DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

Stability of Natural Colorants of Plant Origin

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TURKU, FINLAND – 2014

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ISBN 978-951-29-5962-4 (print)
ISBN 978-951-29-5963-1 (pdf)
ISSN 2323-9395 (print)
ISSN 2323-9409 (pdf)
Painosalama Oy – Turku, Finland 2014

TABLE OF CONTENTS

ABSTRACT

In recent years, there have been studies that show a correlation between the hyperactivity of children and use of artificial food additives, including colorants. This has, in part, led to preference of natural products over products with artificial additives. Consumers have also become more aware of health issues. Natural food colorants have many bioactive functions, mainly vitamin A activity of carotenoids and antioxidativity, and therefore they could be more easily accepted by the consumers. However, natural colorant compounds are usually unstable, which restricts their usage. Microencapsulation could be one way to enhance the stability of natural colorant compounds and thus enable better usage for them as food colorants. Microencapsulation is a term used for processes in which the active material is totally enveloped in a coating or capsule, and thus it is separated and protected from the surrounding environment. In addition to protection by the capsule, microencapsulation can also be used to modify solubility and other properties of the encapsulated material, for example, to incorporate fat-soluble compounds into aqueous matrices.

The aim of this thesis work was to study the stability of two natural pigments, lutein (carotenoid) and betanin (betalain), and to determine possible ways to enhance their stability with different microencapsulation techniques. Another aim was the extraction of pigments without the use of organic solvents and the development of previously used extraction methods. Stability of pigments in microencapsulated pigment preparations and model foods containing these were studied by measuring the pigment content after storage in different conditions. Preliminary studies on the bioavailability of microencapsulated pigments and sensory evaluation for consumer acceptance of model foods containing microencapsulated pigments were also carried out.

Enzyme-assisted oil extraction was used to extract lutein from marigold (*Tagetes erecta*) flower without organic solvents, and the yield was comparable to solvent extraction of lutein from the same flowers. The effects of temperature, extraction time, and beet:water ratio on extraction efficiency of betanin from red beet (*Beta vulgaris*) were studied and the optimal conditions for maximum yield and maximum betanin concentration were determined. In both cases, extraction at 40 °C was better than extraction at 80 °C and the extraction for five minutes was as efficient as 15 or 30 minutes. For maximum betanin yield, the beet:water ratio of 1:2 was better, with possibly repeated extraction, but for maximum betanin concentration, a ratio of 1:1 was better.

Lutein was incorporated into oil-in-water (o/w) emulsions with a polar oil fraction from oat (*Avena sativa*) as an emulsifier and mixtures of guar gum and

xanthan gum or locust bean gum and xanthan gum as stabilizers to retard creaming. The stability of lutein in these emulsions was quite good, with 77 to 91 percent of lutein being left after storage in the dark at 20 to 22°C for 10 weeks whereas in spray dried emulsions the retention of lutein was 67 to 75 percent. The retention of lutein in oil was also good at 85 percent.

Betanin was incorporated into the inner w_1 water phase of a $water_1$-in-oil-in-$water_2$ ($w_1/o/w_2$) double emulsion with primary w_1/o emulsion droplet size of 0.34 μm and secondary $w_1/o/w_2$ emulsion droplet size of 5.5 μm and encapsulation efficiency of betanin of 89 percent. *In vitro* intestinal lipid digestion was performed on the double emulsion, and during the first two hours, coalescence of the inner water phase droplets was observed, and the sizes of the double emulsion droplets increased quickly because of aggregation. This period also corresponded to gradual release of betanin, with a final release of 35 percent. The double emulsion structure was retained throughout the three-hour experiment.

Betanin was also spray dried and incorporated into model juices with different pH and dry matter content. Model juices were stored in the dark at -20, 4, 20–24 or 60 °C (accelerated test) for several months. Betanin degraded quite rapidly in all of the samples and higher temperature and a lower pH accelerated degradation. Stability of betanin was much better in the spray dried powder, with practically no degradation during six months of storage in the dark at 20 to 24 °C and good stability also for six months in the dark at 60 °C with 60 percent retention. Consumer acceptance of model juices colored with spray dried betanin was compared with similar model juices colored with anthocyanins or beet extract. Consumers preferred beet extract and anthocyanin colored model juices over juices colored with spray dried betanin. However, spray dried betanin did not impart any off-odors or off-flavors into the model juices contrary to the beet extract.

In conclusion, this thesis describes novel solvent-free extraction and encapsulation processes for lutein and betanin from plant sources. Lutein showed good stability in oil and in o/w emulsions, but slightly inferior in spray dried emulsions. *In vitro* intestinal lipid digestion showed a good stability of $w_1/o/w_2$ double emulsion and quite high retention of betanin during digestion. Consumer acceptance of model juices colored with spray dried betanin was not as good as model juices colored with anthocyanins, but addition of betanin to real berry juice could produce better results with mixture of added betanin and natural berry anthocyanins could produce a more acceptable color. Overall, further studies are needed to obtain natural colorants with good stability for the use in food products.

SUOMENKIELINEN ABSTRAKTI

Viime vuosien tutkimuksissa on havaittu yhteys lasten yliaktiivisuuden ja keinotekoisten elintarvikelisäaineiden, kuten väriaineiden, kanssa. Tämä on osaltaan vaikuttanut luonnollisten tuotteiden, jotka eivät sisällä keinotekoisia lisäaineita, suosion kasvuun. Lisäksi kuluttajat ovat entistä paremmin tietoisia ruoan terveysvaikutuksista. Luonnonväreillä on monia bioaktiivisia ominaisuuksia, kuten karotenoidien A-vitamiiniaktiivisuus, ja monet luonnonvärit myös toimivat antioksidantteina. Tämän vuoksi luonnonvärit voisivat olla kuluttajien hyväksyttävissä elintarvikkeiden väriaineina. Tosin monet luonnonvärit hajoavat herkästi, mikä rajoittaa niiden käyttöä elintarvikkeissa. Mikrokapseloinnilla voidaan parantaa luonnonvärien pysyvyyttä ja siten niiden käyttömahdollisuuksia elintarvikeväreinä. Mikrokapselointi tarkoittaa jonkin aktiivisen aineen päällystämistä kuoreen tai kapseliin siten, että se on täysin eristetty ympäristöstään. Suojaamisen lisäksi mikrokapselointia voidaan käyttää aineen liukoisuus- ja muiden ominaisuuksien muokkaamiseen.

Tämän väitöskirjatyön tarkoituksena oli tutkia kahden luonnonvärin, luteiinin (karotenoidi) ja betaniinin (betalaiini) pysyvyyttä ja mahdollisuuksia parantaa niiden pysyvyyttä erilaisilla mikrokapselointitekniikoilla. Lisäksi tavoitteena oli väriaineiden uutto kasvimateriaalista ilman orgaanisia liuottimia ja aiemmin kehitettyjen uuttomenetelmien jatkokehitys tehokkaammiksi. Väriaineiden pysyvyyttä mikrokapseloiduissa valmisteissa ja niitä sisältävissä mallielintarvikkeissa tutkittiin määrittämällä väriaineiden pitoisuudet erimittaisten säilytysaikojen jälkeen eri olosuhteissa. Työssä tehtiin myös alustavia esikokeita mikrokapseloitujen väriaineiden biosaatavuudesta ja mallielintarvikkeiden aistinvaraisista ominaisuuksista.

Luteiinia uutettiin samettikukista (*Tagetes erecta*) entsyymiavusteisella öljyuutolla, ja uuttosaanto oli vertailukelpoinen liuotinuuton kanssa. Lämpötilan, uuttoajan ja punajuuri:vesi-suhteen vaikutusta betaniinin uuttotehokkuuteen punajuuresta (*Beta vulgaris*) tutkittiin ja parhaimmat olosuhteet määritettiin suurimmalle betaniinisaannolle sekä suurimmalle uutteen betaniinipitoisuudelle. Molemmissa tapauksissa uutto 40 °C:ssa oli parempi kuin 80 °C:ssa, ja jo viiden minuutin uutto oli yhtä tehokas kuin 15 tai 30 minuutin uutto. Suurin betaniinin saanto saatiin punajuuri:vesi-suhteella 1:2, mahdollisesti vielä toistetulla uutolla, kun taas suurin betaniinipitoisuus uutteelle saatiin suhteella 1:1.

Luteiinia lisättiin öljy vedessä (o/w) -emulsioon, jossa oli kauran (*Avena sativa*) polaarista lipidijaetta emulgaattorina ja guarkumin ja ksantaanikumin tai johanneksenleipäpuujauheen ja ksantaanikumin seoksia stabilointiaineina

hidastamaan emulsion kermoittumista. Luteiinin pysyvyys emulsioissa oli melko hyvä; 10 viikkoa pimeässä 20–22 °C:ssa säilytetyissä emulsioissa oli vielä 77–91 prosenttia luteiinia jäljellä, kun taas sumutuskuivatuissa emulsioissa luteiinia oli säilytyksen jälkeen jäljellä 67–75 prosenttia. Öljyssä säilytettynä luteiinia oli 10 viikon jälkeen jäljellä 85 prosenttia.

Betaniinia lisättiin vesi öljyssä vedessä (w/o/w) -kaksoisemulsion sisempään vesifaasiin. Sisemmän vesi öljyssä -emulsion pisarakoko oli 0.34 µm ja ulomman w/o/w-kaksoisemulsion pisarakoko oli 5.5 µm ja betaniinin kapselointitehokkuus oli 89 prosenttia. Kaksoisemulsiolle tehtiin *in vitro* ruoansulatuskoe (pelkkä suolisto-osa), ja kahden ensimmäisen tunnin aikana sisemmän emulsion vesipisarat sulautuivat osittain yhteen ja ulomman emulsion öljypisarat aggregoituivat. Tässä ajassa tapahtui myös betaniinin vapautuminen sisemmästä vesifaasista, ja lopullinen betaniinin häviö oli 35 prosenttia. Kaksoisemulsion rakenne säilyi kuitenkin koko kokeen ajan.

Betaniinia sumutuskuivattiin ja lisättiin mallimehuihin, joissa oli eri pH ja kuiva-ainepitoisuus. Mallimehuja säilytettiin pimeässä -20 °C:ssa, 4 °C:ssa, 20–24 °C:ssa tai 60 °C:ssa (nopeutettu koe) useita kuukausia. Betaniini hajosi melko nopeasti kaikissa näytteissä ja korkea lämpötila sekä matala pH nopeuttivat hajoamista. Betaniini säilyi paljon paremmin kuivattuna jauheena säilytettynä; huoneenlämmössä säilytettynä käytännössä mitään muutoksia ei tapahtunut 6 kuukauden aikana, ja 60 °C:ssa säilytettynäkin 60 prosenttia betaniinista oli jäljellä 6 kuukauden jälkeen. Mallimehuille tehtiin myös kuluttajatesti aistinvaraisella arviolla, jossa sumutuskuivatulla betaniinilla värjätyn mallimehun ulkonäön, hajun ja maun miellyttävyyttä verrattiin muuten samanlaiseen, mutta antosyaaneilla tai punajuuriuutteella värjättyyn mallimehuun. Kuluttajat pitivät enemmän punajuuriuutteella tai antosyaaneilla värjätyistä mallimehuista kuin sumutuskuivatulla betaniinilla värjätyistä mallimehuista, mutta sumutuskuivatusta betaniinista ei tullut mallimehuihin mitään punajuureen viittaavia virhehajuja tai –makuja.

Tässä väitöskirjassa kuvataan luteiinin ja betaniinin uuttomenetelmiä kasvimateriaalista ilman orgaanisia liuottimia sekä väriaineiden mikrokapselointiprosesseja. Luteiinin pysyvyys öljyssä ja emulsiossa oli hyvä, mutta sumutuskuivatuissa emulsioissa hieman huonompi. *In vitro* ruoansulatuskoe osoitti w/o/w kaksoisemulsion hyvän pysyvyyden ja betaniinin tehokkaan säilymisen kaksoisemulsiossa ruoansulatuskokeen aikana. Kuluttajatesti osoitti, että kuluttajat pitävät enemmän antosyaaneilla värjätyistä mallimehuista kuin sumutuskuivatulla betaniinilla värjätyistä mallimehuista, mutta tilanne voisi olla toinen oikeilla marjamehuilla, jolloin lisätyn betaniinin ja marjojen antosyaanien seos voisi muodostaa hyväksyttävämmän värin. Kaiken kaikkiaan, lisätutkimuksia tarvitaan, jotta luonnonvärien pysyvyys saadaan riittäväksi elintarvikekäyttöä varten.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
a_w	water activity
BHT	butylated hydroxytoluene
D3,2	surface mean diameter (Sauter mean diameter)
D4,3	volume mean diameter (De Brouckere mean diameter)
DE	dextrose equivalent
DGDG	digalactosyldiacylglycerol
DLS	dynamic light scattering
DP	degree of polymerization
GG	guar gum
HPLC	high performance liquid chromatograph(y)
LBG	locust bean gum
LD	(Fraunhofer) laser diffraction
MD	maltodextrin
MGDG	monogalactosyldiacylglycerol
o/w (emulsion)	oil-in-water (emulsion)
o/w/o (emulsion)	oil-in-water-in-oil (emulsion)
PC	phosphatidyl choline
PCA	principal component analysis
PCR	principal component regression
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PGPR	polyglycerol polyricinoleate
PI	phosphatidyl inositol
PP	polypropylene
PS	phosphatidyl serine
PTFE	polytetrafluoroethylene
RH	relative humidity
ROS	reactive oxygen species
SG	steryl glucoside
UV-VIS	ultraviolet and visible (light)
w/o (emulsion)	water-in-oil (emulsion)
w/o/w (emulsion)	water-in-oil-in-water (emulsion)
XG	xanthan gum

LIST OF ORIGINAL PUBLICATIONS

I Kaimainen, M.; Ahvenainen, S.; Kaariste, M.; Järvenpää, E.; Kaasalainen, M.; Salomäki, M.; Salonen, J.; Huopalahti, R. Polar lipid fraction from oat (*Avena sativa*): characterization and use as an o/w emulsifier. *Eur. Food Res. Technol.* **2012**, *235*, 507–515, DOI: 10.1007/s00217-012-1780-1.

