Enzyme-Assisted Oil Extraction of Lutein from Marigold (*Tagetes erecta*) Flowers and Stability of Lutein during Storage

Mika Kaimainen[1*], Eila Järvenpää[2] and Rainer Huopalahti[1]

[1]Food Chemistry and Food Development, Department of Biochemistry, University of Turku, 20014 Turku, Finland

[2]Biotechnology and Food Research, MTT Agrifood Research Finland, 31600 Jokioinen, Finland

Abstract

Marigold (*Tagetes erecta*) flowers are a rich source of lutein pigment, which is usually extracted with organic solvents. In this study, lutein was extracted from marigold flowers with enzyme-assisted oil extraction without using organic solvents. Extraction produced oil that contained 0.36 mg/ml lutein (present as lutein esters but calculated as free lutein), and the yield was comparable to solvent extraction. The oil containing lutein was used to produce oil-in-water emulsions with different polysaccharide mixtures as stabilizers and some emulsions were also spray dried. Wet emulsions, dry emulsions, and oil containing lutein were stored in the dark at 20–22°C for 10 weeks, and the amount of lutein esters was monitored during storage. Stability of lutein was good in oil (85% of the initial amount) and in wet emulsions (77–91%) but slightly worse in spray-dried emulsions (67–75%). Enzyme-assisted oil extraction of lutein from marigold flowers is a potential alternative to solvent extraction with comparable efficiency. In addition, there are no solvent residues in the lutein preparation. Preliminary storage tests showed good stability of lutein in oil or in emulsions stored in the dark at room temperature, and these preparations could be used in different food products.

Keywords: *lutein, oil extraction, emulsion, stability, polysaccharides, storage test*

Introduction

Carotenoids are red, orange, or yellow colored terpenoid compounds present in the chloroplasts of plants and they have many functions, including light harvesting and photoprotection (Bartley and Scolnik 1995, Frank and Cogdell 1996). Carotenoids have vitamin A activity, and they have been shown to have antioxidative properties related to many health benefits as reviewed by Kiokias and Gordon (2004) and Jomova and Valko (2013). Lutein is an oxygen-containing carotenoid, and like other carotenoids, it has antioxidative properties, but it is also associated with eye health (Bhattacharyya et al. 2010, Abdel-Aal et al. 2013).

Marigold flowers (*Tagetes sp.*) contain high amounts of carotenoids, which are predominantly fatty acid esters and diesters of lutein, which makes these flowers a good source of lutein pigment (Bhattacharyya et al. 2010, Hadden et al. 1999, Lapshova et al. 2013). Lutein is usually extracted from marigold flowers either as fatty acid esters and diesters, or, if the extraction is combined with saponification, it is extracted as free lutein. In either case, different kinds of organic solvents are used for extraction, and these have to be removed completely by evaporation if the extracted lutein or lutein esters are to be used in food applications.

* Corresponding author: mika.kaimainen@utu.fi

In the European Union, Directive 95/45/EU defines the raw materials and the purity criteria of the food colorants (OJ L 22.9.1995). For lutein pigments (E161b), the directive lists the solvents that can be used in the extraction of free and esterified lutein from certain plant materials. Of these solvents, lutein is most soluble to dichloromethane and acetone and, to a lesser extent, to hexanes and alcohols (Craft and Soares 1992) where free lutein is concerned. Barzana et al. (2002) showed that free lutein is less prone to isomerisation and degradation in hexane, which might be a benefit, although the solubility is lower. It has to be noted, that the solubility of free lutein differs immensely from the solubility of lutein esters in some solvent systems (Amar et al. 2003). Hojnik et al. (2008) conducted modeling of the extraction process using solvents combined with alkaline ethanolysis, resulting in free lutein preparations. In their model, the extraction yield was most affected by the particle size of the biomass. Such benefits can be obtained using careful grinding but also enzyme-assisted extraction. Barzana et al. (2002) tested some combinations of plant cell wall degrading enzymes and hexane extraction, and they reported extraction efficiencies of up to 97 percent.

