancer or diabetes. Dietary antioxidants can decrease the damaging effects of ROS and for this reason have been the focus of recent research.

1.1.1 Carotenoids

Carotenoids are a family of pigmented compounds that are synthesized by plants and microorganisms, but not animals and humans who have to depend on dietary supply. The structure of carotenoids is based on a C_{40} isoprenoid backbone that may be acyclic or have one or both ends modified into rings. The hydrocarbon carotenoids are classified as carotenes and xanthophylls, this rather group containing at least one oxygen atom.

More than 600 carotenoids have so far been identified in nature. Of these, only about 40 are present in a typical human diet and absorbed. However only six are present in the blood in notable amounts and have been associated with health benefits. These are β -carotene, α -carotene and lycopene of the carotene group, and lutein, β -cryptoxanthin and zeaxanthin of the xanthophylls group (Figure 1) [1-2].

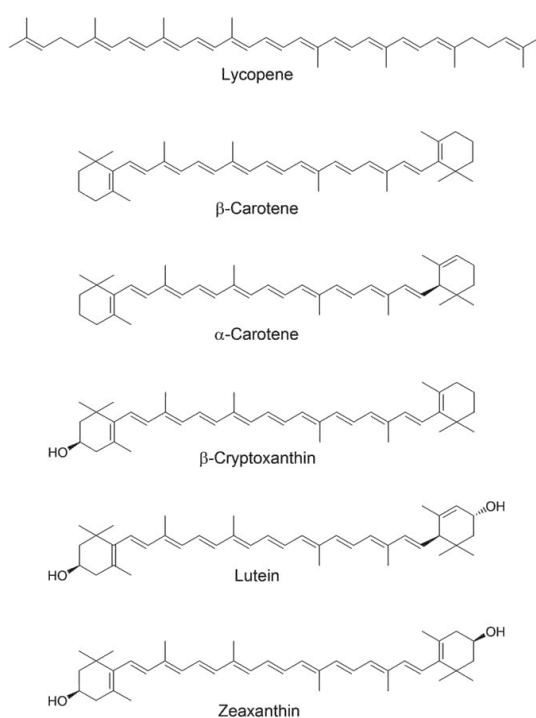


Figure 1. Chemical structure of carotenoids present in human plasma

i. Absorption and metabolism of carotenoids

Carotenoids are released from the food matrix by heat, mechanical and enzymatic treatments during food processing, and in the mouth, by mastication and the action of enzymes in the saliva.

The released carotenoids incorporate into the lipid phase, and emulsify into small lipid droplets in the stomach. From the lipid droplets, carotenoids are transferred to mixed micelles formed by the action of bile salts, biliary phospholipids, dietary lipids, and their hydrolysis products in the small intestine. The mixed micelles migrate to the brush border, where carotenoids are absorbed by the intestinal enterocytes and incorporated into chylomicrons and secreted to the lymphatic system. They are then taken up by the liver, incorporated into lipoproteins (LDL, HDL and VLDL) and released into the blood stream for transport to different tissues.

The uptake of carotenoids from the intestinal lumen takes place by simple diffusion down a concentration gradient through the brush border membrane into the cytoplasm of the enterocytes. However, some reports have suggested the existence of carotenoid transport mechanisms mediated by SR-BI (Figure 2) [2].

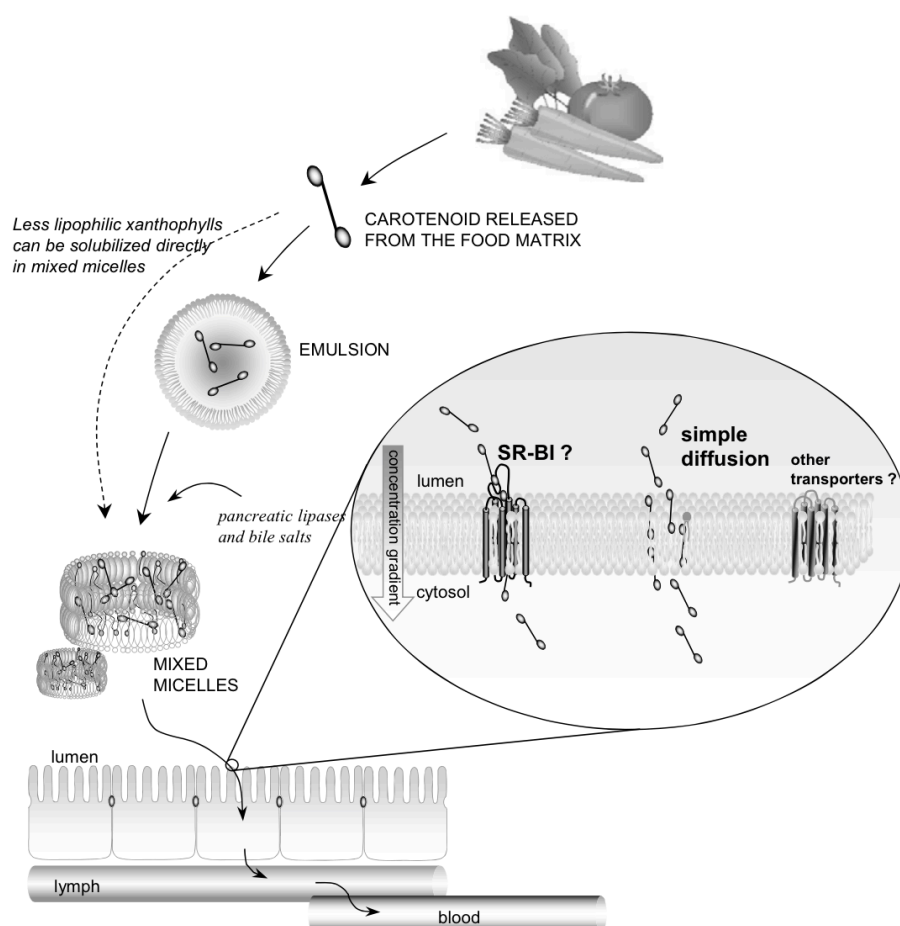


Figure 2. Scheme of dietary carotenoid absorption

Different tissues absorb carotenoids differentially. The major site of tissue storage of carotenoids is the adipose tissue (80-85%), followed by the liver (8-12%), and muscle (2-3%) [3].

The bioavailability, which is defined as the fraction of a dietary component capable of being absorbed and available for use or storage, of carotenoids is extremely variable, and is influenced by dietary and physiological factors. Some of these factors are: molecular linkage, the amount of carotenoids consumed in a meal, matrix in which the carotenoid is incorporated, effects of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors, and interactions. The bioavailability of different carotenoid types is also different. For example, the bioavailability of lutein (67%) is higher than the bioavailability of β -carotene (14%). This difference is due to some β -carotene being converted to vitamin A in the lumen and because lutein is more polar and is consequently more easily incorporated into lipid micelles [4].

The most important dietary factors that affect carotenoid absorption are the amount of dietary fat and the dietary fibre. Increasing the amounts of dietary fat does not influence all carotenoids species equally. They enhance the absorption of highly lipophilic carotenes (β -carotene, α -carotene and lycopene) to a larger extent than that of less lipophilic xanthophylls (lutein and zeaxanthin). This absorption of lipophilic carotenes is higher when a person consumes full-fat meals compared to low-fat meals.

On the other hand, dietary fibres are contributing to lowering the bioavailability of carotenoids. This is because the intake of dietary fibre, especially those classified as soluble, has been associated with cholesterol-lowering effects. Similar to cholesterol, carotenoids are of lipophilic nature, thus their absorption can also be affected by some types of fibre [2].

ii. The role of carotenoids in disease prevention

Carotenoids can serve several important biological activities in humans [5]. The main benefits of carotenoids are through their effects as antioxidants. Reactive oxygen and nitrogen species are generated during aerobic metabolism and pathological processes. They damage biologically important molecules like lipids, DNA and proteins and are involved in the pathobiochemistry of degenerative disease. The antioxidant effects of carotenoids are based on protecting cells and tissues from the damaging effects of free radicals and singlet oxygen.