II Kaimainen, M.; Järvenpää, E.; Huopalahti, R. Enzyme-assisted oil extraction of lutein from marigold (*Tagetes erecta*) flowers and stability of lutein during storage. *Submitted.*

III Kaimainen, M.; Marze, S.; Järvenpää, E.; Anton, M.; Huopalahti, R. Encapsulation of betalain into w/o/w double emulsion and release during *in vitro* intestinal lipid digestion. *LWT – Food Science and technology,* **2015**, 60, 899–904, DOI: 10.1016/j.lwt.2014.10.016

IV Kaimainen, M.; Laaksonen, O.; Järvenpää, E.; Sandell, M.; Huopalahti, R. Consumer acceptance and stability of spray dried betanin in model juices. *Submitted*.

1 INTRODUCTION

Plants can be found in a multitude of colors and color hues. These many colors are due to different pigment compounds, which mainly fall into four compound classes: anthocyanins, betalains, carotenoids, and chlorophylls. A notable exception is the enzymatic browning caused by polymerization of phenols, usually as a result of tissue damage. However, it can be argued that while strictly speaking natural, this kind of coloration is not native to the plant. Natural pigment compounds have other functions in plants as well, and many of them have for example, antioxidative properties.

The color of food is a very important factor for consumers. This is because usually visual appearance is the first attribute to evaluate the quality of food, and color is a major part of visual appearance (van der Laan et al. 2011, van der Laan et al. 2015). When consumers are selecting and purchasing foods, visual appearance may be the only factor they can evaluate before making a purchase decision. Therefore, food producers should pay attention to the visual appearance (e.g. color) of the foods they produce and sell. Some food products are packed into opaque packages, but even in these products, the visual appearance of the food when taken out of the package is important to the consumer, and it may affect future purchase decisions.

Another quite important factor that consumers can evaluate before making purchase decisions is the image or impression of the product, for example, ethical and environmental considerations and the healthiness of the food. Regarding this, a large part of consumers seems to appreciate naturalness of products. Usually this means, for example, that no additives are used, but if they are needed, natural additives may be favored over artificial ones. Especially, reports of relation between artificial additives and hyperactivity in children has decreased consumer acceptance of artificial additives (McCann et al. 2007, Nigg et al. 2012). Thus, natural colorants have the potential to be used as ‘acceptable’ additives in foods as they are natural and they also have potential health benefits. However, natural pigment compounds usually have poor stability as compared to artificial food colorants, which hinders their usage.

Microencapsulation is a term used for processes in which the active ingredient is enveloped into a coating or capsule in a micrometer scale (typically less than 300 μm). If the scale is in submicron range, the term nanoencapsulation can be used. Usually, the technique involves also controlled release. This means that the active ingredient can be released from the capsule when a suitable triggering condition (for example, temperature or pH) is reached. Microencapsulation could be used to enhance the stability of natural pigments and thus allow their better utilization.

The following literature review focuses on the naturally occurring pigment compounds in plants with emphasis on their stability, and microencapsulation techniques used in the food industry. Special attention is given to microencapsulation studies conducted with pigment compounds studied in this thesis, namely carotenoids and betalains.

The experimental part of this thesis work is focused on two natural pigments: lutein from marigold (*Tagetes erecta*) flowers (a hydrophobic carotenoid pigment) and betanin (a hydrophilic betalain pigment) from red beet (*Beta vulgaris*). Pigments were extracted from plant material without organic solvents and extraction methods were developed to be more efficient. Emulsification and spray drying were used to encapsulate these pigments and the stability of pigments was studied in encapsulated pigment preparations and in model foods containing them. This thesis work also included preliminary studies on the bioavailability of encapsulated pigments and sensory evaluations of model foods containing encapsulated pigments to assess their consumer acceptance.

2 REVIEW OF THE LITERATURE

2.1 Natural pigments in plants

The various colors of plants are typically due to four pigment compound classes: carotenoids, betalains, anthocyanins, and chlorophylls. In addition to these four major pigment classes, there are some less common pigments, which are not discussed in this thesis work. Usually pigments from several classes are present in the same plant, but in various amounts in different parts of the plant. Different pigment compounds have variable colors but in plants these compounds are important for their other properties and functions. Possibly for the plant, color is only important for the coloration of flowers and fruits to attract insects and other animals for pollination and dispersion of seeds.

2.1.1 Chlorophylls

Chlorophylls are the major light-harvesting green pigments in photosynthetic organisms, including green plants, algae and bacteria (Schwartz et al. 2008). Chlorophylls are essential in photosynthesis, the pathway to transform energy from the sun into chemical energy (in the form of glucose), and thus they have enabled life as we know to originate and evolve.

2.1.1.1 Structure

Chlorophylls are tetrapyrrole compounds similar to heme pigments in meat. They have a substituted tetrapyrrole porphyrin ring with a coordinated magnesium ion in the middle and an esterified 20-carbon monounsaturated phytol group that makes these compounds partially oil-soluble, although the porphyrin ring gives the molecule polar characteristics as well. Several chlorophylls are found in nature, which differ in the porphyrin ring substituents, but in green plants only two variants, chlorophylls *a* and *b*, are found in an approximate ratio of 3:1. Structures of chlorophylls *a* and *b* differ only on one substituent and they are shown in figure 1. (Scheer 1991, Schwartz et al. 2008)

Chlorophylls have typically two absorption bands in the UV-VIS spectrum: one in the red region between 600 and 700 nm and the other in blue region between 400 and 500 nm. These absorption bands can be altered slightly by removing the coordinated magnesium (producing pheophytins) or replacing it with some other metal ion such as zinc or copper. The removal of the esterified phytol group (producing chlorophyllides) has only a very little effect on the UV-VIS absorption of chlorophyll compounds. (Schwartz et al. 2008)

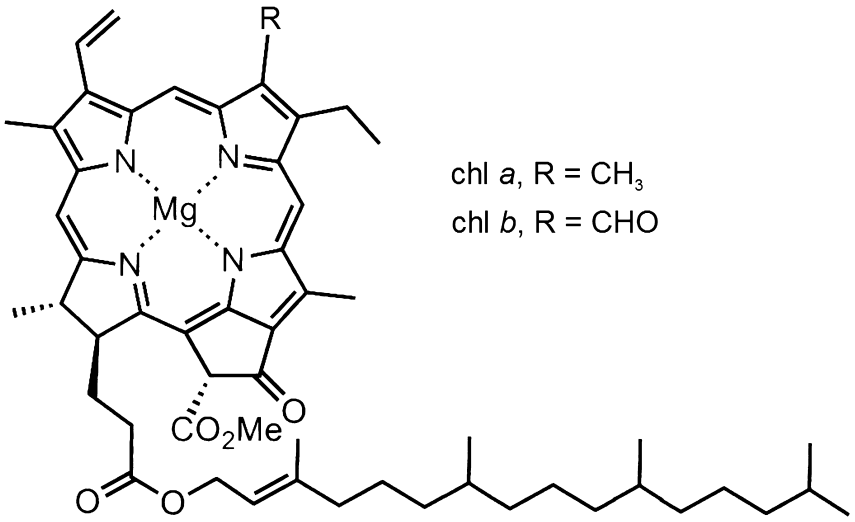


Fig. 1 Structure of chlorophylls *a* and *b*. Adapted from Schwartz et al. 2008.

2.1.1.2 Functions and bioactivity

The main function of chlorophylls is undoubtedly energy production *via* photosynthesis. The basic mechanism of photosynthesis has been known for a long time (Warburg 1958, Whittingham 1970, Hendry 1996), but the details of the mechanism are still studied nowadays (Sztatelman et al. 2010, Galka et al. 2012, Ashraf & Harris 2013). The role of chlorophyll in photosynthesis and the role of photosynthesis to the plant's well-being are so important that the amount of chlorophylls can be used to provide estimates on the photosynthetic potential and physiological status of the plant (Curran et al. 1990, Gitelson et al. 2003, Xiong et al. 2013).

Chlorophylls are abundant in all green plants and therefore they are consumed in considerable quantities by humans. Surprisingly, their fate in the gastrointestinal tract and possible health effects are not as widely studied as, for example, those of carotenoids. However, there are some reports on potential health effects of chlorophylls and their derivatives. Chlorophylls and their derivatives seem to have anticarcinogenic (Blum et al. 2003, de Vogel et al. 2005a, de Vogel et al. 2005b, Ferruzzi & Blakeslee 2007) and antimutagenic (Chernomorsky et al. 1999, Ferruzzi et al. 2002) properties. Chlorophylls and structurally related tetrapyrroles activate cytoprotective genes (Fahey et al. 2005) and natural chlorophylls have chemoprotective properties (Harttig & Bailey 1998). Chlorophylls seem to also have antioxidant activity or at least they are abundant components in plant extracts with antioxidant activity (Cervantes-Paz et al. 2014, Viacava et al. 2014). Recently, pheophorbide *a*, a water-soluble degradation product of chlorophyll *a*, has been reported to have antiviral effect against hepatitis C virus (Ratnoglik et al. 2014).

2.1.1.3 Stability

Vast amounts of chlorophyll are degraded every autumn as most plants undergo autumn senescence and the leaves turn from green to yellow or red. In addition to this autumn degradation, chlorophyll is constantly synthetized and degraded during the plant's whole life and it seems that the half-life of chlorophyll lengthens as the plant matures (Hendry et al. 1987, Hendry 1996). Chlorophyll breakdown in autumn has been widely studied for a long time (Hendry et al. 1987, Merzlyak & Gitelson 1995, Sytykiewicz et al. 2013, Christ & Hortensteiner 2014, Kraeutler 2014). It has been found that chlorophylls are broken into linear tetrapyrrole structures that are stored in vacuoles, but the exact mechanism of this process is still not fully known (Christ & Hortensteiner 2014, Kraeutler 2014).

Stability of chlorophylls in foods has also been studied, widely. Heat and acidity are two major process conditions that enhance chlorophyll degradation (Schanderl et al. 1962, Schwartz et al. 1981, LaBorde & von Elbe 1994, Schwartz et al. 2008). The first change promoted by heat is the inversion of the carbomethoxy group at C10, and this isomerization does not affect the color of the molecule (Schwartz et al. 1981, Schwartz et al. 2008). The loss of magnesium happens easily in an acidic environment with heat, and this produces olive-brown pheophytins (Schanderl et al. 1962, Bacon & Holden 1967, Schwartz et al. 1981, LaBorde & von Elbe 1994, Schwartz et al. 2008). Although the conversion to pheophytins does not occur in alkaline environment ($pH \geq 9$), during heating of plant tissues, the cells may break down and the pH decreases as acids are released (Schwartz et al. 2008). In acidic media, the released magnesium ion can be replaced with zinc or copper ions, and the resulting complexes have similar green color to natural chlorophylls and they are stable in an acidic environment (LaBorde & von Elbe 1994, Hendry 1996, Schwartz et al. 2008).

2.1.2 Carotenoids

Carotenoids are red, orange, or yellow colored oil-soluble terpenoid compounds located in the chloroplasts and chromoplasts of plants and they have many functions in plants, including light-harvesting and photoprotection (Bauernfeind 1972, Bartley & Scolnik 1995, Frank & Cogdell 1996). Carotenoids are found in all photosynthetic plant tissues and they are probably most widespread pigments in nature (Schwartz et al. 2008).

2.1.2.1 Structure

The basic carotenoid structure consists of eight isoprene units that form a 40-carbon backbone (Schwartz et al. 2008) as can be seen in figure 2. There are detailed rules for carotenoid numbering and nomenclature (IUPAC 1975), and the numbering of carotenoid carbons is also shown in figure 2. Despite the accurate systematic names, trivial names of carotenoids are used commonly. Carotenoids have extensive conjugated diene system almost throughout the whole molecule, and in nature the double bonds are often in *trans* (E) configuration, but also *cis* (Z) configurations exist in nature (Bauernfeind 1972, Britton 1996). Figure 2 shows the conjugated double bond system that is common to all carotenoids, but usually there are more double bonds in the end groups (carbons 1-6 and 1'-6'), which may not be part of the conjugated system.

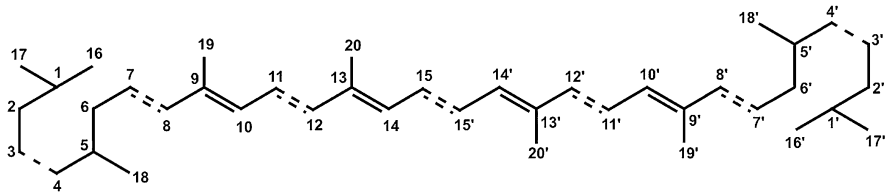


Fig. 2 Basic carotenoid backbone with dashed bonds between isoprenoid units for clarity. General carbon numbering for carotenoids and conjugated double bond system of the backbone are also shown. Adapted and combined from IUPAC 1975 and Schwartz et al. 2008.