In our previous studies we extracted carotenoids from chopped Marigold flower heads and chopped whole plants using supercritical carbon dioxide. Both esterified and free lutein in the extracts of green parts and mainly esterified lutein in the extracts of flower heads was obtained (Järvenpää et al. 2008). The extraction was not very efficient, and Ma et al. (2008) demonstrated a supercritical fluid extraction method for lutein where vegetable oil was used as an extraction enhancer. This approach yields lutein in oil where lutein is less prone to degradation than in a concentrated pasta obtained using pure carbon dioxide (Järvenpää et al. 2008). As an example of overall potentiality of obtaining carotenoids from a biomass using new processes, Gouveia et al. (2007) demonstrated the algal oil and carotenoid recovery from microalgae using acetone, vegetable oil, and supercritical carbon dioxide fluid. They reported the best recovery using vegetable oil at room temperature. The recovery correlated directly with how well the biomass was crushed and ground before extraction.

Encapsulation or microencapsulation is a term used for processes in which an active material is totally enveloped in a coating material. Thus, the active material is isolated and protected from the environment. Also the physico-chemical properties, such as solubility characteristics, of the encapsulated material can differ from those of the material without encapsulation. Different microencapsulation techniques, such as emulsification and spray drying, have been used to enhance the stability of carotenoids (Rodriguez-Huezo et al. 2004, Khalil et al. 2012). As carotenoids are mostly oil-soluble, simple oil-in-water (o/w) emulsions can be used to incorporate carotenoids into aqueous foods. Emulsification can also increase the bioavailability of lutein (Vishwanathan et al. 2009).

In our present study, the biomass was first ground, but the release of carotenoids from the biomass was aided using a macerating enzyme mixture containing cellulolysic and pectinolysic activities. Vegetable oil was chosen as an extractant in order to yield true solvent-free lutein preparation useful for the encapsulation studies. Separation of aqueous and oil phases can be conducted using economical techniques also in larger scale, thus the process could be easily scaled-up. We have previously used a polar lipid fraction from oat as an emulsifier for o/w emulsions (Kaimainen et al. 2012). In that study we had some problems with the creaming of emulsions, but we have since managed to retard the creaming by adding small amounts of stabilizing polysaccharides into the emulsion (unpublished data). The aim of the present study was to extract lutein esters from Marigold flowers without organic solvents using enzyme-assisted oil extraction, to produce o/w emulsions with lutein in the oil phase and different stabilizing polysaccharides in the water phase, and to study the stability of lutein during storage. We also spray dried some of the emulsions because dry powders would be easier to store than wet emulsions, and drying could also have an effect on the stability of lutein.

Materials and Methods

In all stages of the experiment, precautions were made to protect the samples from light and oxidation. These included using dim lighting in the laboratory, using opaque containers or covering

transparent containers with aluminum foil, and using an antioxidant during the extraction of lutein.

Materials

The oat polar lipid fraction used as an emulsifier was extracted from oat flakes (*Avena sativa*) using a supercritical fluid process described by Aro et al. (2007), and it consists mainly of different glycollipids (monogalactosyldiacylglycerol, digalactosyldiacylglycerol and steryl glucoside) and phospholipids (phosphatidyl choline) (Kaimainen et al. 2012, Aro et al. 2007). Marigold flowers (*T. erecta L.*) were grown in Rymättylä, Finland. Cellulase enzyme Econase CE (activity 2330 U/ml) was supplied by AB Enzymes (Darmstadt, Germany) and pectinase enzyme Pectinex Ultra SP-L (activity 9500 U/ml) by Novozymes (Bagswaerd, Denmark). Citric acid monohydrate and disodium hydrogen phosphate dihydrate were supplied by J.T. Baker (Deveuter, Netherlands), sodium chloride by Riedel-de Haën (Seelze, Germany), and potassium chloride by Merck (Darmstadt, Germany). All salts were of analytical grade. Free lutein standard was supplied by Extrasynthese (Lyon, France). Guar gum (GG) and locust bean gum (LBG) from *Ceratonia siliqua* seeds were supplied by Sigma Aldrich (St. Louis, MO, United States). Butylated hydroxytoluene (BHT) was supplied by Supelco (Bellefonte, PA, United States). Maltodextrin (MD) C*Sperse 01314 (DE 12.5-15.5) was supplied by Cerestar (Holte, Denmark). Methanol and n-hexane were supplied by J.T. Baker (Deveuter, Netherlands), Acetone by VWR (Fontenay-sous-Bois, France), and Ethyl Acetate by Lab-scan (Sowinskiego, Poland). All solvents were of HPLC grade. Water used was purified reverse osmosis water (Milli-Q Plus ultra-pure water system, Millipore, Molsheim, France). Commercial rapeseed oil (Bunge Finland Oy, Raisio, Finland) and xanthan gum (XG) (Vuohelan Herkkupuoti Ky, Hartola, Finland) were bought at a local grocery store.