Other health benefits of carotenoids related to their antioxidant potential are the inhibition of the development of certain type of cancer and the protection of cardiovascular disease.

Regarding cancer, several studies suggest that β -carotene and vitamin A may prevent lung cancer. However, two intervention trials [7-8] show that the combination of β -carotene and vitamin A as a supplement had no benefit and may have had an adverse effect on the incidence of lung cancer. The subjects of these interventions were exposed to smoking, and this could be a reason for the observed adverse effects.

A review of epidemiological studies that assessed the relationship between carotenoids and prostate cancer [9] suggested that decreased risk of prostate cancer was associated with high lycopene consumption from tomatoes and tomato products.

With regards to cardiovascular disease, data for β -carotene are conflicting but many studies propose that this compound is capable of inhibiting lipid peroxidation in LDL, a process suggested to be involved in the pathogenesis of atherosclerosis [10].

Another nutritional role of carotenoids is their provitamin A activity. The major provitamin A carotenoid in the diet is β -carotene, whilst α -carotene and β -cryptoxanthin demonstrate lower activity levels. Vitamin A is essential for growth, embryonal development and visual function.

In the intestinal mucosa β -carotene is converted to retinal by β -carotene 15,15' oxygenase 1 (BCO1) and the retinal is then reduced to retinol by a retinal reductase. The retinol is incorporated with the intact carotenoids into chylomicrons and absorbed via the lymphatic system. The two major sites of conversion of β -carotene to vitamin A in humans are the intestine and liver. To obtain 1 μ g of retinol is required 12 μ g of β -carotene, or 24 μ g of α -carotene or β -cryptoxanthin [6].

Studies show that lutein and zeaxanthin preferentially accumulate in the macula of the human retina, and are responsible for the coloration of this tissue. The macula is one part of the retina and the area of maximal visual acuity. These carotenoids help to protect the retina from oxidative damage and reduce the risk of developing age-related macular degeneration (AMD). AMD is a degeneration of the retina and the retinal pigment epithelium in the macular region, and occurs in those above the age of 65 and causes a loss of acute vision of the fovea [11].

The age-related eye disease study 2 (AREDS2) [12] showed that lutein and zeaxanthin may play a role in protecting against AMD, but they are not beneficial for the treatment of advanced AMD.

iii. Carotenoid content in foods

Carotenoids are present in many foods, and the major sources in the human diet are vegetables and fruits. They are present as micro-components in fruits and vegetables and are responsible for their yellow, orange and red colours.

In general, yellow-orange vegetables and fruit mostly contain β -carotene and α -carotene, orange fruits provide α -cryptoxanthin, dark green vegetables provide lutein and tomatoes are a major source of lycopene [13].

Table 1 shows the content of carotenoids in some common fruits and vegetables [14]. It demonstrates how lycopene is predominant in tomato and tomato products, whilst α -carotene is prevalent in carrots. Spinach and broccoli are rich in lutein and zeaxanthin.

Table 1. Data for the content of major carotenoids in selected foods ($\mu\text{g}/100\text{g}$)¹

Foods	β -carotene	α -carotene	Lycopene	Lutein	Zeaxanthin	β -cryptoxanthin
Banana	43-131	60-156	n.d.-247	86-192	-	n.d.-5
Broccoli	291-1750	n.d.	n.d.	707-3300	-	n.d.
Carrot	4350-8840	2840-4960	n.d.	254-510	il	n.d.
Kiwi	<20	-	<10	-	-	-
Lettuce	870-2960	-	-	1000-4780	-	-
Orange	171-476	n.d.	n.d.	-	-	74-141
Spinach	3100-4810	n.d.	n.d.	5930-7900	il	n.d.
Tomato	320-1500	n.d.	850-12700	46-213	il	n.d.
Tomato, ketchup	135-500	n.d.	4710-23400	n.d.	n.d.	n.d.

(Mol. Nutr. Food Res. 2009, 53)

¹ n.d.: not detected or quantified

il: included in lutein

- : not included in the reference

iv. Effects of food processing on the stability of carotenoids

The degradation of carotenoids in foods is a complex process due to factors and mechanisms, which could lead to a decreasing, increasing or no variation in the content of carotenoids. Some of these mechanisms are processing treatments (i.e. the time/temperatures conditions used), the storage conditions and the chemical structure of carotenoids. Factors such as light, pH and oxygen concentration also influence degradation.

The chemical structure of the carotenoid chain is the cause of instability of these compounds, including their susceptibility to oxidation and geometric isomerization. Carotenoids are capable of forming different *cis/trans* geometric isomers. Most natural carotenoids have a *trans* configuration, but small amounts of *cis* isomers are also present naturally or are transformed from the all-*trans* forms during processing. Although *trans* isomers are more stable than *cis* isomer, the *cis* configuration could have a better solubility. Also of note that *cis* isomers have a lower provitamin A activity [15-16].

Thermal treatment during cooking showed to have a negative impact on the carotenoid content although it has a positive effect on the micellarisation of carotenes. Food processing facilitates the disruption of the cell wall, releasing the carotenoids from the food matrix resulting in an increased carotenoid bioavailability. The study by Hornero-Méndez [17] demonstrated with carrot samples that heat treatments caused a degradation of carotenoids after cooking compared to fresh samples. This study also showed a clear effect of cooking on the bioavailability of carotenoids, facilitating their incorporation to mixed micelles ready for absorption.

In general the most common household cooking methods, such as microwave cooking, steaming and boiling, and with low temperatures (60-100°C) do not drastically alter the carotenoid contents of vegetables. However, extreme heat with temperatures above 100°C and excessive cooking time result in the oxidative destruction of carotenoids, and a decreased in total carotenoid contents [14].

Higher storage temperatures have also been shown to exacerbate destruction of the carotenoids. Similarly frozen storage, also causes a reduction in carotenoid contents. Chen, H.E. et al studied the degradation of various carotenoids and changes in vitamin A content during storage of carrot juice in dark and light conditions. They concluded that the concentration changes of lutein, α -carotene, β -carotene and vitamin A in the carrot juice decreased with increasing storage temperature. Light storage can be more destructive to each carotenoid and vitamin A than dark storage [18].

The pH of intestine may influence carotenoid absorption. If intestinal pH becomes too low (pH < 4,5) the solubilisation of carotenoids into micelles decreases, therefore decreasing carotenoid absorption. It seems that β -carotene is absorbed best under slightly acid conditions [19]. With regards to oxygen, carotenoids act as catalysts by deactivating singlet molecular oxygen (O_2^*). The carotenoids transfer an electron to O_2 , which leads to its destruction. However, CAR^* can easily return to the ground state [20].

2. Background

This project focuses on the CAROTFoods study that is conducted by the natural product group of the Rowett Institute of Nutrition and Health of the University of Aberdeen. This study focuses on a specific group of antioxidants, carotenoids. The CAROTFood study is investigating the acute bioavailability of carotenoids that have been incorporated into processed food (bread and mayonnaise).

Some recent work suggests that carotenoid bioavailability improves when fat is present in the form of mayonnaise compared to plain oil. Takeda *et al* [21] fed healthy subjects with broccoli as a control meal, broccoli with oil or broccoli with mayonnaise and found that serum carotenoids were significantly higher when the vegetables were co-ingested with mayonnaise. The same group [22] found that β -carotene from carrot transferred more easily into mayonnaise than into oil, and that the mayonnaise also dispersed more easily in gastric juice.

The two studies suggested that the greater solubility of carotenoids in mayonnaise might be due to its emulsion properties. Carotenoids may be more miscible in oil-water emulsions (such as mayonnaise) due to its amphiphilic nature. The increased carotenoid absorption in the presence of mayonnaise may also be due to the activity of phosphatidylcholine (from egg yolk), which was shown to enhance absorption of carotenoids. Mayonnaise was also shown to be more miscible in gastric juices compared to plain oil and this too may be contributing to the greater bioavailability of carotenoids.