Carotenoids can be divided into two structural groups based on the elements present in them: carotenes contain only carbon and hydrogen atoms (i.e., they are hydrocarbons) and xanthophylls contain also oxygen in the form of hydroxyl, epoxy, aldehyde and/or keto groups. In nature, xanthophylls containing hydroxyl groups can be present also as fatty acid esters. Many carotenoids contain cyclic end groups, but some carotenoids (for example, lycopene) are acyclic (IUPAC 1975, Britton 1996, Schwartz et al. 2008). Carotenoids can also have shorter or cleaved backbone (for example, bixin), in which case, they are referred to as apocarotenoids (IUPAC 1975). Examples of common carotenoids with different structures are shown in figure 3.

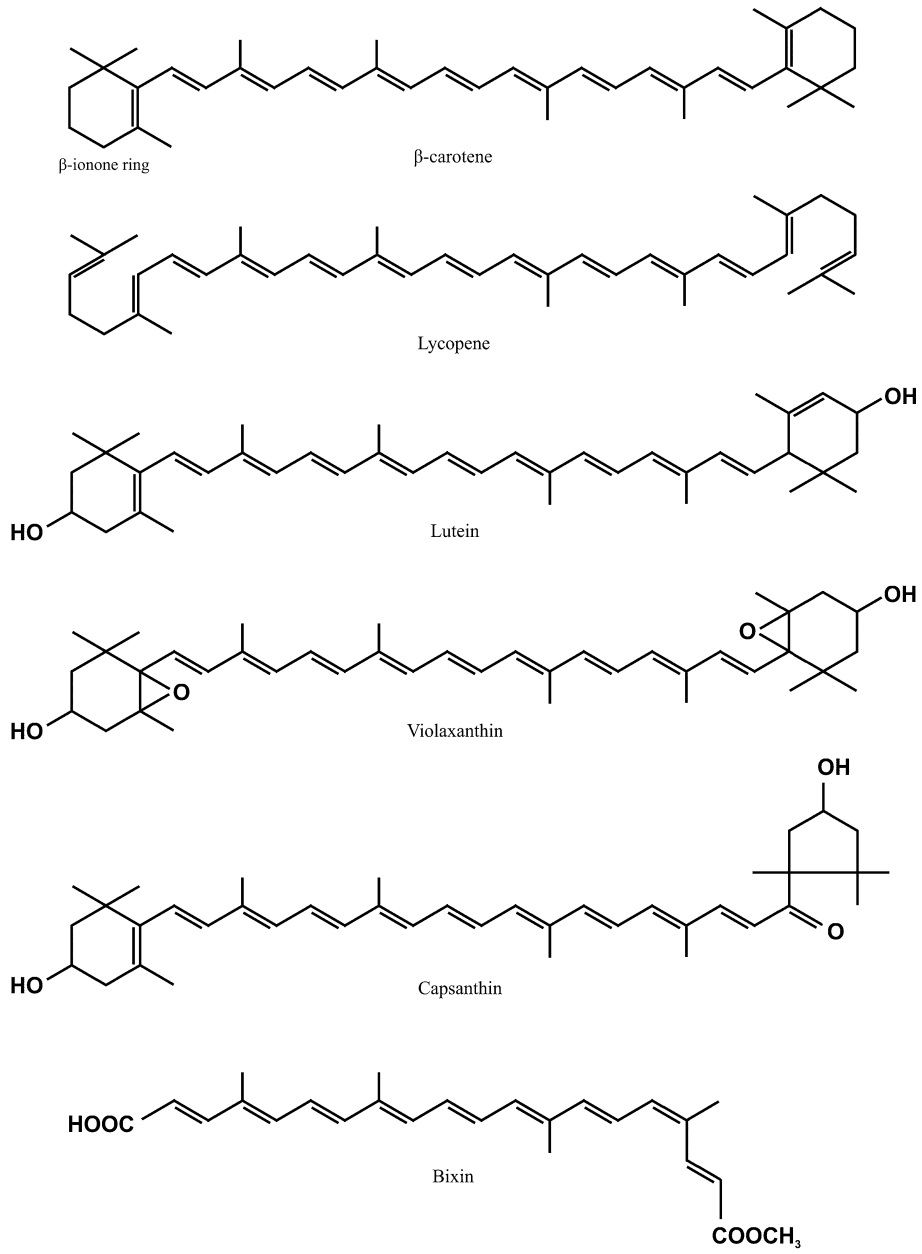


Fig. 3 Structures of common carotenoids. Adapted from Schwartz et al. 2008.

2.1.2.2 Functions and bioactivity

The primary functions of carotenoids in plants depend on the location: in photosynthetic tissues, the main functions are light-harvesting and photoprotection, and in other tissues mainly contribution to color of flowers and fruits and act as antioxidants (Bartley & Scolnik 1995, Britton 1996,

Vershinin 1999, Howitt & Pogson 2006). Carotenoids absorb light at the blue-green region of the spectrum (400-600 nm), which is in the middle of the two absorption regions of chlorophylls, and they can transfer the absorbed energy to chlorophylls in chloroplasts (Bartley & Scolnik 1995, Frank & Cogdell 1996, Vershinin 1999). This makes it possible for plants to use a broader part of the light spectrum for photosynthesis and is the basis of the light-harvesting function of carotenoids. The photoprotection function of carotenoids in photosynthetic tissues involves an energy transfer from excited triplet state chlorophylls to carotenoids and quenching of singlet oxygen to avoid irreparable damage to tissues (Bartley & Scolnik 1995, Britton 1996, Frank & Cogdell 1996, Vershinin 1999). The presence of carotenoids has also been shown to be critical for the plant's survival, as mutations inhibiting carotenoid biosynthesis results in plants that can only live in dim lighting and this is mainly attributed to the photoprotection function of carotenoids (Bartley & Scolnik 1995, Demmig-Adams et al. 1996, Vershinin 1999). In non-photosynthetic tissues, carotenoids usually accumulate in chromoplasts and their primary function seems to be to produce color of fruits and flowers to attract insects and animals for pollination and dispersion of seeds (Bartley & Scolnik 1995, Vershinin 1999, Howitt & Pogson 2006). In addition, they also act as antioxidants in these tissues and are precursors to scent molecules (Howitt & Pogson 2006). The antioxidant properties of carotenoids are due to their ability to quench free radicals and reactive oxygen species (ROS) and the mechanism is quite similar to their photoprotective function. Recently, it has also been shown that carotenoid oxidation products can act as stress signal molecules in plants (Havaux 2013). When carotenoids quench ROS, they can oxidize and become cleaved into carotenoid derivatives, part of which are bioactive compounds that can affect gene expression and thus help the plant to acclimate to stress conditions.

When ingested, carotenoids have vitamin A activity, and they have antioxidative properties related to many health benefits, as reviewed by, for example, Krinsky (1989), Kiokias & Gordon (2004) and Jomova & Valko (2013). Vitamin A activity requires that the carotenoid has at least one β-ionone ring (see β-carotene in figure 3 for structure) so that it can act as a retinoid precursor and thus β-carotene, which has two β-ionone rings, has the greatest vitamin A activity of all carotenoids (Bauernfeind 1972, Britton 1996, Schwartz et al. 2008, Tang & Russell 2009). As already discussed in functions of carotenoids section, they are powerful antioxidants, and they continue to have these properties when ingested. The antioxidant properties repair oxidative DNA damage and a high intake of carotenoids is correlated to reduced risk of certain diseases, such as coronary heart disease and different types of cancer (Glei et al. 2000, Kiokias & Gordon 2004, Amiot-Carlin et al.

2008, Johnson & Krinsky 2009, Rock 2009, Yeum et al. 2009). Two xanthophylls, lutein and zeaxanthin, accumulate in the retina of eye and they are strongly correlated with eye health and prevention of age-related macular degeneration (Alves-Rodrigues & Shao 2004, Amiot-Carlin et al. 2008, Schalch et al. 2009, Bhattacharyya et al. 2010, Ma & Lin 2010, Abdel-Aal et al. 2013). It is hypothesized that these xanthophylls protect the retina by absorbing highly energetic blue light *via* their antioxidant properties but the role of these carotenoids in the human eye is not fully understood (Alves-Rodrigues & Shao 2004, Schalch et al. 2009, Ma & Lin 2010).

2.1.2.3 Stability

The same extensive conjugated double bond system that makes carotenoids powerful antioxidants also makes them susceptible to oxidation (Schwartz et al. 2008, Boon et al. 2010). In intact plant tissues, carotenoids are usually well protected from oxidation but when tissues are damaged, the cells break down and the environment changes and is more favorable to oxidation (Schwartz et al. 2008). Oxidation of carotenoids can be initiated by many oxidizing agents and the oxidation may process *via* different mechanisms, which are shown in figure 4 (Boon et al. 2010). Due to the many double bonds in carotenoid structure and variability in oxidation mechanisms, the oxidation may result in a multitude of products from isomerization to extensive cleavage of the molecule (Schwartz et al. 2008, Boon et al. 2010). Isomerization of *trans* double bonds to *cis* isomers occurs readily due to many conditions such as heat, organic solvents, acid, and light (Schwartz et al. 2008). Isomerization to *cis* isomers does not affect the spectral properties of the carotenoid compound but the possible provitamin A activity is decreased or lost (Schwartz et al. 2008). Isomerization is thought to proceed *via* carbocation carotenoid intermediates (Konovalov & Kispert 1999, Boon et al. 2010). Studies of Craft and Soares (1992) and Henry et al. (1998) showed that lutein is slightly more stable than β-carotene when stored in various solvents at room temperature or in safflower seed oil at elevated temperatures. Subagio et al. (1999) also showed that esterification of the hydroxyl groups of lutein with myristic acid improved the stability of lutein against UV light degradation. In food products, carotenoids are very sensitive to heat so processes involving heat treatments should be kept at minimum both for temperature and time, and storage in frozen temperatures is advised for maximum carotenoid stability (Schwartz et al. 2008, Abdel-Aal et al. 2010, Cerón-García et al. 2010, Rubio-Diaz et al. 2010, Wenzel et al. 2010, Cervantes-Paz et al. 2014).

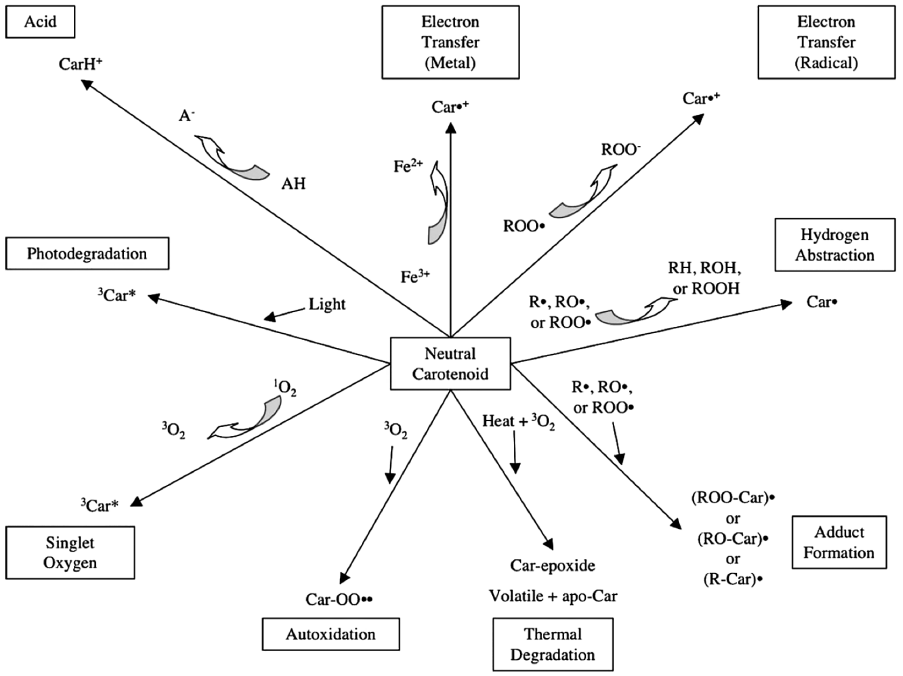


Fig. 4 Carotenoid oxidation pathways. Reprinted from Boon et al. 2010 with permission. Copyright 2010 Taylor and Francis Group, LLC.

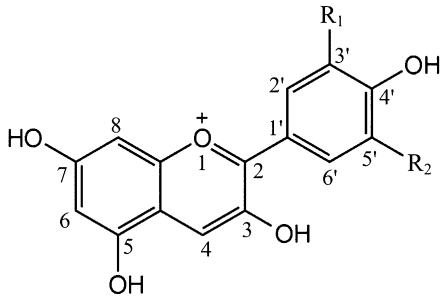
2.1.3 Anthocyanins

Anthocyanins are widely distributed water-soluble pigment compounds in plants and they contribute to a broad range of colors including: blue, violet, and red. Anthocyanins belong in the flavonoid group of phenolic compounds and flavonoids are reported to have many potential health effects. (Jackman & Smith 1996, Andersen & Jordheim 2006, Schwartz et al. 2008)

2.1.3.1 Structure

Anthocyanins are glycosylated forms of anthocyanidins, which have the characteristic $C_6C_3C_6$ structure of flavonoids (Brouillard 1982, Jackman & Smith 1996, Clifford 2000, Andersen & Jordheim 2006, Schwartz et al. 2008). The aglycon anthocyanidins are less water-soluble than their glycosides, and generally they are not found free in nature (Brouillard 1982, Clifford 2000, Andersen & Jordheim 2006, Schwartz et al. 2008). There are more than 30 anthocyanidin structures found in nature but the six most common anthocyanidins, pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin comprise around 90 percent of all anthocyanidins found in nature (Andersen & Jordheim 2006). Structures of these six common anthocyanidins

are shown in figure 5. Hydroxyl groups shift the color of the molecule towards blue and methoxyl groups towards red (Schwartz et al. 2008). The hydroxyl group at C3 is always glycosylated in nature, and additional glycosylation can occur on hydroxyl groups at C5 (most likely), C7, C3', C4' and/or C5' (Schwartz et al. 2008). The most common sugars are monosaccharides: glucose, rhamnose, galactose, xylose, disaccharides: rutinose (6-O-α-L-rhamnosyl-D-glucose) and sophorose (2-O-β-D-glucosyl-D-glucose) and some trisaccharides (Brouillard 1982, Clifford 2000, Andersen & Jordheim 2006, Schwartz et al. 2008). The sugar units can furthermore be acylated by different aliphatic (malonic, acetic, malic, succinid and oxalic acids) or aromatic (caffeic, *p*-coumaric, ferulic, sinapic *p*-hydroxybenzoic and gallic acids) acids (Brouillard 1982, Clifford 2000, Andersen & Jordheim 2006, Schwartz et al. 2008). These variations in glycosylation and acylation give rise to hundreds of different anthocyanins all based on the six common anthocyanidin aglycones.