Oil Extraction of Lutein Esters from Marigold Flowers

Marigold flowers were suspended in water at proportion 1:9 and treated with cellulase and pectinase (both enzymes 10 ml/l) at room temperature overnight (20–25°C, 18–24 hours) with magnetic stirring. After the enzymatic breakdown of flowers (observed by the loss of solid matter in the suspension), the water suspension was mixed with rapeseed oil at proportion 5:1 at room temperature for one hour with magnetic stirring to extract lutein esters into the rapeseed oil. After extraction, the oil phase was separated from the water phase by centrifuging aliquots of the mixture at 1050 x g for 10 minutes twice and collecting the separated oil after each centrifugation. The oil extraction was repeated with fresh oil mixed with the same water suspension. The amount of lutein esters in oil was determined by making a 20 mg/ml dilution in acetone and analyzing it with HPLC using the same method as in the storage test described below. The oil extracts of the repeated extractions were combined and stored in the dark at 4°C until used for emulsion preparation and storage test.

Preparation of Emulsions and Spray Drying

The emulsions were prepared similarly to our previous research (Kaimainen et al. 2012) with slight modifications. We used a pH of 5.8 instead of 2.6 because even though the oat lipid emulsifier showed higher solubility in lower pH, the polysaccharides used as stabilizers are stable from pH 4 to 10 (Dreher 1999). A pH 5.8 citrate-phosphate buffer was prepared by mixing 0.1 M citric acid solution, 0.2 M disodium hydrogen phosphate solution, and water in proportion 197:303:500, respectively. Oat lipid emulsifier (5 mg/ml) and polysaccharides (3 mg/ml) were dissolved in the buffer with magnetic stirring at 50–60°C for two hours. Four different polysaccharide mixtures were used as stabilizers: guar gum + xanthan gum 1:3 and 3:1 and locust bean gum + xanthan gum 1:3 and 3:1. Emulsions were made by adding 20 mg/ml of oil containing lutein esters and homogenizing with a high speed mixer. Two different equipment was used for emulsification: either an Ultra-Turrax® T 25 Digital disperser with a S 25 N – 10 G dispersing tool (IKA®, Staufen, Germany) at 24 000 rpm for 5 minutes or a Magic LAB® dispersing system with micro-plant assembly and 6F blade (IKA®, Staufen, Germany) at 16 000 rpm for 5 minutes. The Magic LAB equipment was used for producing a slightly larger volume of the emulsions for spray drying for two stabilizer compositions. After emulsification of these larger batches, 200 mg/ml maltodextrin (MD) was added to the emulsion, a small sample was taken for storage test to see the effect of added MD, and the rest of the batch was spray dried with a Mini Spray Dryer B290 (Büchi, Flawil,

Switzerland). The drying conditions were as follows: inlet air temperature 150°C, outlet temperature 84°C, air flow 450 l/h, feed flow 7 ml/min, aspirator 37 m^3/h and nozzle orifice 1.50 mm. A summary of stabilizer compositions used for emulsions and equipment used for emulsification along with code letters is shown in Table 1.

Table 1

Sample codes for emulsion samples, composition of stabilizers used in them and the equipment used for their emulsification. GG = guar gum, XG = xanthan gum, LBG = locust bean gum, MD = maltodextrin.

Sample Code	Stabilizer Composition	Emulsification Equipment
A	GG + XG 3:1	Ultra-Turrax®
B	GG + XG 1:3	Ultra-Turrax®
C	LBG + XG 3:1	Ultra-Turrax®
D	LBG + XG 1:3	Ultra-Turrax®
E	GG + XG 3:1 + MD	Magic LAB®
F	LBG + XG 1:3 + MD	Magic LAB®
G	GG + XG 3:1 + MD, spray dried	Magic LAB®
H	LBG + XG 1:3 + MD, spray dried	Magic LAB®