Another study [23] showed similar results. Subjects were provided three test salads that had identical vegetable compositions but with dressings containing different fat contents (0, 6 or 25 g canola oil). They found that when the salads were ingested with fat-free salad dressing, the absorption of carotenoids was negligible. When the salads were ingested with reduced-fat salad dressing, the added fat promoted α -carotene, β -carotene, and lycopene absorption. Similarly, when salads were ingested with a full-fat salad dressing there was more absorption of carotenoids than when it was consumed with reduced-fat salad dressing.

Similarly, another study [24] observed that adding avocado fruit significantly enhanced carotenoid absorption, which is attributed to the lipids present in this fruit.

The CAROTFood study compares carotenoid bioavailability when these are incorporated either into bread or mayonnaise. Furthermore, the study investigates how mayonnaise affects the bioavailability of carotenoids incorporated into a processed food (bread). These carotenoids were

sourced from carrots and tomato. The study is focused on the bioavailability of five carotenoids found in foods: lutein/zeaxanthin, β -cryptoxanthin, lycopene, α -carotene and β -carotene.

According to the Scottish health survey (2008) conducted by the Scottish Government, Scottish people don't consume sufficient amount of fruit and vegetables. The Scottish Government recommend changing eating habits by reducing the consumption of saturated fats, salt and sugar and eating more fruits and vegetables.

Therefore the findings of the study would have practical potential both in the health food product development (carotenoid containing mayonnaise and bread) and public health nutrition (dietary advice for increasing carotenoid intake in humans) spheres.

3. Hypothesis

Carotenoids bioavailability is influenced by the processed food matrix in which they are contained.

Fat in the form of mayonnaise increases carotenoid bioavailability.

4. Objective

The aims of this project are:

1. To compare the acute-phase bioavailability (systemic and gut absorption) of targeted carotenoids (lutein/zeaxanthin, beta-cryptoxanthin, lycopene, α -carotene and β -carotene) when they are incorporated into different processed food matrices (bread and mayonnaise) in a bread-mayonnaise meal.
2. To study how mayonnaise affects the acute bioavailability of carotenoids incorporated into a processed food.
3. To analyse carotenoids in plasma and in the chylomicron-rich fractions using HPLC.
4. To analyse food diaries of volunteers using NetWISP software.

5. Methods and materials

5.1 Sample size and subject characteristics

The study used healthy volunteers. Before inclusion, anthropometric measurements were taken, and health questionnaires were administered to potential participants to assess suitability. These were carried out during the screening visit. The participants were required to fulfil the following inclusion and exclusion criteria to be included in the study:

- Inclusion criteria:

- Healthy males and females aged between 18-75 years
- Body mass index between 18,5-40 kg/m²
- Blood pressure \leq 139/89Hgmm
- HbA1C² \leq 6,5%
- Blood total cholesterol < 6mmol/L

- Exclusion criteria:

- Having following diseases: diabetes, kidney disease, hepatic disease, gout, gastrointestinal disorders, thromboembolic o coagulation disease, hypertension, thyroid disorders, hypercholesterolemia
- On prescription medications: orlistat, digoxin, anti-arrhythmics, tricyclic anti-depressants, neuroleptics, oral anti-diabetic medication, insulin, anti-inflammatories, anti-pyretics, statins
- Allergic or intolerant to any of the foods in the study
- Vegetarian o vegan
- Restricted eating and/or eating disorders
- Alcohol and/or other substance abuse
- Regularly take nutrition supplements (one a day)
- Smoking
- Poor venous access, and having veins that are difficult to cannulate

Volunteers were recruited by poster advertisement (annex 1) on notice boards of the Rowett Institute of Nutrition and Health (RINH), the Foresterhill and King's campus' of the University of Aberdeen and Robert Gordon University, and public places such as notice boards, libraries, gyms, GP clinics, supermarkets and shops.

² HbA1C: glycated hemoglobin – normal values: 5-6%

Fourteen people were screened for the study, three of them were excluded and eleven fulfilled inclusion criteria, of these three are males and eight are females aged between 20-70. So far, eight volunteers are complete, two are running and one is waiting to start.

5.2 Study design and research methodology

This acute interventional study adopted a randomised controlled non-blind crossover design. Each participant returned for four sessions with a minimum washout period of fourteen days between test sessions. The study design is illustrated in Figure 3.

The volunteers were advised to eat a diet low in carotenoids during the two days running up to each study session. They were advised on what foods to avoid and were given a leaflet with guidelines to use as a reference (annex 2). They were also asked to limit alcohol consumption to <3 units/day for men and <2 units/day for women (based on UK DOH³ guidelines). Food diaries were obtained to confirm compliance. Female participants were advised to attend test sessions only on days when they are not menstruating. Participants were provided a standardised dinner to consume the evening before each study session (5.3.1). They were instructed to eat the entire meal, and finish eating by 10 pm. No restriction on water was made.

Each of the study sessions lasted approximately nine hours and was identical in all respects except for the test meal consumed (5.3.2). The order for the test meals for each volunteer was based on a randomisation plan provided by BLOSS⁴.

Volunteers arrived at the Human Nutrition Unit in the morning in overnight fasted state. An indwelling catheter was inserted into the ante-cubital vein of the left or right forearm at the ante-cubital fossa and kept patent during the test session.

A baseline blood sample was subsequently obtained and the volunteers were given the test meal to consume at a comfortable pace within 20 minutes. Following the meal the volunteers were given a questionnaire to complete on the taste and palatability of the test food. Further blood samples (5 mL each time) were taken hourly for the next eight hours.

During the test period one snack was provided to the volunteers; this consisted of 28 g of graham crackers and 122 g of fat-free milk.

At the end of testing the catheter was removed. The volunteers were served lunch (5.3.3) after which they were free to leave. They were instructed to eat a diet low in carotenoids for the rest of

³ DOH: Department of Health

⁴ BLOSS: Biomathematics & Statics Scotland

the day and finish eating by 10 pm in the evening. The volunteers were instructed to return to the Human Nutrition Unit the following morning in an overnight fasted state. A single blood sample (5 mL) was drawn in the fasted state using venepuncture at the time corresponding to 24 hours of eating the test meal.