R_1	R_2	Anthocyanidin
H	H	Pelargonidin
OH	H	Cyanidin
OH	OH	Delphinidin
OCH_3	H	Peonidin
OCH_3	OH	Petunidin
OCH_3	OCH_3	Malvidin

Fig. 5 Structure of anthocyanidins in acidic environments at the flavylium cation state. Adapted from Schwartz et al. 2008.

2.1.3.2 Functions and bioactivity

Coloration of flowers, berries, and fruits attracts insects and other animals for pollination and seed dispersal and anthocyanins played a role in this coloration. However, in some plants, also the leaves or roots contain anthocyanins, which suggests that these pigments have other functions as well. They seem to take part in plant defense functions and some anthocyanins have antifungal properties, although they are not toxic to most animals whereas some other flavonoid compounds are (Stintzing & Carle 2004, Gould & Lister 2006). There is no direct evidence on the anthocyanin defense function against herbivores but at least some insects avoid eating red-colored leaves (Gould & Lister 2006). It has also been proposed that plant pigments and defensive compounds share biosynthetic routes, and therefore herbivores have learned to avoid plant parts with bright coloration as they are likely to contain high

amounts of defensive compounds (Schaefer & Rolshausen 2006). Anthocyanins have also been hypothesized to protect the plant from UV radiation but there is also some conflicting evidence such as most anthocyanins not absorbing UV light and not being located in the optimal location for UV absorption (Takahashi et al. 1991, Stintzing & Carle 2004, Gould & Lister 2006). Direct protection from UV radiation by absorption may well be a function of other flavonoid compounds but anthocyanins may take part indirectly by repairing the DNA damage caused by UV radiation through their antioxidative properties (Stintzing & Carle 2004, Gould & Lister 2006). Anthocyanins have also a role in photoprotection to absorb excess light that could cause photoinhibition to photosynthesis (Jackman & Smith 1996, Stintzing & Carle 2004, Gould & Lister 2006). Recently, it has also been found that in addition to generally enhancing anthocyanin biosynthesis, different plant stress conditions also produce different mixtures of anthocyanin pigments indicating that individual anthocyanins may have different functions in responses to varying stress conditions (Kovinich et al. 2014).

Similarly to carotenoids, anthocyanins have antioxidative functions in plants, and they have the same antioxidative properties when ingested (Tsuda et al. 1994, Tsuda et al. 1996, Gómez-Cordovés et al. 2000, Nielsen et al. 2003, Awika et al. 2004, Stintzing & Carle 2004, Clifford & Brown 2006, Amiot-Carlin et al. 2008, Pojer et al. 2013). The antioxidative properties of anthocyanins and other flavonoids have been studied widely and they are related to many health benefits such as reduced risk of cardiovascular disease and some cancers (Nielsen et al. 2003, Stintzing & Carle 2004, Clifford & Brown 2006, Amiot-Carlin et al. 2008, Pojer et al. 2013). A correlation between antioxidant activity and anthocyanin content in chokeberry has been reported, together with inhibition of tumor cell proliferation by cyaniding glycosides (Rugina et al. 2012). However, the absorption of anthocyanins has been reported to be low and they are quickly excreted in the urine, so the *in vivo* effects may be smaller than predicted (Clifford 2000, Stintzing & Carle 2004, Clifford & Brown 2006, Lehtonen et al. 2009, Pojer et al. 2013). It has also been found out that different glycosylation (amount and position of sugar units) affects the bioavailability and anticancer properties of anthocyanins (Zhao et al. 2014).

2.1.3.3 Stability

Anthocyanins are quite unstable compounds and they have maximum stability at acidic conditions (Brouillard 1982, Markakis 1982, Jackman & Smith 1996, Andersen & Jordheim 2006, Schwartz et al. 2008). Contrary to many other natural colorants, pH has a great effect on the color of anthocyanins because protonation and deprotonation of the molecule changes and breaks the

conjugated double bond system that gives the molecule its color. In acidic environments, the anthocyanins are predominantly in the red flavylium cation state, but as the pH rises to neutral, the deprotonated blue quinonoidal base, or the colorless hydroxyl adducts carbinol (pseudo)base or chalcone is predominant (Jackman & Smith 1996, Andersen & Jordheim 2006, Schwartz et al. 2008). Structures of these different anthocyanin forms are shown in figure 6. The predominant form in neutral pH depends on the anthocyanin structure and substituents (hydroxyl, methoxyl, or glycosyl) (Jackman & Smith 1996, Andersen & Jordheim 2006, Schwartz et al. 2008). In addition to color changes, anthocyanins are also degraded at high pH (Markakis 1982, Jackman & Smith 1996, Fossen et al. 1998, Nielsen et al. 2003, Andersen & Jordheim 2006, Schwartz et al. 2008). Hydroxyl groups react easily to changes in pH, and methoxylated and/or glycosylated anthocyanins are more stable to changes in pH than anthocyanins with many free hydroxyl groups (Jackman & Smith 1996, Andersen & Jordheim 2006, Schwartz et al. 2008).

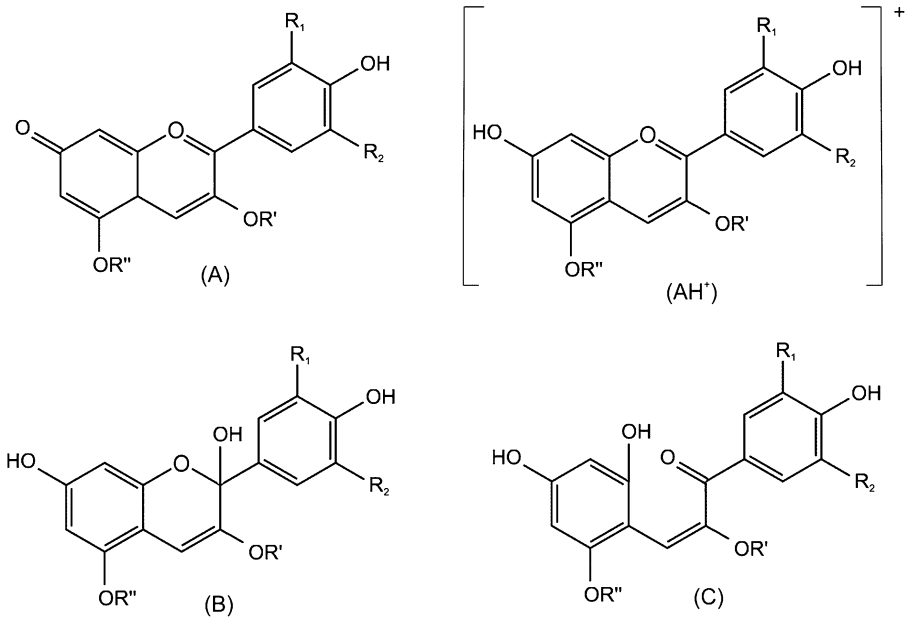


Fig. 6 Anthocyanin structures present in aqueous solution at different pH: A, quinonoidal base (blue); AH^+, flavylium cation (red); B, carbinol (pseudo)base (colorless); C, chalcone (colorless). Adapted from Schwartz et al. 2008.

High temperature, oxygen, and light enhance anthocyanin degradation (Markakis 1982, Jackman & Smith 1996, Andersen & Jordheim 2006, Schwartz et al. 2008). Especially the effect of temperature on anthocyanin stability has been studied widely (Ahmed et al. 2004, Kirca et al. 2006,

Sadilova et al. 2006, Sadilova et al. 2007, Yang et al. 2008, Patras et al. 2010, Hellström et al. 2013). Thermal degradation usually follows first-order reaction kinetics (Jackman & Smith 1996, Ahmed et al. 2004, Kirca et al. 2006, Yang et al. 2008, Patras et al. 2010). However, the exact mechanisms are not fully understood, and it seems that the degradation is affected by many variables including the anthocyanin involved, temperature, and protective effects of other compounds in the mixture (Jackman & Smith 1996, Schwartz et al. 2008). Hydroxyl, methoxyl, or glycosyl substituents have the same effect on thermal stability as they have for pH stability (Jackman & Smith 1996, Schwartz et al. 2008, Zhao et al. 2014). Oxygen degrades anthocyanins either by direct oxidation or indirect oxidation of other compounds in the mixture, which then react with anthocyanins (Jackman & Smith 1996, Schwartz et al. 2008). Light degrades anthocyanins and it also increases thermal degradation rates (Jackman & Smith 1996, Schwartz et al. 2008). Substitution at the C5 hydroxyl group increases the anthocyanin's susceptibility to photodegradation (Jackman & Smith 1996, Schwartz et al. 2008).

Sugars and their degradation products accelerate anthocyanin degradation when present in relatively low concentrations. The rate of anthocyanin degradation in these conditions seems to be very similar to rate of sugar degradation to furfural. The mechanism of this reaction is not known but the reaction produces colorless or brown compounds. When the concentration of sugars is high, they seem to stabilize anthocyanins and this effect is thought to be a result of lowered water activity. Also, complexation of anthocyanins with metal ions such as calcium, iron, aluminum, and tin seems to stabilize anthocyanins and change their spectral properties. (Markakis 1982, Jackman & Smith 1996, Schwartz et al. 2008)

Anthocyanins form complexes with themselves and other organic compounds such as other flavonoids, proteins, tannins, and polysaccharides and this process is called copigmentation. In these complexes, the anthocyanin color may be altered by shifts of the absorption maxima and also the maximum absorption may be increased. Copigmentation usually increases anthocyanin stability during processing and storage. Especially in winemaking, the copigmentation of anthocyanins with themselves produces covalent linkages between the molecules and the resulting anthocyanin polymers are more stable than monomeric anthocyanins. Also, covalent linkage to other compounds, for example, pectin or starches, may produce more stable colored compounds but condensation reactions can also result in a loss of color if the conjugated double bond system is disrupted. (Osawa 1982, Jackman & Smith 1996, Schwartz et al. 2008)

2.1.4 Betalains

Betalains are N-heterocyclic water-soluble pigments found in certain plant families of the order Caryophyllales or the older name Centrospermae. Betalains and anthocyanins have never been found in the same plant and it seems that the biosynthetic routes of these two pigment groups are mutually exclusive. (Jackman & Smith 1996, Strack et al. 2003, Schwartz et al. 2008)

2.1.4.1 Structure

Betalains can be described as condensation products of a primary or secondary amine with betalamic acid. The general structure of betalains and betalamic acid are shown in figure 7. Betalains can be divided into two groups depending on the substitution of the amine group. In betacyanins, there is an aromatic ring in the form of a cyclodopa group and in betaxanthins, the substitution is an amine or amino acid. The aromatic ring in betacyanins extends the conjugation of the chromophore and their absorption maximum is around 540 nm (red or violet), whereas the absorption maximum of betaxanthins is about 480 nm (yellow). (Jackman & Smith 1996, Schwartz et al. 2008)

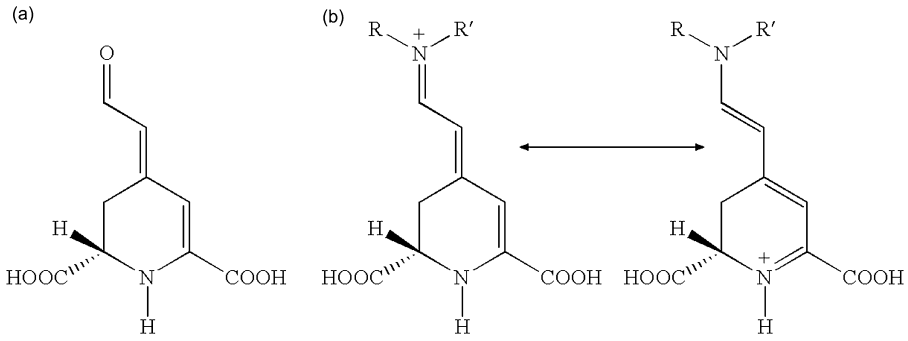


Fig. 7 Structure of betalamic acid (a) and general structure of betalains showing the structural transformation of the chromophore (b). Adapted and combined from Jackman & Smith 1996, Schwartz et al. 2008.