Storage Test and Extraction of Lutein and Lutein Esters

A total of six wet emulsion samples, two dried emulsion samples (listed in Table 1), and one oil sample was used in the storage test. Samples were stored in dark at 20–22°C in plastic tubes, and the amount of lutein in samples was determined in triplicate after 0, 1, 2, 5 and 10 weeks of storage. The storage conditions were selected based on preliminary tests made with lutein esters in oil, which showed that the stability was good in dark and low temperature and poor in light (unpublished data). Emulsions were also inspected visually at the same time points. Before taking a sample for lutein extraction, the emulsions were gently mixed by turning the containers a few times. For the extraction of lutein and lutein esters, 1 ml of emulsion sample, 200 mg of dried emulsion sample + 1 ml of water or 20 mg of oil + 1 ml of water were transferred into a glass tube with a screw cap. 500 µl of saturated NaCl solution and 100 µl of 2 M citric acid solution was added to break the emulsion and help the extraction process,

as the stabilizing polysaccharides are degraded if pH is lower than 3 (Dreher 1999). The tube was thoroughly mixed with a vortex mixer, after which 2 ml of hexane containing 0.2 mg/ml BHT was added, and the tube was shaken at 500 rpm for 10 minutes. The hexane and water phases were separated by centrifuging at 966 x g for 5 minutes, after which the hexane phase was transferred to another tube containing 2 ml of 8.8 mg/ml KCl solution. The second tube was shaken at 500 rpm for 2 minutes and centrifuged at 966 x g for 5 minutes, after which the washed hexane phase was collected to a third tube. The contents of the first tube were extracted again with 2 ml of hexane containing 0.2 mg/ml BHT, and the procedure was repeated using the same tube and KCl solution in tube 2, and the hexane phases were combined in tube 3. Hexane was evaporated under a stream of nitrogen at 25–30°C, and the residue was dissolved in 1 ml of acetone and filtered through a 0.45 µm PTFE membrane. If not analyzed immediately after extraction, the samples were stored at -80°C until HPLC analysis.

HPLC Analysis of Lutein and Lutein Esters

HPLC analysis of lutein content was performed using a combined and modified method described by Cano (1991) and Monreal et al. (1999). A liquid chromatograph (Shimadzu corporation, Kyoto, Japan) consisting of a GT-154 vacuum degasser, two LC-10AT VP solvent delivery modules, an SIL-10A Auto injector with sample cooler, a CTO-10A column oven, an SPD-M10AVP diode array detector, and an SCL-10 A VP system controller was used with a reversed phase column Luna 5u C18 (length 150 mm, inner diameter 3.00 mm, particle size 5 µm and pore size 95Å, Phenomenex, Torrance, CA, United States). The system was computer-controlled with a Labsolutions version 1.23 SP1 software (Shimadzu Corporation, Kyoto, Japan). A gradient mixture of methanol and water (75:25) as eluent A and ethyl acetate as eluent B was used, raising percentage of B from 0% to 70% during 0–10 minutes, followed by a further rise to 100% during 10–14 minutes, after which the initial conditions of 0% B were restored during 14–20 minutes, and the system was equilibrated for the following run during 20–35 minutes. The column was operated at 40°C, flow rate was kept constant at 1.0 ml/min, injection volume was 10 µl, and samples were kept at 8°C in the sample cooler.

Detection wavelength was 445 nm, and free lutein and lutein esters were quantified as free lutein using a calibration curve made using different concentrations of an external standard of free lutein in acetone. Quantification could be done with free lutein standard, as the esterified fatty acids do not affect the molar absorptivity of lutein.

Statistical Analysis

The external lutein standard calibration curve was made with eight concentration points ranging from 0.70 to 15.6 µg/ml, and each point was calculated as mean value of five replicate analyses. The correlation coefficient of the calibration curve was 0.9996. The amount of lutein in the samples at each time point during the storage test was calculated as mean value of triplicate analyses and compared to the initial amount of each sample. Due to the different sample matrices (wet emulsion, spray dried emulsion or plain oil), the quantitative amounts of lutein varied between samples, and comparison to the initial amount allowed for easier comparison of lutein stability between samples. Statistical differences between samples and time points were analyzed with one-way analysis of variance (ANOVA) together with Tukey's t-test. Differences between time points were also verified with repeated measures ANOVA together with least significant difference test. IBM® SPSS® Statistics version 21 software (IBM Corporation, Armonk, NY, United States) was used in the statistical analyses.