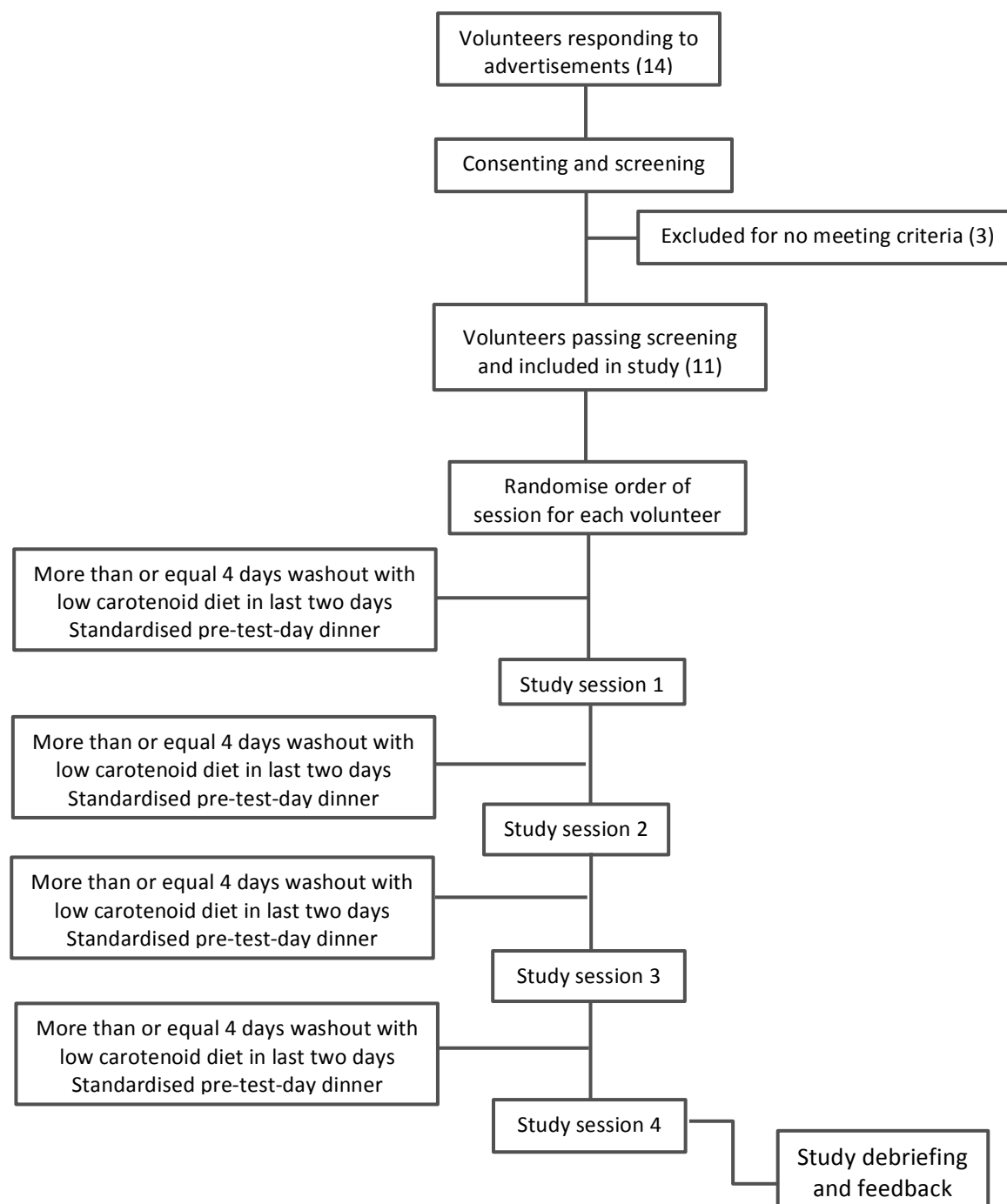


Figure 3. Schematic of study

5.3 Meals

5.3.1 Pre-test day dinner

Volunteers were given a meal consisting of a main and dessert (Table 2). The meal contained 960 kcal of energy, of which 24%, 31% and 46% came from carbohydrates, proteins and fat respectively. There weren't restrictions on water.

Table 2. Details of meal choices for pre-test day dinner

Meal Component	Type	Weight (g)
Mains	Chicken with Italian pancetta and mozzarella	450
Dessert	Vanilla cheesecake	100

5.3.2 Test meals

The volunteers were consuming four test meals (Table 3) in random order at the four study sessions.

Table 3. Description of test meal

Test meal	Food
1	Plain white bread + plain mayonnaise
2	Plain white bread + mayonnaise containing vegetable powder
3	Bread containing vegetable powder + plain mayonnaise
4	Bread containing vegetable powder

The vegetable powder was made by mixing together freeze-dried and ground carrots and tomato (1:1).

The recipes for the plain and vegetable breads are presented in table 4, and those for the plain and vegetable mayonnaises are presented in table 5.

Both were made at the Human Nutrition Unit. The vegetable bread and mayonnaise was made by incorporating the vegetable powder into the plain recipe at the time of production. The bread and the mayonnaise were served separately to the volunteers.

Table 4. Recipe from the plain and vegetable bread

Ingredients	Quantity (g)
Flour (Allison strong white bread flour)	120 / 108,5*
Water	75
Yeast (Tesco instant yeast)	3,6
Salt	2,4
Vegetable powder (50:50 carrot and tomato powder)	11,5
<i>TOTAL</i>	<i>201</i>
<i>*Quantity in vegetable bread</i> 1. Warm the water to 40°C 2. Disperse the yeast in the warm water 3. Mix the flour and salt. And the vegetable powder to make the vegetable bread 4. Add the water + yeast to the dry ingredients, mix and form a ball 5. Knead dough for 5 minutes until firm, non-sticky and resistant denting 6. If preparing multiple portions then divide dough into 201g portions and form into individuals balls 7. Knock back, make up and transfer to floured baking sheet 8. Second prove for 40 minutes 9. Transfer to pre-heated oven and bake for 12 minutes at 200°C 10. Cool and wire rack, wrap in foil, place in freezer bag and freeze (individually)	

Table 5. Recipe from the plain and vegetable mayonnaise

Ingredients	Quantity (g)
Rapeseed oil	120 / 140*
Water	38/40*
Egg powder	6/8*
Vinegar	5/10*
Salt	1/2*
Vegetable powder (50:50 carrot and tomato powder)	30
<i>TOTAL</i>	<i>200</i>
<i>*Quantity in vegetable mayonnaise</i> 1. Weigh all the ingredients 2. Mix the water, egg powder, salt and vinegar (A) 3. Mix the oil with the vegetable powders (B) 4. Homogenise the sample using a food processor by adding slowly and steadily the oil phase (B) to the water phase (A). 5. Store at 4°C 6. Shelf life: 2 weeks	

5.3.3 Post-test-session snack

The post-test-session lunch was macaroni cheese. One portion provided 650 kcal of energy, of which 43%, 18% and 39% came from carbohydrates, protein and fat respectively.

5.4 Biochemical analyses

The blood samples were collected into K-EDTA coated tubes, stored in ice immediately after collection and centrifuged to separate plasma. The total volume collected at each test session was 50 mL.

The volunteers were asked to provide two faecal samples at each study session. They were asked to provide the first faecal sample from either the night before the test day, or the morning of the test day (before the start of testing). For the second sample the participants were instructed to collect all the faeces they void between 12-24 hours from the time of eating the test meal. In the event that they were unable to void during this period the volunteers were instructed to collect the faeces they void at the first subsequent instance.

Volunteers were also asked to provide urine samples at each test session. They were asked to provide hourly samples in the first 8 hours following the test meal. They were also asked to collect an overnight sample i.e. all the urine they void from the time they leave the Human Nutrition Unit until the time they return the following morning.

5.4.1 Analysis of carotenoids in plasma samples

The analysis of carotenoids in blood was carried out on two of its components. Firstly, the amount of free carotenoids in plasma was quantified by directly measuring amounts present in the plasma. Secondly, carotenoid concentrations in the chylomicron-rich fraction of plasma were measured. As carotenoids are fat soluble compounds their absorption from the gut lumen follows that of lipids i.e. via the inclusion in chylomicrons. Therefore the amount of carotenoids in the chylomicron-rich fraction of plasma could be a good indicator of the bioavailability of dietary carotenoids as they represent only those originating from dietary sources, and not those that are endogenously produced.

To separate the chylomicron-rich fraction the plasma was layered with a liquid having a density of 1.006 g/mL and ultracentrifuged at approximately 28,000 x g. The supernatant containing the chylomicrons was aspirated out and used for analysing carotenoids.

The quantification of carotenoids in the samples was carried out using HPLC (high-performance liquid chromatography). Specifically reverse phase HPLC was used. This method allows the simultaneous measurement of retinol, five carotenoids (lutein/zeaxanthin, β -cryptoxanthin, lycopene, α -carotene and β -carotene), α -tocopherol and γ -tocopherol in plasma using fluorescence and visible detection. This thesis will focus only on the data obtained for the carotenoids.

The HPLC system consists of a Waters 515 pump, a Waters 717plus autosampler, a 2487 programmable multiwavelength UV/V15 detector and a 2475 multi λ fluorescence detector run on two channels. Channel 1: 298 Ex/328 Emm allows the measurement of α and γ tocopherols. And channel 2: 450 Ex/470 Emm for measurement of retinol.

For the detection of the carotenoids λ 450 nm, 0-11 minutes allows the measurement of lutein/zeaxanthin, β -cryptoxanthin and echinone. At 11-16 minutes λ 470 nm allows the measurement of lycopene, and at 16-30 minutes 450 nm for α -carotene and β -carotene.