Similar to anthocyanins, betacyanins exist in nature as glycosides, and the most common aglycones are betanidin and its C15 epimer, isobetanidin. The most common glycosyl group is glucose, which when attached to betanidin or isobetanidin, forms betanin or isobetanin. Other common sugars are sophorose and rhamnose. As with anthocyanins, the sugar residues can also be acylated, by, for example, sulfuric, malonic, citric, ferulic, caffeic, *p*-coumaric or sinapic acids. Structures of betanidin, betanin, and amaranthin are shown in figure 8. Betanin is the dominant pigment in red beet (B*eta vulgaris*) and amaranthin in

amaranth (*Amaranthus sp*.). Contrary to betacyanins, betaxanthins are usually present as free aglycones in nature. As the R group in betaxanthins is an amine or amino acid, there are potentially hundreds of possible structures, although only a few have been characterized so far. The first characterized betaxanthin was indicaxanthin from cactus pear (*Opuntia ficus-indica*), in which the amino acid is proline. Two betaxanthins have been found in red beet, vulgaxanthins I and II, in which the condensed amino acids are glutamine and glutamic acid, respectively. Structures of these three common betaxanthins are shown in figure 9. (Jackman & Smith 1996, Schwartz et al. 2008)

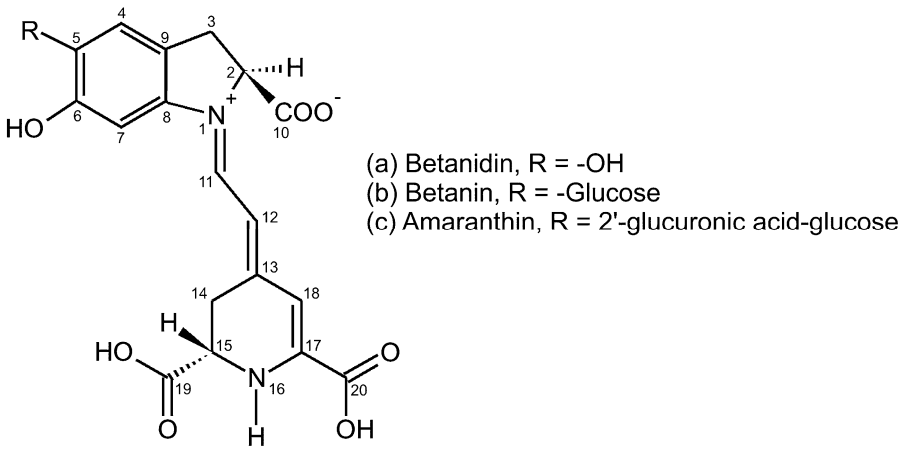


Fig. 8 Structures of betanidin, betanin, and amaranthin. C15 epimers are also found in nature, and they are isobetanidin, isobetanin, and isoamaranthin, respectively. Adapted from Schwartz et al. 2008.

2.1.4.2 Functions and bioactivity

As betalains seem to replace anthocyanins in plants that produce them, it could be reasonable to assume that they share similar functions. Indeed this seems to be true and betalains have the probable main function of producing color to flowers and fruits (Stafford 1994, Jackman & Smith 1996, Stintzing & Carle 2004, Stintzing & Carle 2008). They also have other functions similar to anthocyanins such as taking part in plant defense mechanisms, photoprotection, and acting as antioxidants (Stafford 1994, Jackman & Smith 1996, Stintzing & Carle 2004). Especially in red beet, the root contains high amounts of betanin, which is concentrated on the peel of the root, and here the function of betanin could be increased pathogen resistance (Stintzing & Carle 2004). One proposed function of betalains is that they could act as nitrogen reservoirs, and this hypothesis is supported by the presence of betalain-specific decolorizing enzymes in some plants (Kumon et al. 1990, Jackman & Smith 1996).

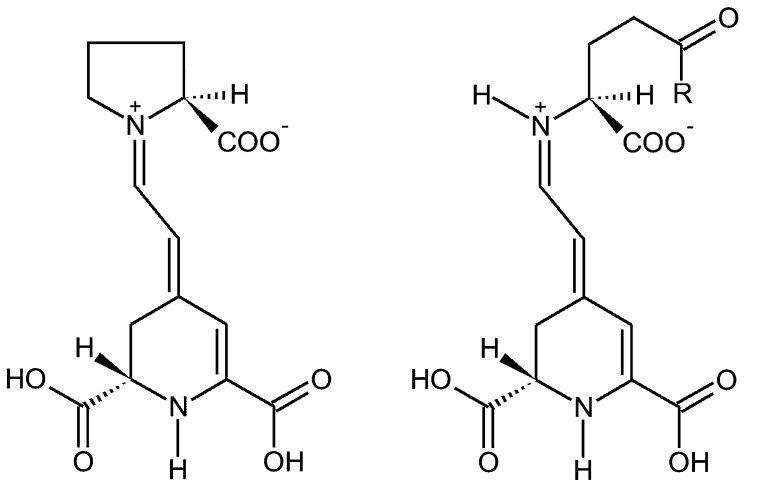


Fig. 9 Structures of indicaxanthin (a) and vulgaxanthins I and II (b). Adapted from Schwartz et al. 2008.

Just like with other plant colorants, betalains continue to have antioxidative properties also when ingested. Especially antioxidant properties of beet extracts and betalains from beet have been studied (Stintzing & Carle 2004, Suganyadevi et al. 2010, Pavokovic & Krsnik-Rasol 2011, Wroblewska et al. 2011, Kazimierczak et al. 2014, Vulic et al. 2014). In a physiological-based study, red beet crisps fed to rats on dyslipidaemic diets showed clear positive effects on the serum cholesterol and triacylglycerol levels (Wroblewska et al. 2011). Beetroot juice has also been found to have anticancer activity (Kazimierczak et al. 2014). However, in some studies, the antioxidant activities of red beet extracts have not been attributed specifically to betalains but possibly on flavonoid compounds present in the extracts (Suganyadevi et al. 2010). Also antioxidant properties of betalains from other plants, such as amaranth and cactus pear, have been studied (Cai et al. 2003, Stintzing & Carle 2004). Some relations with betalain structure and free radical scavenging ability have also been studied and hydroxyl, imino, and glycosyl groups seem to have effect on the activity (Cai et al. 2003).

2.1.4.3 Stability

The stability of betalain pigments is influenced by many factors including pH, temperature, oxygen, water activity, and light, similarly to other natural pigments (Von Elbe et al. 1974, Jackman & Smith 1996, Herbach et al. 2006, Schwartz et al. 2008). A majority of research has been done with betacyanins

(and mainly betanin from red beet), and only a few studies focus on betaxanthins. Contrary to anthocyanins, pH values between three and seven do not markedly affect the color of betacyanins (Jackman & Smith 1996, Herbach et al. 2006, Schwartz et al. 2008). Below pH 3, the color of betanin shifts towards violet and above pH 7 the color shifts towards blue (Von Elbe et al. 1974, Jackman & Smith 1996, Schwartz et al. 2008). In alkaline conditions (above pH 10), betanin degrades to yellow betalamic acid and colorless cyclodopa-5-*O*-glucoside (Jackman & Smith 1996, Herbach et al. 2006, Schwartz et al. 2008). This reaction is reversible and the pigment may be partially regenerated by mild heating at optimum pH of 4–5 (Jackman & Smith 1996, Schwartz et al. 2008). Betanin is also degraded at very acidic conditions (Herbach et al. 2006). Betaxanthins from *Celosia argentea* were found to be stable in a similar pH range than betacyanins with maximum stability at pH 5.5 (Cai et al. 2001).

Thermal degradation of betalains above temperatures of 50 °C is probably the main problem that restricts their use as food colorants. Thermal degradation of betacyanins (mainly betanin from red beet) has been studied widely (Von Elbe et al. 1974, Cai et al. 1998, Han et al. 1998, Cai et al. 2001, Herbach et al. 2004, Herbach et al. 2006). Some thermal degradation pathways of betanin are quite well known, and these pathways are shown in figure 10. Heating of betanin causes the same degradation to betalamic acid and cyclodopa-5-*O*-glucoside than alkaline pH, and the reaction is similarly reversible (Jackman & Smith 1996, Herbach et al. 2004, Herbach et al. 2006, Schwartz et al. 2008). Other thermal degradation pathways are various decarboxylation reactions and removal of the glycoside unit (Jackman & Smith 1996, Herbach et al. 2004, Herbach et al. 2006, Schwartz et al. 2008). It has also been found out that pH affects thermal degradation rates with maximum betanin thermal stability at pH 4–5 in anaerobic conditions and at pH 5–6 in aerobic conditions (Von Elbe et al. 1974, Jackman & Smith 1996, Herbach et al. 2006).

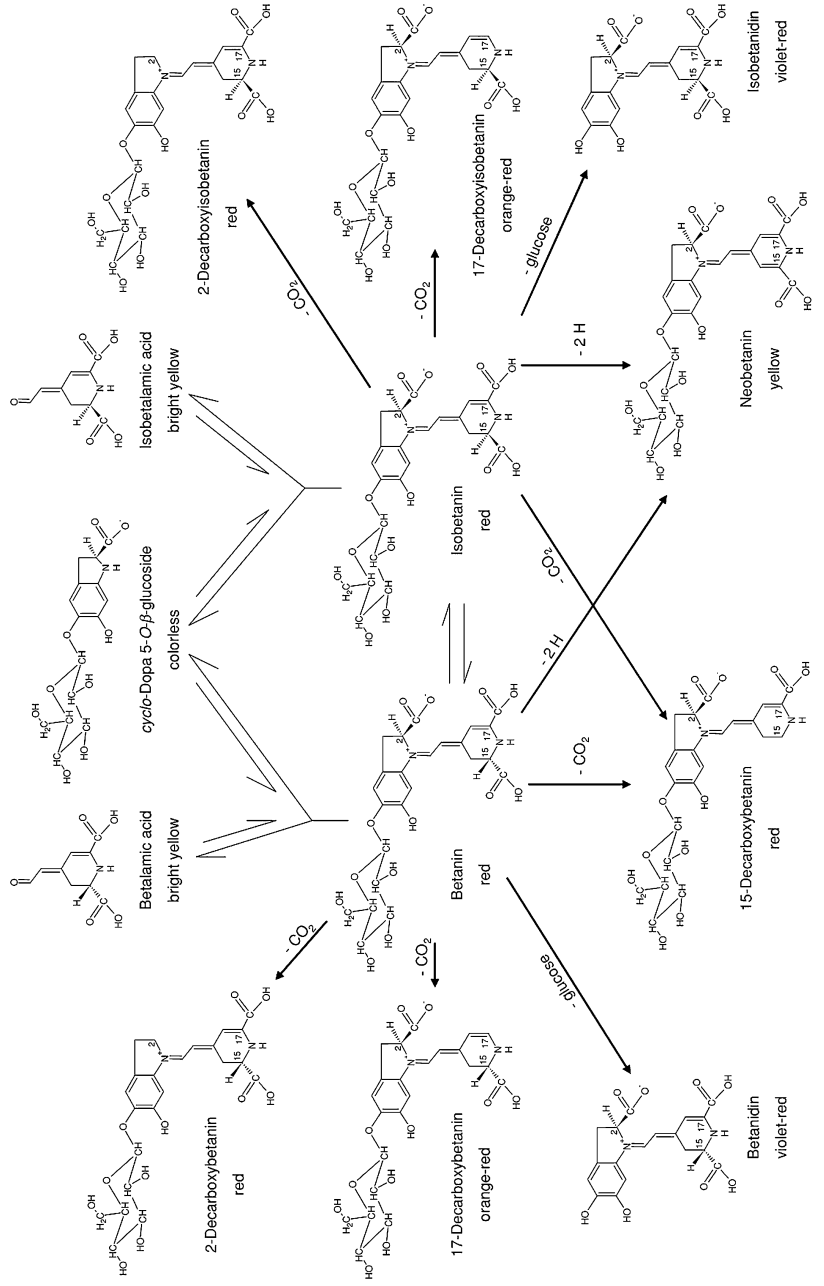


Fig. 10 Initial steps of betanin thermal degradation and colors of degradation products. Reprinted from Herbach et al. 2006 (modified from Herbach et al. 2004) with permission. Copyright 2006 John Wiley and Sons.

The effect of water activity on betanin stability has been studied and drying to water activities of 0.37 or below greatly increases betanin stability (Pasch & Von Elbe 1975, Jackman & Smith 1996, Cai et al. 1998, Cai & Corke 2001, Herbach et al. 2006, Schwartz et al. 2008). The drying method has also an effect on betanin stability with freeze-drying or fast-drying in an oven at 70–80 °C and this produces more stable dried pigments than by natural air drying or solar drying (Cai & Corke 2001). Light and oxygen have both been shown to degrade betanin by around 15 or 16 percent, respectively, in six days at 15 °C and these effects are additive for a combined loss of 29 percent (Von Elbe et al. 1974). Photooxidation of betanin seems to be also dependent on the pH with degradation being greater at pH 3 than at pH 5 (Jackman & Smith 1996). Matrix effects also play a role in betanin stability, as sucrose at concentrations above 16 percent increased degradation of Amaranth betacyanin pigments (Cai & Corke 1999). On the other hand, addition of ascorbic acid in betanin juice at pH 3.8 or metaphosphoric acid or gluconic acid at pH 6.2 enhanced regeneration of thermally degraded betanin pigments (Han et al. 1998). Also betanin in red beet juice and red beet puree showed better thermal stability than betanin in buffer solution, which suggests a protective matrix effect (Von Elbe et al. 1974).