Results and Discussion

The oil extraction of lutein esters from marigold flowers was successful, with a lutein yield (present as lutein esters but calculated as free lutein) of 1.1 mg/g of marigold flowers (fresh weight). The lutein contents of oil extracts were 0.50 mg/ml for the first extract, 0.23 mg/ml for the second extract, and 0.36 mg/ml for the combined extract. The yield of lutein was quite similar to what we extracted from the same marigold flowers using acetone (unpublished data). In literature, the lutein content of marigold flowers is usually reported for dry weight, and it varies widely depending on the variety. Examples of reported lutein contents (per dry matter) for different varieties of *Tagetes erecta* are 0.77–14.4 mg/g (Khalil et al. 2012), 1.61–6.11 mg/g (Li et al. 2007), 0.18–3.0 mg/g (Piccaglia et

al. 1998), and 10.3–17.0 mg/g (Deineka et al. 2007). Deineka et al. (2007) also measured the lutein content for fresh weight, and it was 2.10–2.97 mg/g with approximately 5–6 fold difference to content per dry weight. We do not have the variety data for our marigold flowers, but comparing to these previously reported lutein contents, the lutein yield of 1.1 mg/g flowers (fresh weight) is quite similar.

Initial appearances of the emulsions mixed by different equipment were similar. Spray drying of emulsions produced slightly yellowish powders, which had moisture contents of 60 mg/g. Visual inspection of wet emulsions revealed only one difference during the storage test. The emulsion with GG + XG 3:1 + MD (sample E) had slight creaming after 5 and 10 weeks. The creaming could be due to MD modifying the structure of the water phase or increasing the density difference between the oil and water phases. It could also be an effect of lower speed of mixing of the emulsions made with MD. The visual appearance of the other samples did not change during the 10 week storage period.

The HPLC analysis of the samples revealed peaks for free lutein and lutein esters in all of the samples. The amount of free lutein was 0.5–1.0% of the amount of lutein esters, and the ratio remained constant during the whole storage period. The amount of lutein decreased in all samples during the 10 week storage period, as can be seen in Table 2. Only in sample B was the difference between initial amount and amount after 10 weeks not statistically significant, but even in that sample, the amount after 5 weeks was significantly lower than the initial amount. Retention of lutein during 10 weeks varied between 67% and 91%.

There could be some analysis error in the measurements of samples B, D, and G, as the amount of lutein after one week is nearly 12%, 9% or 6% greater than in the beginning, respectively. The error could be in the measurements of the one week samples but also in the initial amount. If the error is in the initial amount, then it is carried on to other relative values as well. The retention of lutein was worst in the spray dried samples, 75% for sample G and 67% for sample H, although sample G did not differ significantly from the majority of the samples.

Table 2

Retention of lutein during 10 week storage period with standard deviations of triplicate measurements presented as% lutein left compared to initial amount.

Sample	0 weeks	1 week	2 weeks	5 weeks	10 weeks
A	100 ± 1%[a,b]	99 ± 2%[a,1,2]	97 ± 1%[b,1]	83 ± 3%[c,1,2,3]	77 ± 3%[d,1,2]
B	100 ± 5%[a,b]	112 ± 6%[c,d,4]	99 ± 6%[a,c,e,1]	91 ± 5%[e,f,3,4]	91 ± 7%[b,d,f,3]
C	100 ± 2%[a]	101 ± 3%[b,1,2,3]	97 ± 2%[a,b,1]	87 ± 3%[c,2,3,4]	82 ± 3%[d,1,2]
D	100 ± 3%[a,b]	109 ± 3%[a,3,4]	94 ± 3%[b,1,2]	83 ± 3%[c,1,2]	77 ± 3%[d,1]
E	100 ± 2%[a]	98 ± 3%[a,1,2]	91 ± 2%[b,1,2]	81 ± 3%[c,1,2]	80 ± 2%[c,1,2]
F	100 ± 3%[a]	98 ± 4%[a,b,1]	97 ± 3%[a,1]	81 ± 3%[c,1,2]	82 ± 6%[b,c,1,2]
G	100 ± 5%[a,b]	106 ± 5%[a,2,3,4]	94 ± 7%[b,1,2]	79 ± 8%[b,c,1,2]	75 ± 5%[c,1,4]
H	100 ± 1%[a]	99 ± 3%[a,1,2]	88 ± 5%[b,2]	79 ± 2%[b,1]	67 ± 4%[c,4]
oil	100 ± 1%[a]	101 ± 2%[a,1,2,3]	96 ± 2%[b,1]	91 ± 1%[c,4]	85 ± 1%[c,2,3]

Time points not sharing at least one same superscript letter (a-f) are statistically different from each other for each sample ($p \leq$ 0.05, comparison by rows) and samples not sharing at least one same superscript number (1-4) are statistically different from each other in each time point ($p \leq$ 0.05, comparison by columns). See table 1 for sample codes.