Figure 4 shows the chromatographic profiles of the carotenoids corresponding to a plasma extract. Echinone was used as an internal standard to measure carotenoid recovery rates. Echinone is a synthetic carotenoid that has a structure and chemical properties very similar to the naturally occurring carotenoids present in serum. On the chromatogram echinone appears between β -cryptoxanthin and lycopene and does not interfere with the other carotenoids analysed. [25]

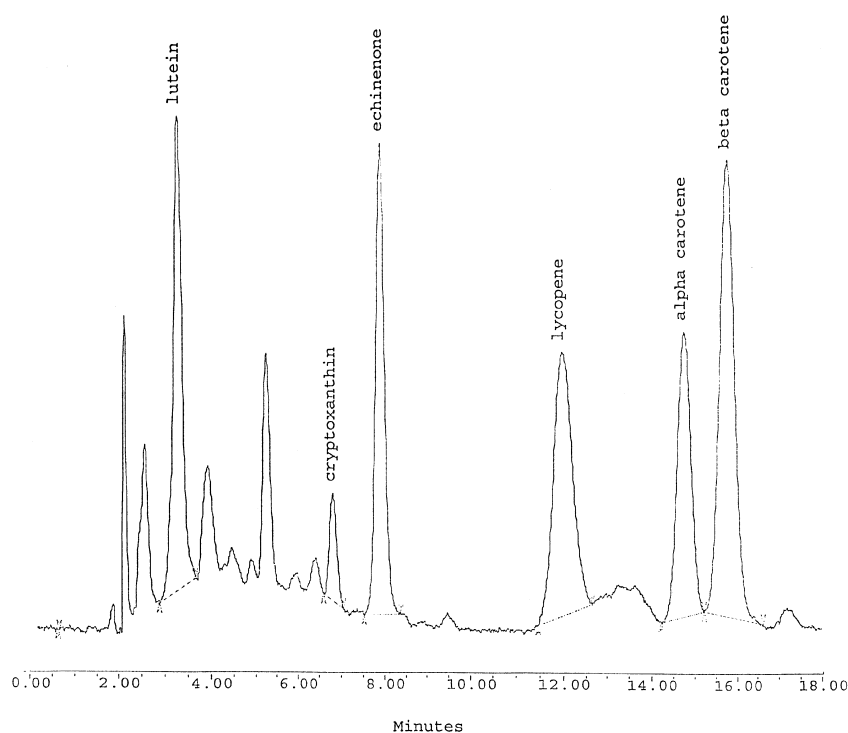


Figure 4. Chromatogram of channel 2: detection at 450 nm for carotenoids

Before running the HPLC analysis samples were prepared. Into a 2ml microtube, 100µl plasma or 200 µl chylomicrons + 300µl water + 400µl ethanol were pipetted, whilimixed for 10 seconds. Then, 700µl of hexane (containing BHT) + 100 echinone were added. The microtube was shaken for 10 minutes on the vortex genie, and then centrifuged for 5 minutes. Continuing the hexane layer (600 µl) was removed and dried down on the speed vac for 10 minutes. To finish, this sample was dissolved in 200µl of DEA, and was shaken for 5-10 minutes before application on the HPLC column [26]. We did for each hour twice.

Chromatograms allowed obtaining the quantity of carotenoids in each volunteer for each hour in µg/µl. Data were filled into an excel spreadsheet and corrected with the percentage of echinone recovered. Average values from two replicate measurements were calculated. Overall average and standard deviation values were graphically represented for each carotenoid.

The area under the curve (AUC) for the 8 hours postprandial period was calculated using the trapezoidal method. The trapezoidal method involves dividing the AUC into individual trapezoids and calculating their area. The analysis of variance procedure (ANOVA) was used to statistically analyse differences in AUC between treatments. The ANOVA procedure was also used to analyse differences in carotenoid data over time. The p value was set at 0.05. The statistics programme that was used was IBM SPSS version 22.

5.5 Analyses of food diaries using NetWISP software

The food diaries were designed according to a standard template. Each volunteer had to record at each session all the food and drink consumed on the two days before the study and on the day of the study. The volunteers had to describe the food, the method of cooking and the weight, and if at the end of the meal there were any leftovers.

NetWISP software (Weighed Intake Software Program; Tinuviel Software, Warrington, UK) was used to analyse the food diaries. NetWISP is a fully-featured, powerful nutritional analysis package that converts dietary information to food quantities and nutrient values. The software uses an extensive data base of the most common foods in UK.

In the software, a template for each volunteer and for each treatment was created. The templates were then filled with foods that most resembled those indicated by the volunteer and the corresponding weight. If the volunteer did not indicate the weight, average portion sizes (*Food Portion Sizes, from the Food Standards Agency*) were assumed.

6. Results

6.1 Characteristics of subjects

This thesis focuses on the data from eight volunteers who have completed the four sessions of the study. The characteristics of the study volunteers are presented in table 6. The subjects were all in good health and fulfilled all the inclusion and exclusion criteria.

All these volunteers do not smoke, do not have any allergies or intolerances and are not vegetarian or vegan. They are not on prescription medications, do not have any important disease and don't abuse of alcohol. All of them have good venous access.

Table 6. Screening of the volunteers (inclusion criteria)

Volunteer	Age	Gender	Height	Weight	BMI	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	HbA1C (%)	Total cholesterol (mmol/L)
V2	33	Male	1.67	90.6	32.49	124	72	4.8	4.81
V3	48	Female	1.67	71.9	25.78	109	62	5.1	5.48
V5	72	Female	1.59	69.9	27.65	160	71	5.4	5.94
V7	49	Female	1.52	55.25	23.91	131	84	5.4	6.05
V9	20	Female	1.63	50.65	19.06	108	70	5.2	3.94
V10	58	Male	1.76	80.45	25.97	108	65	5	4.69
V11	70	Female	1.59	57.5	22.74	120	76	4.8	6.84
V13	58	Male	1.77	87.9	28.06	138	89	4.9	4.97
Average	51±18	-	1.65±0.09	70.52±15.1	25.71±4	124.75±18.09	73.6±9.1	5.08±0.24	5.34±0.92

6.2 Analyses of food diaries using NetWISP software

Food diaries were obtained to confirm compliance to eating low amounts of carotenoids before the studies sessions in order to show clearer effects of the meals provided.

The amount of energy, carbohydrates, fat, proteins and carotenoids that the volunteers consumed during this wash-out period are summarized in table 7. The data shows the average of each treatment from six volunteers.

Table 7. Average of energy, carbohydrates, fat, proteins and carotenoids for each treatment.

Treatments	Kcal	Carbohydrates (g)	Fat (g)	Proteins (g)	Carotenoids (mg)
1	1538,89 ± 252,7	142,03 ± 30,12 (36,92%)	72,29 ± 18,38 (42,28%)	83,20 ± 18,17 (21,63%)	51,59 ± 51,85
2	1789,5 ± 571,70	174,33 ± 75,25 (38,97%)	80,97 ± 31,38 (40,72%)	95,79 ± 35,95 (21,41%)	104,07 ± 138,45
3	1479,78 ± 245,11	142,75 ± 37,74 (38,59%)	66,59 ± 21,39 (40,50%)	79,77 ± 25,6 (21,56%)	43,73 ± 51,7
4	1378,5 ± 372,01	122,82 ± 39,41 (35,64%)	60,64 ± 18,8 (39,59%)	87,28 ± 33,24 (25,33%)	56,42 ± 92,96

Results expressed as mean ± standard deviation.

Mean values were calculated considering the daily intake on the two days before the study and on the day of the study session.

Values between brackets correspond to the contribution of each nutrient in terms of energy.

As the volunteers were advised to eat a diet low in carotenoids during the two days before the study session they had to minimise the amount of fruits and vegetables they ate. Therefore their meals consisted of predominantly carbohydrate and protein and this is reflected in the higher levels of fat, protein and carbohydrates.

The data shows that the volunteers followed a low carotenoid diet before the test sessions. The maximum average total amount of carotenoids volunteers ate over the two days was 104 micrograms and this was in the case of treatment 2. The other three test sessions showed even lower values.

6.3 Free carotenoids in plasma

Changes in plasma of lutein/zeaxanthin, beta-cryptoxanthin, lycopene, α -carotene and β -carotene following the consumption of the four treatments are shown in Figure 5, 6, 7, 8, and 9 respectively.