2.2 Microencapsulation of food ingredients

Microencapsulation in its many forms has been used in the food industry for several decades (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). Earlier, the reasons for microencapsulation of food ingredients were quite simple. Microencapsulation merely masks unpleasant flavors and converts liquid ingredients into solid form (Gouin 2004). However, in recent years, the techniques have been developed further and possibilities have expanded greatly. Nowadays, there are multiple reasons to apply microencapsulation (Desai & Park 2005):

- to protect the ingredient from degradation
- to decrease the transfer rate of ingredient to the outside environment
- to modify the physical properties of the ingredient
- controlled release of the ingredient from the capsule
- to mask unpleasant flavors
- to dilute the ingredient but still achieve uniform dispersion
- to separate ingredients from each other and prevent their reactions

Especially, controlled release has emerged as an interesting concept allowing the release of ingredients where and when needed (Gouin 2004). This concept has much more potential in the drug industry where microencapsulation is also used widely. Nevertheless, it also broadens the application range of various

food ingredients and it is also a way to produce totally new ingredients with unique properties (Gouin 2004).

Microcapsules can be classified based on their structure and morphology. One widely used classification is based on the number of core particles and wall layers. The simplest form is a spherical core particle, which has a uniform layer of wall material surrounding it. The core may also have multiple layers of the same or different wall materials, in which case it is called a multi-wall or multi-layer capsule. The capsule can also have multiple cores, and usually in these kinds of multi-core or aggregate capsules the cores are embedded in a continuous matrix of wall material. Examples of these different types of microcapsules are shown in figure 11. The capsules may also be non-spherical, in which case they are usually irregular-shaped. The shape and type of capsule depends on the properties of core and wall materials and also on the encapsulation method used. (Gibbs et al. 1999, Desai & Park 2005)

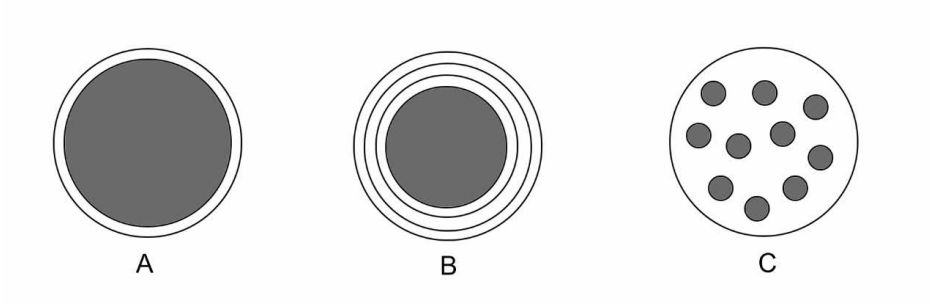


Fig. 11 Different types of microcapsules: A, single-wall or simple capsule; B, multi-wall or multi-layer capsule; C, multi-core or aggregate capsule. Core material is colored in grey and wall material in white. Adapted and combined from Gibbs et al. 1999 and Desai and Park 2005.

2.2.1 Microencapsulation techniques

Many different microencapsulation techniques are available for the food industry, and each has its own advantages and disadvantages. They have different requirements for the core and wall materials, and they can produce different sized microcapsules. A brief description of techniques most commonly used in food industry is presented here. It should also be noted that some techniques can be combined, for example, an active ingredient can first be incorporated into emulsion, which is then spray dried to produce a dry powder, and this powder can even be coated in a fluidized bed reactor. Obviously, the total cost-effectiveness has to be considered when using multiple techniques.

2.2.1.1 Spray drying and spray chilling

Spray drying has been used in the food industry for more than 50 years and it still the most widely used technique for encapsulation of food ingredients (Gouin 2004, Desai & Park 2005). The reason why spray drying is so widely used is that it is a relatively simple and economical technique (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005, Gharsallaoui et al. 2007). In principle, the core and wall materials are mixed in a liquid solvent, which is then atomized in a small nozzle and hot gas is blown at the same time to evaporate the solvent and produce a dry powder product (Gibbs et al. 1999, Desai & Park 2005, Gharsallaoui et al. 2007).

In the food industry, the liquid solvent is nearly always water and the drying gas is usually air or sometimes an inert gas like nitrogen (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005, Gharsallaoui et al. 2007). The main process parameters that can be optimized are feed temperature, feed flow rate, air inlet temperature, air flow, and air outlet temperature (Gharsallaoui et al. 2007). Feed temperature affects the viscosity of the solutions and in that way the atomization of the solution when sprayed, which is also affected by the feed flow rate (Gharsallaoui et al. 2007). Inlet air temperature and air flow affect the evaporation efficiency and product moisture content (Gharsallaoui et al. 2007). Low evaporation efficiency causes formation of dense walls and the particles retain high moisture contents and they may agglomerate or stick into the walls of the spraying chamber (Gharsallaoui et al. 2007). If the inlet air temperature is too high, water evaporates too quickly and the walls of particles crack and the core material is not completely encapsulated (Gharsallaoui et al. 2007). The outlet air temperature cannot be controlled directly but it can be used to monitor the process, as it depends on the air inlet temperature and the drying process, and ideally it should be 50–80 °C (Gharsallaoui et al. 2007). Recently, techniques called single droplet drying methods have been used to monitor the drying process and to help achieving optimal drying conditions (Schutyser et al. 2012).

Because water is the most common solvent in spray drying, suitable wall materials are restricted to compounds that are soluble in water at acceptable concentrations (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005, Gharsallaoui et al. 2007). If the solid content of the mixture is low, there is more water to evaporate, which needs a lot of energy and increases the cost of the process (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005, Gharsallaoui et al. 2007). Commonly used wall materials are thus water-soluble carbohydrates such as maltodextrins, gum acasia, chitosan, and modified starches or mixtures of these (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005, Gharsallaoui et al. 2007, Estevinho et al. 2013). Other polysaccharides and some proteins can also be used as wall materials, and especially if they are mixed with some high

solubility compound, they can produce microcapsules with good properties through their better film-forming and other functional properties and the high solubility (low molecular weight) component produces adequate solids content in the mixture (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005, Gharsallaoui et al. 2007, Estevinho et al. 2013).

Spray chilling or spray cooling can be thought as a variant of spray drying. The process is very similar but instead of evaporating the solvent with hot air, microcapsules are formed by using wall material heated to its melting point as solvent, and cool air is used to solidify the wall material after atomization (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). Spray chilling or spray cooling usually produces aggregate type capsules (see figure 11) and not true layered microcapsules (Gouin 2004). Usually different kinds of vegetable fats or stearin with melting points between 45 and 122 °C are used as wall materials in spray- cooling and hydrogenated or fractionated vegetable oils with melting points between 32 and 42 °C in spray chilling (Gibbs et al. 1999, Desai & Park 2005). The low melting points of wall materials in spray chilling may require special handling and storage conditions for the product to maintain the microcapsules in solid state (Gibbs et al. 1999). Due to the fatty wall materials used, the microcapsules are not soluble in water (Desai & Park 2005). However, the particles tend to have some active material also on their surfaces, so the release of water soluble core material begins almost immediately when the capsules are brought into aqueous environment (Gouin 2004).

2.2.1.2 Fluidized bed coating

Fluidized bed coating was developed originally for encapsulation of pharmaceuticals (Dewettinck & Huyghebaert 1999, Desai & Park 2005). The process is not as economical as, for example, in spray drying. In the food industry, the cost is a more restrictive factor than in the pharmaceutical industry, so some compromises need to be done in food applications to keep the cost acceptable (Dewettinck & Huyghebaert 1999, Desai & Park 2005). In fluidized bed coating, the core material has to be in powder form, and it is fluidized by blowing air upwards through the core material layer (Dewettinck & Huyghebaert 1999, Gibbs et al. 1999, Guignon et al. 2002, Gouin 2004, Desai & Park 2005). Coating or wall material is sprayed into the chamber either as a hot melt, which solidifies onto the core material particles or as an aqueous solution and hot air is used to evaporate the water (Dewettinck & Huyghebaert 1999, Gibbs et al. 1999, Guignon et al. 2002, Gouin 2004, Desai & Park 2005). The coating material can be sprayed from above (top spray), from below (bottom spray or Wurster system) or from the side (tangential spray) (Dewettinck & Huyghebaert 1999, Guignon et al. 2002, Gouin 2004, Desai & Park 2005). Important process control variables are inlet air temperature and

velocity, coating material spray rate, and temperature, and atomization pressure, and environmental factors such as ambient air temperature and humidity also affect the process (Dewettinck & Huyghebaert 1999, Guignon et al. 2002, Desai & Park 2005). The particle size and especially particle size distribution of the core material before coating process are important factors because they affect how the fluidized bed layer is formed (Gibbs et al. 1999). Because the coating material can be applied either as a hot melt or as an aqueous solution, the variety of suitable coating materials is much greater than in many other encapsulation methods, but in food application carbohydrates, proteins, or fats are most commonly used (Dewettinck & Huyghebaert 1999, Guignon et al. 2002, Gouin 2004, Desai & Park 2005).

2.2.1.3 Extrusion

Extrusion as a microencapsulation method should not be mixed with cooking and texturing processes of cereal products. Extrusion has been used mainly for encapsulation of flavor oils into a carbohydrate matrix (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). In the process, the core material is mixed into a molten carbohydrate mass, and the mixture is forced through a nozzle (extruder die) into a cooling and hardening solution, usually isopropanol, after which the extrusion product is dried and cut or ground to smaller pieces (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). Advantages of the method are that during the washing any residual core material not completely coated by the carbohydrate is removed and studies have reported stability of more than five years for citrus oil compared to about one year for spray dried product (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). Disadvantages are typically quite low payloads (less than 20 percent), which means that a substantial amount of carbohydrate is added to the food product, and relatively high particle size (up to 1 mm), which may be a hindrance as such large particles affect the mouth-feel of the food product (Gouin 2004).

2.2.1.4 Emulsification and liposomes

Emulsions are mixtures of two (or more) immiscible liquid phases in which one liquid (the dispersed phase) is dispersed as small droplets in the other (the continuous phase). The two immiscible liquids are usually oil and water. Two types of simple emulsions are possible: oil-in-water (o/w) or direct emulsions, in which oil is dispersed in continuous water phase, and water-in-oil (w/o) or inverse emulsions, in which water is dispersed in continuous oil phase (Leal-Calderon et al. 2007b, Tadros 2009). If the system contains three (or more) liquid phases, it is a double (or multiple) emulsion, which can be described as an emulsion within an emulsion (Leal-Calderon et al. 2007a, Jiao & Burgess

2008). Two major types of double emulsions can be classified – water-in-oil-in-water (w/o/w) emulsions, which have water droplets dispersed into oil droplets dispersed into a continuous water phase, and oil-in-water-in-oil (o/w/o) emulsions, which have oil droplets dispersed into water droplets dispersed into a continuous oil phase (Leal-Calderon et al. 2007a, Jiao & Burgess 2008). As most foods are aqueous systems, simple o/w emulsions and double w/o/w emulsions that both have a continuous water phase offer the most potential in food applications. Emulsions are typically unstable or metastable systems because the immiscible liquid phases are forced in contact with each other and the small droplets of the dispersed phase require a lot of energy to produce and maintain (Leal-Calderon et al. 2007b, Tadros 2009). Emulsifiers are amphipathic molecules that position themselves in the interface of the emulsion phases and stabilize the interface through lowering the surface tension (Leal-Calderon et al. 2007b, Hasenhuettl 2008, Tadros 2009). Emulsifiers also prevent the small droplets from merging together, which would eventually result in total separation of the liquid phases (Tadros 2009).

Emulsification is usually a step in many other encapsulation methods if oil-soluble and water-soluble components need to be mixed together. However, in some cases, no emulsifier is needed as when the process continues immediately and the emulsion only needs to be stable for a short period of time. This is the case for example in extrusion of flavor oils, where solidification of the carbohydrate matrix prevents phase separation and the system is no longer an emulsion. Emulsification can also be used as an encapsulation method itself.

Liposomes are vesicles formed by phospholipids and they have an aqueous core surrounded by a double phospholipid layer, very much like the plasma membrane of cells (Gibbs et al. 1999, Desai & Park 2005). Liposomes can be used to encapsulate both hydrophilic (in the aqueous core) and hydrophobic (in the phospholipid bilayer) core materials, and they are relatively easy to produce (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). Other advantages of liposomes in food applications are that they are stable in aqueous environments and controlled release with a thermal trigger is easy because, at 50 °C, the phospholipids undergo phase transition and the liposomes are broken (Gouin 2004, Desai & Park 2005). Major drawbacks are high costs involved in scaling up the processes and the lack of suitable and cost-effective drying methods, which means that liposomes usually need to be stored as dilute aqueous solutions (Gouin 2004, Desai & Park 2005). This is problematic for large-scale production and storage as an aqueous solution also raises the issue of microbiological shelf-life (Gouin 2004, Desai & Park 2005).

2.2.1.5 Spinning disk

Spinning disk or centrifugal suspension separation is a relatively new encapsulation method (Gibbs et al. 1999, Desai & Park 2005). In the process, the core material is mixed with liquid or dissolved wall material and the suspension is fed onto a spinning disk in such conditions that the liquid forms a layer with thickness much smaller than the particle size of the core material (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). As the disk spins, the coated particles and much smaller residual particles of pure coating material are atomized at the edge of the disk, and due to their size difference, the microcapsules are easy to separate from the pure wall material particles, for example, by sieving (Gouin 2004). Since the process is continuous and quite fast and the equipment is relatively simple, the method is suitable for food applications (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). However, scaling up the process is challenging due to requirement of multi-head nozzles and the small nozzles would also cause frequent problems with clogging (Gouin 2004).