With these emulsion compositions, spray drying had a negative effect on the stability of lutein. The reason for this is not clear, but it is possible that the conditions in the spray drying process may have initiated lutein degradation reactions, which then continued during storage. This is in spite of the fact that the temperature of the droplet or particle usually does not rise much during the spray drying process, as time in the hot air is very short and most of the energy is used to evaporate the water (Gharsallaoui et al. 2007). Further reasons may arise from the effect of emulsion and powder structure on the proximity of oxygen species and lutein. The surface of the emulsion is constituted of oat lipids, LBG/GG, XG, and MD, and it could be porous after spray drying and thus, more easily penetrated by oxygen than the coating in wet emulsions. The wall materials can affect the porosity of the microcapsules, and more porous microparticles contain more oxygen available for degradation reactions (Tonon et al. 2010, Ferrari et al. 2012). However, the porosity of our spray dried powders should be assessed to verify this hypothesis.

Carotenoids are sensitive to oxidation initiated by oxygen, light, and heat (Boon et al. 2010). The atmosphere of the sample tubes was not controlled, and there was air over the samples in the tubes, so oxygen was available in the storage conditions. As our samples were stored in dark at 20–22°C, light and heat are not probable causes for the loss of lutein in our samples. The effect of water activity (a_w) on carotenoid stability has been studied for dried laver (*Porphyra*) (Oh et al. 2013), freeze-dried carrots (Lavelli et al. 2007), or spray dried multiple emulsions (Rodriguez-Huezo et al. 2004). Oh et al. (2013) reported increasing carotenoid degradation with increasing storage a_w, whereas Lavelli et al. (2007) reported minimum carotenoid degradation at storage a_w between 0.31 and 0.54 with increasing degradation above or below these values. Rodriquez-Huezo et al. (2004) reported maximum carotenoid degradation rate at a_w 0.628 with smaller and practically constant degradation rate at a_w above or below this value. The effect of a_w on carotenoid stability seems to vary with different sample materials, but it could be one reason for the difference in stabilities of spray dried emulsions and wet emulsions. Although we did not measure the a_w of the samples and did not control the a_w of the storage conditions, it seems reasonable to expect very low a_w values for spray dried samples and high a_w values for emulsion samples. The a_w of the oil sample is also probably quite low. These preliminary observations warrant further studies focusing on the usability of oil and emulsions containing lutein in various food applications. Further studies on emulsions should also contain characterization of emulsions (droplet size and droplet size distribution), which was not done in this study.

Conclusions

Enzyme-assisted oil extraction of lutein without organic solvents was quite effective and produced oil with an adequate amount of lutein. This method can be used for food applications, as no organic solvents were used, and so no evaporation step is needed, and there is no risk of solvent residues. Lutein stability during 10 weeks in o/w emulsions stabilized with different polysaccharides and in oil was quite good. This allows incorporation of lutein in both aqueous and fatty matrices. Spray drying of these emulsions with maltodextrin as wall material seemed to weaken lutein stability during storage. Different wall materials could produce better results, but also the effect on emulsion structure need to be considered. For example, increasing the amount of long chain polysaccharides used as stabilizers will greatly increase the viscosity of the emulsion, or even produce a gel, which will also affect the spray drying process.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

References

Abdel-Aal, E.M., Akhtar, H., Zaheer, K. and Ali, R. (2013), "Dietary sources of lutein and zeaxanthin carotenoids and their role in eye health", *Nutrients,* vol. 5, pp. 1169-1185

Amar, I., Aserin, A. and Garti, N. (2003), "Solubilization Patterns of Lutein and Lutein Esters in Food Grade Nonionic Micro-emulsions", *Journal of Agricultural and Food Chemistry,* vol. 51, no. 16, pp. 4775-4781