The AUC for plasma of lutein/zeaxanthin, beta-cryptoxanthin, lycopene, α -carotene and β -carotene for the 8 hour postprandial period was not significantly different between the treatments [F(3,28)=2.613; p=0.071], [F(3,28)=1.826; p=0.165], [F(3,28)=0.625; p=0.605], [F(3,28)=0.410; p=0.747] and [F(3,28)=0.275; p=0.843] respectively.

The AUC for plasma of lutein/zeaxanthin, beta-cryptoxanthin, lycopene, α -carotene and β -carotene as 24 hours was also not significantly different between the four treatments [F(3,27)=1.568; p=0.220], [F(3,27)=1.391; p=0.267], [F(3,27)=0.434; p=0.730], [F(3,27)=0.516; p=0.675] and [F(3,27)=0.264; p=0.851] respectively.

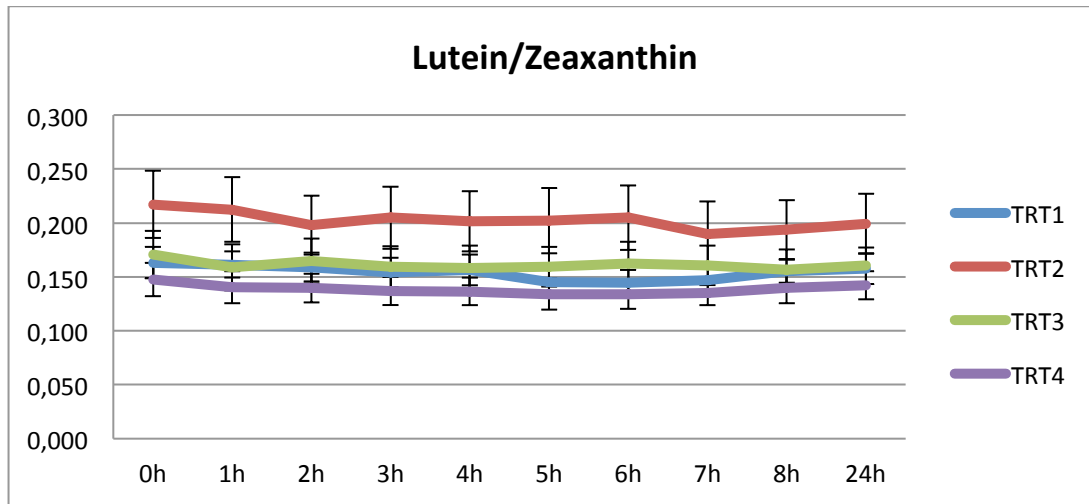


Figure 5. Plasma concentration (µg/ml) of lutein/zeaxanthin after consume each meal provided.
 Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

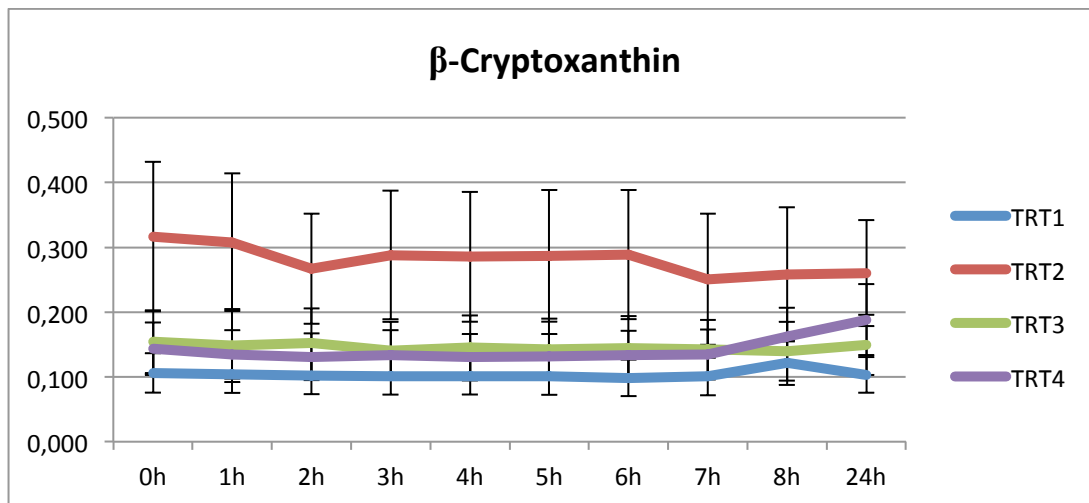


Figure 6. Plasma concentration (µg/ml) of β-cryptoxanthin after consume each meal provided.
 Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

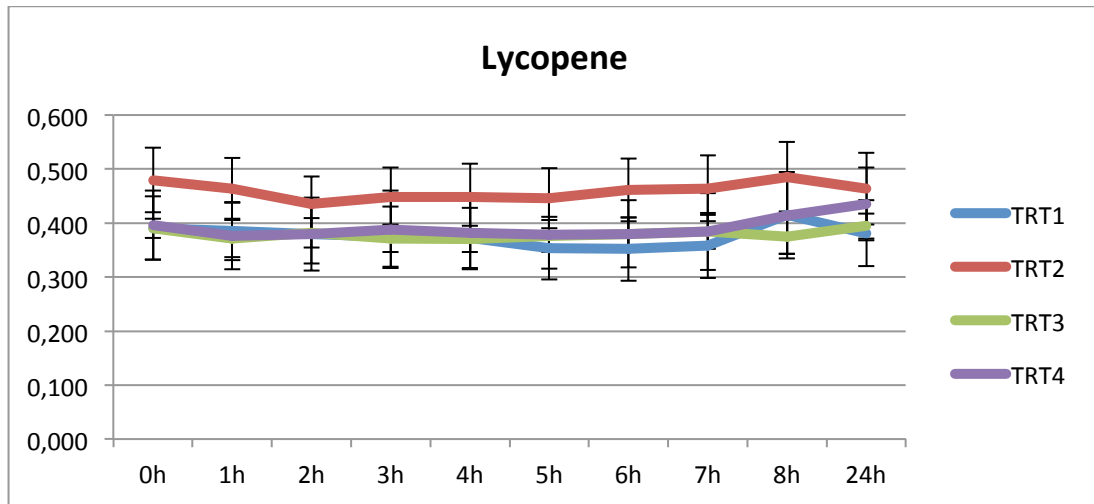


Figure 7. Plasma concentration (µg/ml) of lycopene after consume each meal provided.

Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

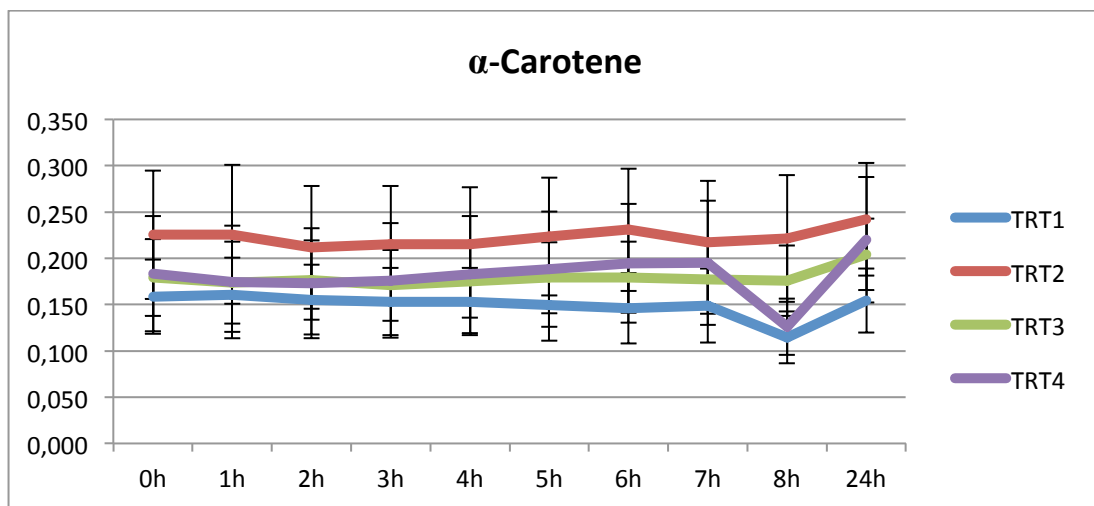


Figure 8. Plasma concentration (µg/ml) of α-carotene after consume each meal provided.

Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

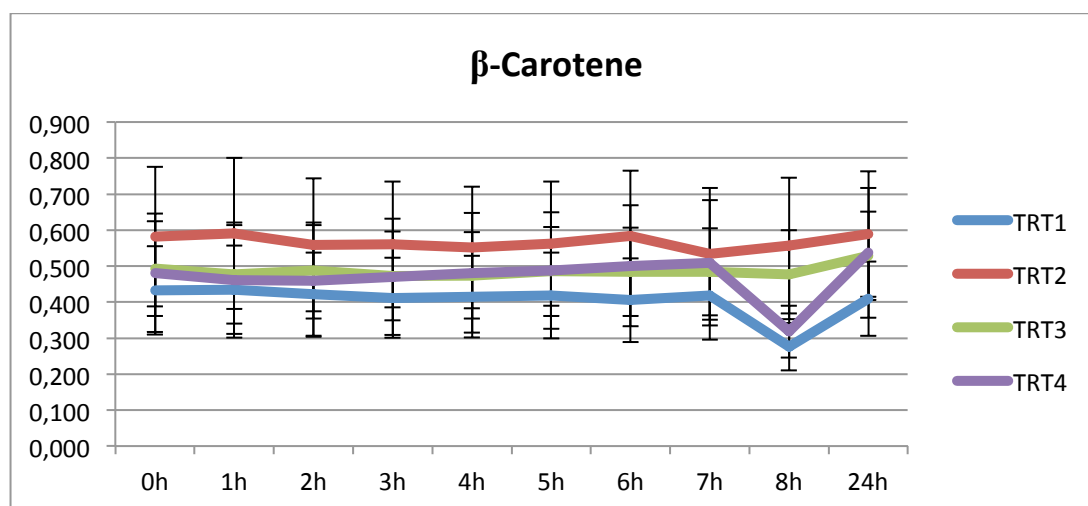


Figure 9. Plasma concentration (µg/ml) of β-carotene after consume each meal provided.

Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

For all the carotenoids the highest plasma levels were observed for treatment 2 (plain bread with vegetable mayonnaise). The treatment that led to the lowest levels of carotenoids was treatment 1 (plain bread with plain mayonnaise).

Of all the carotenoids, lycopene and β-carotene showed the highest concentration.

6.4 Carotenoids in the chylomicron-rich fraction of plasma

Only lutein/zeaxanthin, lycopene, α-carotene and β-carotene were detected in chylomicrons and the changes of these carotenoids following the consumption of the four treatments are shown in Figure 10, 11, 12 and 13 respectively.

The AUC for chylomicrons of lutein/zeaxanthin, lycopene, α-carotene and β-carotene, as estimated by 0-8 h, were not significantly different between the four treatments [$F(3,16)=0.146$; $p=0.931$], [$F(3,16)=0.457$; $p=0.716$], [$F(3,16)=1.303$; $p=0.308$] and [$F(3,16)=1.156$; $p=0.357$] respectively.

The AUC for chylomicrons of lutein/zeaxanthin, lycopene, α-carotene and β-carotene as 24 hours were also not significantly different between the four treatments [$F(3,16)=0.145$; $p=0.933$], [$F(3,16)=0.403$; $p=0.753$], [$F(3,16)=1.401$; $p=0.279$] and [$F(3,16)=0.816$; $p=0.504$] respectively.

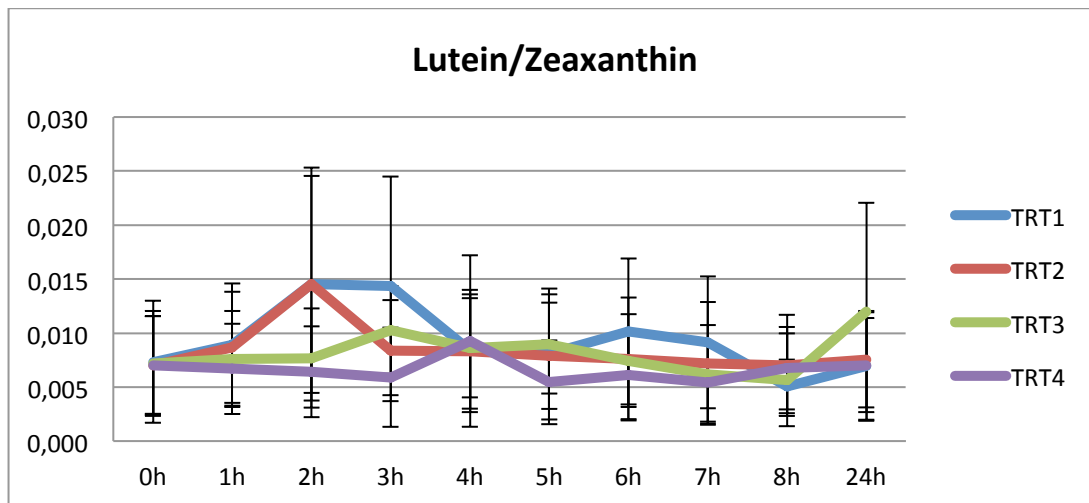


Figure 10. Chylomicron concentration (µg/ml) of lutein/zeaxanthin after consume each meal provided.
 Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

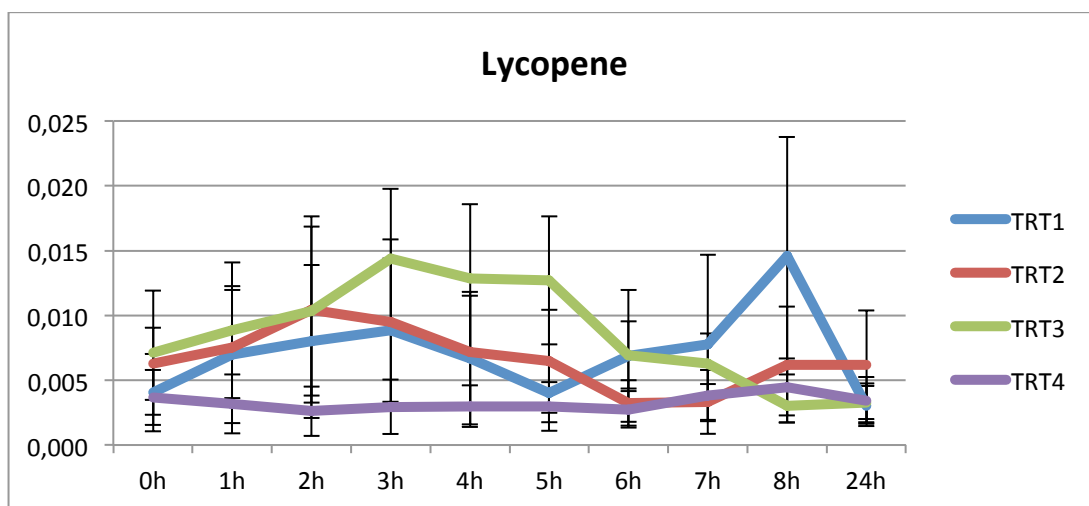


Figure 11. Chylomicron concentration (µg/ml) of lycopene after consume each meal provided.
 Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