2.2.1.6 Coacervation

Coacervation involves mixing of the core material (typically flavor oil in food applications) and the coating polymer in the manufacturing solution followed by solidification of the wall material and subsequent drying of formed microcapsules (Gibbs et al. 1999, Desai & Park 2005, Ezhilarasi et al. 2013). There are many polymers available as wall materials but the most studied and well-known system is the gelatin/gum acacia system, which is based on opposite charges of the two polymers at low pH values causing the polymers to associate with each other (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005). Solidification of the coating material can be achieved by adjusting the temperature (as in the gelatin/gum acacia system) or cross-linking through some chemical reaction (Gibbs et al. 1999, Gouin 2004, Desai & Park 2005, Ezhilarasi et al. 2013). Some problems with the technique in food industry are the relatively high cost and complexity of the method and the use of toxic glutaraldehyde in cross-linking of the wall material (Gouin 2004, Desai & Park 2005, Ezhilarasi et al. 2013). The glutaraldehyde could be replaced with enzymatic cross-linking methods but these are yet very long reactions that increase the production cost (Gouin 2004, Desai & Park 2005).

2.2.2 Microencapsulation of natural colorants

Natural colorants have been a target for microencapsulation for some time. Due to the low cost and efficiency of the process, spray drying is the most used method for encapsulating natural colorants (Kandansamy & Somasundaram 2012). The pigment classes that have been studied most are carotenoids and anthocyanins, but also betalains have been encapsulated. The following review is focused on carotenoids and betalains because these colorants were studied in this thesis work. However, microencapsulation of anthocyanins is also an important research area as reviewed by Kandansamy and Somasundaram (2012) and Mahdavi et al. (2014).

2.2.2.1 Microencapsulation of carotenoids

Microencapsulation of carotenoids has been studied widely. Donhowe and Kong (2014) have reviewed β-carotene research with microencapsulation as one part of their review. As carotenoids are mostly oil soluble and most foods are aqueous systems, emulsification is a common method or part of it in carotenoid microencapsulation. Khalil et al. (2012) extracted lutein from marigold flowers with medium chain triacylglycerol oil and emulsified it with β-lactoglobulin as emulsifier and the emulsified lutein showed better stability in UV light at 365 nm than non-emulsified lutein. Losso et al. (2005) used random centroid optimization to determine optimal compositions for o/w emulsion containing lutein and lutein in the emulsion showed good storage and heat stability. The research group of Socaciu has studied incorporation of carotenoids into liposomes for many years and they have shown that carotenoids have an effect on the properties of the phospholipid membrane such as membrane fluidity and size of the liposomes (Socaciu et al. 2000, Socaciu et al. 2002, Pintea et al. 2005). They have also shown that carotenoids compete with cholesterol on incorporation into liposome membranes (Socaciu et al. 2000) and that incorporation yields are higher for free carotenoids than for carotenoid esters (Pintea et al. 2005).

Some studies have used spray drying or freeze-drying in addition to emulsification to produce carotenoid-containing microcapsules. Rodriques-Huezo et al. (2004) prepared double w/o/w emulsions containing carotenoid oleoresin in the oil phase and saponified carotenoids in the inner water phase, and the double emulsions were spray dried using various polysaccharides as wall material to produce microcapsules. They showed that microcapsules with higher polysaccharide:primary emulsion ratios had better morphology and encapsulation efficiency and larger particle size, but also higher carotenoid degradation kinetics than microcapsules with a lower polysaccharide:primary emulsion ratio (Rodriguez-Huezo et al. 2004). They also found out that

microcapsules stored at a water activity of 0.628 had lowest carotenoid stability with carotenoid stability being better at higher or lower water activity. Guo et al. (2014) used ultrasonic homogenization to incorporate lycopene in o/w emulsion, which was then freeze-dried with mannitol as wall material. The freeze-dried microcapsules retained 80 percent of lycopene after storage at 4 °C for two months whereas the control (free lycopene) retained less than 10 percent of lycopene (Guo et al. 2014). Wenzel et al. (2010) spray dried and freeze-dried egg yolk (a natural emulsion) containing carotenoids and stored the microcapsules at 20 °C and -18 °C for six months. Storage at both temperatures decreased the total carotenoid content of both types of microcapsules to roughly 60–70 percent and the retention of carotenoids at -18 °C was only slightly better than at 20 °C (Wenzel et al. 2010).

Many studies have also been made on bioavailability of carotenoids in emulsions. Salvia-Trujillo et al. (2013) studied the effect of droplet size on simulated lipid digestion and bioaccessibility of β-carotene in o/w emulsions. The droplet sizes increased during the simulated lipid digestion and the rate and extent of digestions as well as bioaccessibility of β-carotene increased with decreasing initial emulsion droplet size (Salvia-Trujillo et al. 2013). Khalil et al. (2012) studied bioavailability of lutein dispersed in oil and lutein in spray dried emulsion by analyzing lutein levels in blood after ingesting the preparates. They showed a better lutein bioavailability from oil than from spray dried emulsion, and speculated about possible reasons for a lower bioavailability from spray dried emulsion either as changes in the absorption or instability of the microcapsules in the gastrointestinal system that are mainly due instability of the emulsion in low pH (Khalil et al. 2012). Gorusupudi and Vallikannan (2012) and Sato et al. (2011) have studied lutein bioavailability by rat studies. Gorusupudi and Vallikannan (2012) showed a better bioavailability of lutein dispersed with glycolipids than lutein dispersed with phospholipids, neutral lipids, or crude lipids. Sato et al. (2011) showed better lutein absorption from o/w emulsion when fed to rats with food intake as opposed to feeding the lutein emulsion alone and hypothesized that bile acids and some food components play an important role in the absorption mechanism. Vishwanathan et al. (2009) compared the bioavailability of lutein from commercial supplements to lutein in o/w nanoemulsion and showed a better bioavailability of lutein in a nanoemulsion. There are also some patents concerning lutein preparations in emulsions with increased bioavailability (Ornelas Cravioto 2007, Montoya-Olvera & Torres-Quiroga 2012).

2.2.2.2 Microencapsulation of betalains

Although betalains are not as commonly found pigment compounds as carotenoids or anthocyanins, encapsulation of them has still been studied for many years. Since betalains are water-soluble, spray drying of aqueous solutions seems to be a widely used method for encapsulating betalains. Cai and Corke (2000) spray dried betacyanins from Amaranth using different maltodextrins, of dextrose equivalent (DE) 10–25 and starches as wall materials. All wall materials had better pigment retention at five percent relative humidity (RH) than at 32 percent RH (Cai & Corke 2000). At five percent RH the retention increased with increasing DE values, whereas at 32 percent RH the retention increased with decreasing DE values, and this was thought to be due to differnet hygroscopicity of the wall materials (Cai & Corke 2000). Serris and Biliaderis (2001) stored spray dried beetroot pigments with maltodextrin (DE 5 or 20) or pullulan as wall material in various water activity and temperature conditions. They showed the highest betanin degradation at water activity of 0.64 regardless of wall material and storage temperature, and degradation was slower in higher or lower water activities (Serris & Biliaderis 2001). Degradation of betanin occurred also below the glass transition temperatures of the wall materials, although the degradation was slower when the wall material was in glassy state (Serris & Biliaderis 2001). Pitalua et al. (2010) studied the antioxidant activity of spray dried beetroot juice stored at different water activities and they showed higher antioxidant activities but lower betalain concentrations for microcapsules stored for 45 days at water activities of 0.748 and 0.898 compared to microcapsules stored at water activities of 0.110, 0.326, and 0.521. Gandía-Herrero et al. (2013) showed that spray dried betalains from *Lampranthus productus* using maltodextrin or chitosan as wall materials and the spray dried pigments had good stability and retained their antioxidant activity for storage in the dark for six months, especially when stored cold.

Betalains from cactus pear (*Opuntia ficus-indica*) have been spray dried by several research groups (Saénz et al. 2009, Gandía-Herrero et al. 2010, Vergara et al. 2014). Saénz et al. (2009) searched for optimum spray drying conditions for pulp and ethanolic extracts of cactus pear fruits using maltodextrin of DE 10 and inulin of degree of polymerization (DP) greater than 23 as wall materials and stored the microcapsules at 60 °C in the dark. Different core and wall material systems required slightly different spray drying conditions, and betalains in all systems showed slow degradation during storage at 60 °C with indicaxanthins being more stable than betacyanins (Saénz et al. 2009). Gandía-Herrero et al. (2010) described a procedure for spray drying indicaxanthin from cactus pear, and the microcapsules were stable at least for six months when

stored in the dark at 20, 4, or -20 °C. Recently, Vergara et al. (2014) spray dried pulp and ultrafiltered cactus pear extracts using a commercial octenylsuccinate-derivatized starch as wall material and stored the microcapsules at 60 °C in the dark for 25 weeks. Betalains in spray dried ultrafiltered extracts showed better stability than betalains in spray dried pulp, which was attributed to mucilage and/or higher sugar content in pulp causing a difference in hygroscopicity and available water leading to accelerated degradation reactions in pulp microcapsules (Vergara et al. 2014).

2.3 Summary

Plants come in a huge variety of colors, and the compounds producing these colors are almost equally varied. Due to the great variation in structures and properties of these natural colorants, they offer lots of possibilities for use in different kinds of food matrices as added colorants. Although the properties of the four major plant colorant groups, chlorophylls, carotenoids, anthocyanins, and betalains are very different, there are some common properties as well. All of these colorants have antioxidative properties, which are related to many health benefits such as reduced risk of coronary heart disease and different types of cancer. Another common thing is that these compounds tend to be unstable, especially when incorporated in foods.

The antioxidativity and related health benefits make natural plant colorants attractive options for food colorants, preferably replacing artificial food colorants. This would have two benefits as consumers are nowadays quite aware of health issues and artificial food colorants (and other additives) have a negative image. On the other hand, the instability of these natural colorants makes them less attractive choices, as the shelf-life of the products would be shortened, unless the stability could be improved.

Different microencapsulation techniques offer potential ways to improve the stability of natural plant colorants and microencapsulation has been used successfully to improve the stability of natural colors. Furthermore, in some cases, it has been reported to improve the bioavailability as well. However, most of the studies have considered stability of the colorants only in the microcapsules, and not when incorporated into food. For example, most spray dried microcapsules probably lose their protective effect when incorporated in aqueous foods due to the wall materials being soluble in water. Therefore, these studies merely show improved stability for colorant preparations and not for colorants in foodstuffs.

Future studies in the area of microencapsulation of natural colorants should consider also the stability of colorants when the microcapsules are incorporated

in foods. This may in many cases be significantly different than the stability in microcapsules. Also, if one of the reasons to use natural colorants is their potential health effects, they should be bioavailable when ingested. Therefore, the bioavailability of the natural colorants should also be studied, and not only from the microcapsules, but from the food matrix as well. Another important factor that should be studied is taste or flavor. The taste of food is a crucial factor for consumer acceptance, and for example, bitter and astringent phenolic compounds are easily co-extracted with anthocyanins, so the effect of added colorants to the flavor of food should also be studied.

3 AIMS OF THE STUDY

The overall aim of this thesis study was to use microencapsulation techniques to improve the stability of natural colorant compounds. One hydrophobic (lutein) and one hydrophilic (betanin) compound were chosen as model substances. Other objectives were to extract pigments without using organic solvents, to study the composition and suitability of polar lipid fraction from oat as an emulsifier, to study the stability of pigments in model foods, and to perform preliminary studies on bioavailability of pigments and consumer acceptance of model foods containing them. Specific sub-aims for the separate studies were:

I. To study the composition and emulsifier properties of polar lipid fraction from oat (*Avena sativa*) and preliminary production of o/w emulsions containing lutein in the oil phase.

II. To optimize the enzyme assisted oil extraction of lutein from marigold (*Tagetes erecta*) flowers, prepare o/w emulsions and spray dried emulsions with lutein in the oil phase, and to assess the stability of lutein during storage in oil and in wet and dried emulsions.

III. To prepare $w_1/o/w_2$ double emulsions containing betanin from red beet (*Beta vulgaris*) in the inner w_1 phase and carry out a preliminary bioavailability study using a simulated *in vitro* intestinal lipid digestion model.

IV. To optimize the extraction method of betanin from industrial red beet (*Beta vulgaris*) peeling by-product, prepare model juices colored with spray dried betanin, study the stability of betanin in these model juices during storage and study the consumer acceptance of model juices colored with betanin.

4 MATERIALS AND METHODS

4.1 Plant material

Marigold flowers (*Tagetes erecta*) used for lutein extraction (study **II**) were grown in Rymättylä, Finland. Red beet (*Beta vulgaris*) used for betanin extraction were either bought at a grocery store (studies **III** and **IV**) or an industrial red beet peeling by-product received from Orkla Foods Finland Oy (Turku, Finland) was used (study **IV**).