Aro, H., Järvenpää, E., Könkö, K., Huopalahti, R. and Hietaniemi, V. (2007), "The characterization of oat lipids produced by supercritical fluid technologies", *Journal of Cereal Science,* vol. 45, no. 1, pp. 116-119

Bartley, G.E. and Scolnik, P.A. (1995), "Plant carotenoids: pigments for photoprotection, visual attraction, and human health". *Plant Cell,* vol. 7, no. 7, pp. 1027-1038

Barzana, E., Rubio, D., Santamaria, R.I., Garcia-Correa, O., Garcia, F., Ridaura Sanz, V.E. and López-Munguía, A. (2002), "Enzyme-Mediated Solvent Extraction of Carotenoids from Marigold Flower (*Tagetes erecta*)", *Journal of Agricultural and Food Chemistry,* vol. 50, no. 16, pp. 4491-4496

Bhattacharyya, S., Datta, S., Mallick, B., Dhar, P. and Ghosh, S. (2010), "Lutein Content and *in Vitro* Antioxidant Activity of Different Culti-vars of Indian Marigold Flower (*Tagetes patula L.*) Extracts", *Journal of Agricultural and Food Chemistry,* vol. 58, no. 14, pp. 8259-8264

Boon, C.S., McClements, D.J., Weiss, J. and Decker, E.A. (2010), "Factors Influencing the Chemical Stability of Carotenoids in Foods". *Critical reviews in food science and nutrition,* vol. 50, no. 6, pp. 515-532

Cano, M.P. (1991), "HPLC Separation of Chloro-phyll and Carotenoid Pigments of Four Kiwi Fruit Cultivars", *Journal of Agricultural and Food Chemistry,* vol. 39, pp. 1786-1791

Craft, N.E. and Soares, J.H. (1992), "Relative solubility, stability, and absorptivity of lutein and β-carotene in organic solvents", *Journal of Agricultural and Food Chemistry,* vol. 40, no. 3, pp. 431-434

Deineka, V.I., Sorokopudov, V.N., Deineka, L.A. and Tret'yakov, M.Y. (2007), "Flowers of mari-gold (*Tagetes*) species as a source of xantho-phylls", *Pharmaceutical Chemistry Journal,* vol. 41, no. 10, pp. 540-542

Dreher, M. (1999), "Food Sources and Uses of Dietary Fiber" in Cho, S.S., Prosky, L. and Dreher, M. (eds.) *Complex Carbohydrates in Foods*, Marcel Dekker Inc., New York, USA, pp. 327-371

Ferrari, C.C., Germer, S.P.M., Alvim, I.D., Vissotto, F.Z. and de Aguirre, J.M. (2012), "Influence of carrier agents on the physico-chemical properties of blackberry powder produced by spray drying", *International Journal of Food Science and Technology,* vol. 47, no. 6, pp. 1237-1245

Frank, H.A. and Cogdell, R.J. (1996), "Carotenoids in photosynthesis", *Photochemistry and photobiology,* vol. 63, no. 3, pp. 257-264

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A. and Saurel, R. (2007), "Applications of spray-drying in microencapsulation of food ingredients: An overview", *Food Research International,* vol. 40, no. 9, pp. 1107-1121

Gouveia, L., Nobre, B.P., Marcelo, F.M., Mrejen, S., Cardoso, M.T., Palavra, A.F. and Mendes, R.L. (2007), "Functional food oil coloured by pigments extracted from microalgae with supercritical CO_2", *Food Chemistry,* vol. 101, no. 2, pp. 717-723

Hadden, W.L., Watkins, R.H., Levy, L.W., Regalado, E., Rivadeneira, D.M., van Breemen, R.B. and Schwartz, S.J. (1999), "Carotenoid Composition of Marigold (*Tagetes erecta*) Flower Extract Used as Nutritional Supplement", *Journal of Agricultural and Food Chemistry,* vol. 47, no. 10, pp. 4189-4194

Hojnik, M., Škerget, M. and Knez, Ž. (2008), "Extraction of lutein from Marigold flower petals – Experimental kinetics and modelling", *LWT - Food Science and Technology,* vol. 41, no. 10, pp. 2008-2016