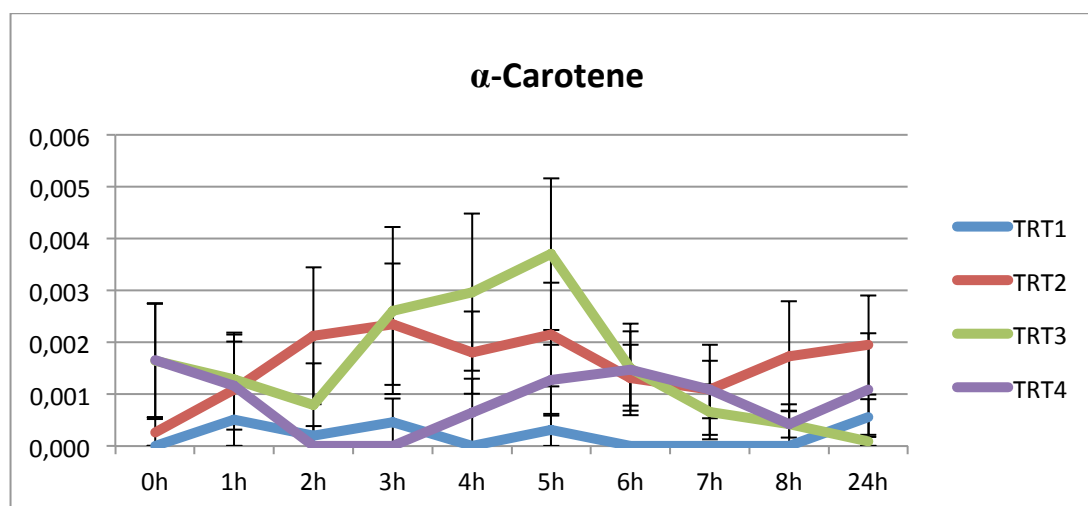


Figure 12. Chylomicron concentration (μg/ml) of α-carotene after consume each meal provided.
 Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

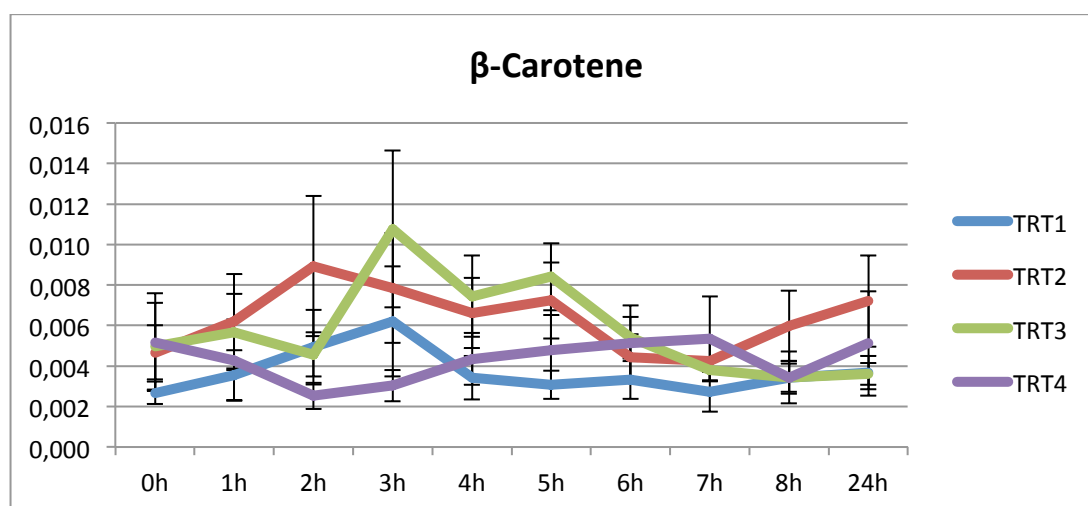


Figure 13. Chylomicron concentration (μg/ml) of β-carotene after consume each meal provided.
 Legend: TRT1= plain bread with plain mayonnaise; TRT2= plain bread with vegetable mayonnaise; TRT3= vegetable bread with plain mayonnaise; TRT4= vegetable bread

For all the carotenoids, except for lutein/zeaxanthin, the highest levels were observed for treatment 3 (vegetable bread with plain mayonnaise) following by treatment 2 (plain bread with vegetable mayonnaise), whose levels rose especially in the case of α and β-carotene. For these two treatments, and for α and β-carotene a notable increase was observed between three and six hours. However lycopene levels exhibited a notable increase during this period only for treatment 3.

As expected, the control meal showed low carotenoid levels. Similar to this the vegetable bread meal (treatment 4) also led to low chylomicron carotenoid levels.

For lutein/zeaxanthin, the carotenoids concentration for all the treatments was approximately the same. Of all the carotenoids, α -carotene showed the lowest concentrations in chylomicrons.

7. Discussion

The data show that carotenoid concentrations both in plasma and in chylomicrons were similar in all four treatments. The lack of significance may be due to the small sub-sample size analysed as part of the thesis. Human data inherently shows large variations and this is evident also in the present data, which shows large standard deviations. Therefore, human studies require larger sample sizes to observe treatment effects and the exact number should be estimated using statistical tools that take into consideration effect sizes and study power.

Regarding to plasma, the data suggest that mayonnaise is related with the absorption of carotenoids. If treatment 4 (vegetable bread) is compared with treatments 3 and 2 (with added mayonnaise in the meal) it is evident that the plasma concentration of carotenoids increases with the incorporation of this emulsion. This agrees with previous studies reported by Takeda S. et al. [21-22] who found that mayonnaise raises the serum β -carotene and lutein/zeaxanthin concentrations. One of the studies attributed this to the emulsifying property of the egg yolk contained in mayonnaise. Furthermore, mayonnaise contains lecithin, which includes phosphatidylcholine (PC). Lysophosphatidylcholine, the lipolysis product of PC, was shown to enhance the β -carotene and lutein concentration. This suggests that lysophosphatidylcholine is important in regulating the absorption of carotenoids (27).

In addition, the data also show that treatments 2, 3, and 4, which have vegetable powder incorporated into the bread or mayonnaise, led to higher plasma carotenoid levels than treatment 1, which does not contain any vegetables. Therefore, it proves that carotenoids can be efficiently absorbed from these foods. Adding vegetable powder to processed foods may be an alternative way of introducing these important compounds into the diet since there is a notable segment of the population who does not consume adequate amounts of fruits and vegetables in UK.

The fact that higher levels of lycopene and β -carotene were observed in plasma may be due to the composition of the vegetable power, which was made up of carrots and tomatoes. The predominant carotenoids in carrots and tomatoes are β -carotene and lycopene respectively.

Carotenoids in the chylomicrons-rich fraction of plasma is considered the gold standard for bioavailability as they represent only those originating from the diet. Plasma typically contains carotenoids, either those endogenously produced or those originating from the diet. Therefore measuring plasma carotenoids alone is insufficient for measuring bioavailability. Carotenoids in the chylomicron-rich fraction provide an accurate picture of how much carotenoid have been absorbed

from the test meals. The data from the study confirm that the chylomicron-rich fraction provides a better picture of carotenoid absorption than whole plasma.

The data from chylomicrons clearly shows that significant increases in the bioavailability of carotenoids occur after the intake of the provided meals. This data also confirms that mayonnaise has a positive impact on the absorption of carotenoids because the two treatments with mayonnaise showed the higher levels (treatment 2 and 3). Therefore these results are in agreement with the importance of fat for carotenoid absorption. Treatment 4, which does not have fat, exhibited low values of carotenoid levels.

The chylomicron-rich fraction data suggests that adding vegetable powder into the bread has more impact on the bioavailability than if it is incorporated into mayonnaise.

As discussed earlier, fiber can lower the bioavailability of carotenoids. In this project white bread was used because it has a low quantity of fiber and therefore did not affect the objectives of the project.

8. Conclusion

Vegetable and fruit consumption has been recommended to prevent the incidence of cancer, coronary heart and another chronic diseases. Epidemiological evidence is compatible with a possible protective role of carotenoids. This project shows that the processed food matrix in which the carotenoids are contained and the consuming of mayonnaise influences their bioavailability.

In this case the food matrix where the carotenoids are contained, bread or mayonnaise, have a positive role on their absorption. The presence of mayonnaise increases the absorption of carotenoids. The data obtained from the chylomicron-rich fraction demonstrates the beneficial effects of mayonnaise better than data obtained from plasma.

All the carotenoids studied were found both plasma and chylomicron-rich fraction, except the β -cryptoxanthin, which does not find in chylomicrons samples.

In conclusion, the study suggests that carotenoids added to processed foods are bioavailable. Furthermore, incorporation of mayonnaise stands as a good vehicle for increasing their bioavailability.

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