Järvenpää, E., Tastula, P., Aro, T., Rostiala, N., Hietaniemi, V. and Huopalahti, R. (2008), Stability of lutein and lutein ester preparations obtained using supercritical fluid extraction of *Tagetes erecta*", *Proceedings of the 5th International Congress on Pigments in Food - For Quality and Health*, Helsinki, pp. 133-136

Jomova, K. and Valko, M. (2013), "Health protective effects of carotenoids and their interactions with other biological antioxidants", *European journal of medicinal chemistry,* vol. 70, pp. 102-110

Kaimainen, M., Ahvenainen, S., Kaariste, M., Järvenpää, E., Kaasalainen, M., Salomäki, M., Salonen, J. and Huopalahti, R. (2012), "Polar lipid fraction from oat (*Avena sativa*): characterization and use as an o/w emulsifier", *European Food Research and Technology,* vol. 235, pp. 507-515

Khalil, M., Raila, J., Ali, M., Islam, K.M.S., Schenk, R., Krause, J.-P., Schweigert, F.J. and Rawel, H. (2012), "Stability and bioavailability of lutein ester supplements from *Tagetes* flower prepared under food processing conditions", *Journal of Functional Foods,* vol. 4, pp. 602-610

Kiokias, S. and Gordon, M.H. (2004), "Antioxidant Properties of Carotenoids *In Vitro* and *In Vivo*", *Food Reviews International,* vol. 20, no. 2, pp. 99-121

Lapshova, M.S., Deineka, V.I., Deineka, L.A., Blinova, I.P. and Tret'yakov, M.Y. (2013), "Identification of xanthophylls in a marigold petal extract", *Journal of Analytical Chemistry,* vol. 68, no. 11, pp. 1014-1019

Lavelli, V., Zanoni, B. and Zaniboni, A. (2007), "Effect of water activity on carotenoid degradation in dehydrated carrots", *Food Chemistry,* vol. 104, no. 4, pp. 1705-1711

Li, W., Gao, Y., Zhao, J. and Wang, Q. (2007), "Phenolic, Flavonoid, and Lutein Ester Content and Antioxidant Activity of 11 Cultivars of Chinese Marigold", *Journal of Agricultural and Food Chemistry,* vol. 55, no. 21, pp. 8478-8484

Ma, Q., Xu, X., Gao, Y., Wang, Q. and Zhao, J. (2008), "Optimisation of supercritical carbon dioxide extraction of lutein esters from marigold (*Tagetes erect* L.) with soybean oil as a co-solvent", *International Journal of Food Science and Technology,* vol. 43, no. 10, pp. 1763-1769

Monreal, M., De Ancos, B. and Cano, M.P. (1999), "Influence of Critical Storage Temperatures on Degradative Pathways of Pigments in Green Beans (*Phaseolus vulgaris* Cvs. Perona and Boby)", *Journal of Agricultural and Food Chemistry,* vol. 47, pp. 19-24

Oh, S., Shin, M., Lee, K. and Choe, E. (2013), "Effects of water activity on pigments in dried laver (Porphyra) during storage", *Food Science*

and Biotechnology, vol. 22, no. 6, pp. 1523-1529

OJ L 22.9.1995, "Commission Directive 95/45/EC of 26 July 1995 laying down specific purity criteria concerning colours for use in food-stuffs", *Official Journal of the European Communities,* vol. 226, pp. 1-45

Piccaglia, R., Marotti, M. and Grandi, S. (1998), "Lutein and lutein ester content in different types of *Tagetes patula* and *T. erecta*", *Industrial Crops and Products,* vol. 8, no. 1, pp. 45-51

Rodriguez-Huezo, M.E., Pedroza-Islas, R., Prado-Barragan, L.A., Beristain, C.I. and Vernon-Carter, E.J. (2004), "Microencapsulation by spray drying of multiple emulsions containing carotenoids", *Journal of Food Science,* vol. 69, no. 7, pp. E351-E359

Tonon, R.V., Brabet, C. and Hubinger, M.D. (2010), "Anthocyanin stability and antioxidant activity of spray-dried açai (*Euterpe oleracea* Mart.) juice produced with different carrier agents", *Food Research International,* vol. 43, no. 3, pp. 907-914

Vishwanathan, R., Wilson, T.A. and Nicolosi, R.J. (2009), "Bioavailability of a nanoemulsion of lutein is greater than a lutein supplement", *Nano Biomedicine and Engineering,* vol. 1, pp. 57-73