The oat polar lipid fraction used as an o/w emulsifier (studies **I**, **II**, and **III**) was extracted from oat flakes (*Avena sativa*, var Roope) using a supercritical fluid process described by Aro et al. (2007b). The composition of the oat polar lipid used as an o/w emulsifier was analyzed in study **I** by HPLC fractionation using a modified method described by Kurvinen et al. (2000). An LC-9A (Shimadzu, Kyoto, Japan) pump with a LiChro-CART® 250-10 Lichrospher® Si 100 (5 µm) column (Merck KGaA, Darmstadt, Germany) was used to fractionate oat polar lipids into six fractions in two steps. The first step was fractionation into three fractions (1, 2, and 3) using an isocratic mixture of chloroform:methanol:2-propanol:water (65:30:1:5) as eluent with a flow rate of 3 ml/min. The second step was a further division of fraction 1 into four subfractions (1a, 1b, 1c, and 1d) using an isocratic mixture of chloroform:methanol:2-propanol (76:13.5:0.5) as eluent with a flow rate of 3 ml/min. Before actual fractionation, a Sedex 55 (S.E.D.E.R.E., Alfortville, France) electronic light scattering detector (ELSD), and a C-R3A Chromatopac integrator (Shimadzu, Kyoto, Japan) were used for method optimization and identification of peaks and retention times. After fractionation, the fractions were evaporated under a stream of nitrogen gas with mild heating, weighed, and dissolved in chloroform:methanol (2:1). Identification of fractions was performed with a Li-ChroCART® 250-4 Lichrospher® Si 60 (5 µm) column (Merck KGaA, Darmstadt, Germany) using the same isocratic solvent mixtures as for fractionation with a flow rate of 0.5 ml/min. Retention times were compared with standards, digalactosyldiacylglycerol (DGDG), and monogalactosyldiacylglycerol (MGDG) from Sigma-Aldrich (St. Louis, Missouri, USA) and steryl glucoside (SG) and phospholipid mixture POL mix 71 (consisting of phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl serine (PS), and phosphatidyl glycerol (PG)) from Larodan AB (Malmö, Sweden). Tentative identifications were verified with coinjections.

4.2 Extraction of pigments

In study **II**, lutein was extracted from marigold flowers using an enzyme-assisted oil extraction procedure, which was first developed in an earlier project funded by TEKES (Aro et al. 2007a). Marigold flowers were suspended in water at a proportion of 1:9 and they were treated with 10 ml/l cellulase (Econase CE, AB Enzymes, Darmstadt, Germany, activity 2330 U/ml) and 10 ml/l pectinase (Pectinex Ultra SP-L, Novozymes, Bagsvaerd, Denmark, activity 9500 U/ml) at 20–25 °C for 18-24 hours with magnetic stirring and covered with aluminum foil. After the enzymes had broken the solid plant material, the water suspension was mixed with commercial rapeseed oil at proportion 5:1 at 20–25 °C for 1 hour with magnetic stirring and covered with aluminum foil. The oil phase containing most of the lutein was separated from the water phase by centrifuging aliquots of the mixture at 1050 x g for 10 minutes, after which the oil was collected. The extraction with oil was repeated with fresh oil mixed with the same suspension. After centrifugation and collection of oil, the two oil extracts were combined and stored in the dark at 4 °C until used in experiments.

In study **III**, betanin was extracted from red beets with water. Whole beets were cut to approximately 1 cm^3 cubes and extracted with water (proportion 1:2) at 70 °C for 30 minutes with magnetic stirring and covered with aluminum foil. After extraction, the solid plant material was filtered out through a filter paper under vacuum and the extract was concentrated to 60 percent of original volume using a rotary evaporator. The concentrated extract was stored frozen in the dark at -20 °C until used for experiments. In study **IV**, the method was further optimized, and betanin was extracted from cut whole beets or industrial peeling by-product with water with magnetic stirring and covered in aluminum foil. Effects of temperature (40 or 80 °C), extraction time (5, 15, 30 or 60 minutes), and proportion beet:water (1:1 or 1:2) on extraction efficiency were studied. After extraction, the suspension was filtered through a cloth and filter paper under vacuum to remove solid plant material. The extract from industrial peeling by-product was concentrated with a rotary evaporator from 2 °brix to 10 °brix to increase solids content for spray drying. The extracts were stored in the dark at -20 °C until used in experiments.

4.3 Encapsulation of pigments

4.3.1 Preparation of o/w emulsions

In studies **I** and **II**, o/w emulsions were prepared with citrate-phosphate buffer as water phase, rapeseed oil as oil phase, and polar lipid fraction from oat as emulsifier but the exact emulsion composition were slightly different. In study **I**, the water phase had a pH of 2.6 and it was prepared by mixing 0.1 M citric

acid solution, 0.2 M disodium hydrogen phosphate solution, and water in proportion 223:27:250, respectively. In preliminary studies (unpublished data), the oat emulsifier showed higher solubility in lower pH (test range 2.6–7.0), and therefore the lowest pH tested was chosen for preparation of emulsions. Oat emulsifier was dissolved in the buffer at 50 °C with magnetic stirring for one hour in three different concentrations: 2.5 mg/ml, 5 mg/ml, or 10 mg/ml. After cooling to room temperature, commercial rapeseed oil in three different concentrations: 10 mg/ml, 20 mg/ml, or 50 mg/ml (50 mg/ml only for the emulsion with 10 mg/ml emulsifier) was added and emulsions were prepared by homogenization with an Ultra-Turrax® T 25 Digital disperser with a S 25 N–10 G dispersing tool (IKA®, Staufen, Germany) at a speed of 24,000 rpm for five minutes. Droplet sizes were measured by a light microscope attached with a digital camera (crude estimate), dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, Worcestershire, United Kingdom) with a scattering angle of 173° (backscattering) and Fraunhofer laser diffraction (LD) using a HELOS/KF with CUVETTE wet dispersing system (Sympatec, Clausthal-Zellerfeld, Germany). From some emulsions stored for three days, it was possible to separate the upper 'cream' phase and the lower 'bulk emulsion' phase, and droplet sizes were measured separately for these two phases.

In study **II**, we used mixtures of different polysaccharides as stabilizers to prevent creaming of emulsions. In preliminary tests, many different polysaccharides and mixtures of them at different concentrations were tested, and mixtures of locust bean gum (LBG) and xanthan gum (XG) or guar gum (GG) and XG at a total concentration of 3 mg/ml (proportions 1:3 and 3:1 for both mixtures) seemed to work best (unpublished data). Because these polysaccharides are stable at pH 4–10 (Dreher 1999), a pH of 5.8 was chosen for the buffer instead of 2.6, and this 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 emulsifier (concentration: 5 mg/ml) and polysaccharide mixtures were dissolved in the buffer with magnetic stirring at 50–60 °C for two hours replacing the evaporated water afterwards. Emulsions were prepared by adding 20 mg/ml of oil containing lutein esters into the water phase and homogenizing with a high speed mixer for five minutes. Two different types of equipment was used: an Ultra-Turrax® T 25 Digital disperser with a S 25 N - 10 G dispersing tool at 24,000 rpm for small scale and a Magic LAB® dispersing system with micro-plant assembly and 6F blade at 16,000 rpm for pilot scale, both from IKA®, Staufen, Germany. After emulsification of the pilot batches, 200 mg/ml maltodextrin (MD) with DE 12.5–15.5 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 used for spray drying. A summary of emulsion and dried emulsion sample compositions,

sample codes, and emulsification equipment used is shown in table 1. In this study, we had no equipment available for particle size measurement, and the droplet sizes of emulsions were evaluated only with light microscope.

Table 1 Sample codes for emulsion samples in study **II**, composition of stabilizers used in them and the equipment used for their emulsification. Total amount of GG + XG or LBG + XG is 3 mg/ml in each sample and the amount of MD is 200 mg/ml. 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®

4.3.2 Preparation of w/o/w emulsions

In study **III**, double $w_1/o/w_2$ emulsions with beet extract as the inner w_1 phase were prepared. The primary w_1/o emulsion was prepared by adding beet extract slowly into rapeseed oil containing 20 mg/g polyglycerol polyricinoleate (PGPR) with mixing at 10,000 rpm with a SilentCrusher M high-speed mixer (Heidolph, Schwabach, Germany) so that the amount of the inner water phase w_1 was 0.3 ml/g of the total w_1/o emulsion. This primary w_1/o emulsion was slowly added to the outer water phase w_2 with mixing at 13,000 rpm so that the amount of w_1/o emulsion was 0.03 ml/g of the total double emulsion, and after the whole amount was added, the double emulsion was homogenized at 18,000 rpm for five minutes. The outer water phase, w_2, was a pH 5.8 citrate-phosphate buffer prepared by mixing 0.1 M citric acid solution, 0.2 M disodium hydrogen phosphate solution, and water in proportion 197:303:500, respectively, containing 5 mg/g oat polar lipid emulsifier, 2 mg/g GG, 2 mg/g XG, and 39 mg/g glucose (for adjusting the osmolarity of the solution). The osmolarities of inner w_1 and outer w_2 water phases were measured with a Micro-Osmometer type 13 Autocal (Roebling, Berlin, Germany) and glucose was added to the outer water phase to balance the osmolarity of the inner and outer water phases.

Encapsulation efficiency was measured by centrifuging a double emulsion sample at 3000 x g for 10 minutes and filtering the outer water phase through a

0.45 µm polytetrafluoroethylene (PTFE) syringe filter. The absorbance at 530 nm of the filtered sample was measured with a Multiskan GO spectrophotometer (Thermo Scientific, Vantaa, Finland) against a blank sample made of a filtered (0.45 µm PTFE syringe filter) outer water phase and the value was compared with a standard curve prepared by adding calculated amounts of beet extract to the filtered (0.45 µm PTFE syringe filter) outer water phase corresponding to 95, 90, 80, 60, 40 or 0 percent encapsulation efficiency. Droplet sizes were measured by light microscope attached with a digital camera (crude estimate) and LD using a Mastersizer S equipped with a 2 mW He–Ne laser of 633 nm and a 300RF lens (Malvern Instruments Ltd., Worcestershire, UK). Back-scattering measurements (scattering angle 173°) of the primary w_1/o emulsion droplet size distribution were obtained by DLS using a Zetasizer Nano ZS equipped with a 4 mW He–Ne laser of wavelength 633 nm (Malvern Instruments Ltd., Worcestershire, UK).

4.3.3 Spray drying

Spray drying was used in studies **II** and **IV**. In study **II**, the pilot scale emulsions were spray dried after adding 200 mg/ml MD (DE 12.5–15.5) to the emulsions using 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. The moisture contents of spray dried powders were analyzed by heating overnight at 100 °C and measuring the weight loss due to evaporated moisture. In study **IV**, MD (DE 6) was added to the concentrated beet extract as wall material and the solution was filtered through a milk filter bag and pasteurized for 10 minutes at 65 °C. Betanin was spray dried with a Mini Spray Dryer B290 (Büchi, Flavil, Switzerland) using the following conditions: inlet air temperature, 170 °C; outlet temperature, 67 °C; air flow, 450 l/h; feed flow, 6 ml/min; aspirator, 35 m^3/h; and nozzle orifice, 1.50 mm. The moisture contents of spray dried powders were analyzed by heating overnight at 100 °C and measuring the weight loss due to evaporated moisture.

4.4 Storage tests

Storage tests of the encapsulated pigments were conducted in studies **II** and **IV**. In study **II**, the samples (oil, o/w emulsions, and spray dried emulsions, see table 1 for a list of emulsions and dried emulsions) were stored in the 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. 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 were added to break the emulsion and help the extraction process. The tube was thoroughly mixed with a vortex mixer, after which 2 ml of hexane containing 0.2 mg/ml butylated hydroxytoluene (BHT) as an antioxidant 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 aqueous 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.

High performance liquid chromatography (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 reverse phase column Luna 5u C18 (length 150 mm, inner diameter 3.00 mm, particle size 5 µm, and pore size 95 Å, 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 that raised the 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, the 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.

In study **IV**, model juices were prepared and colored with spray dried betanin and stability of betanin and changes in some properties of model juices during storage at different temperatures were studied. Model juices were prepared to mimic real juices but with a very simple composition. The base

mixture of each juice was a solution of 9 g/l citric acid and 0.3 g/l ascorbic acid. The pH of the base mixture was adjusted to either 3.4 or 5.0 with 1 mol/l NaOH solution and spray dried betanin powder was added at a concentration of either 0.7 g/l or 1.4 g/l. Juices were sweetened either with 10 g/l sucrose or 4 g/l commercial stevia product containing 9.1 percent steviol glycosides (Canderel Green, Merisant, Switzerland) to produce two kinds of juices with equal sweetness but different dry matter contents. The juices were pasteurized for 10 minutes at 65 °C before packing into polypropylene (PP) cans with aluminum lids or PP tubes with PP caps. A summary of different juice compositions and storage packages is shown in table 2. The juices were stored in the dark at -20 °C, 4 °C, 20–24 °C (later referred as 20 °C), and 60 °C (accelerated test) and samples were taken for analysis weekly during the first month and then once a month for juices stored at 20 °C or 60 °C. For juices in cold storage, samples were taken after two, three, four, or five months. The spray dried betanin powder was also stored at 20 °C and 60 °C in a sealed plastic bag and a 0.7 g/l aqueous solution was prepared and analyzed similarly to juice samples after four or six months.

The pH, °brix, UV spectrum (350–600 nm) and betanin content were measured at each time point. Betanin content was measured from a filtered (0.45 µm PTFE syringe filter) sample with HPLC analysis using a modified method from Herbach et al. (2004). 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 reverse phase column Luna 5u C18 (length 150 mm, inner diameter 3.00 mm, particle size 5 µm and pore size 95 Å, 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 0.2 % aqueous formic acid as eluent A and acetonitrile as eluent B was used, starting with 100 % A for 0–4.9 minutes, then raising percentage of B from 0 % to 7 % during 4.9–7 minutes, followed by further raises to 10 % during 7–10 minutes, to 30 % during 14–17.5 minutes and to 100 % during 17.5–19.6 minutes, after which the initial conditions of 0 % B were restored during 19.6–23.1 minutes and the system was equilibrated for the following run during 23.1–26.6 minutes. The column was operated at 40 °C, the flow rate was kept constant at 1.0 ml/min, the injection volume was 5 µl and samples were kept at 8 °C in the sample cooler. The detection wavelength was 535 nm, and peak areas of betanin and isobetanin in samples were added together and compared between different time points to study betanin degradation during storage.

Table 2 Summary of storage test juices in study **IV**.

Juice	pH	betanin	sweetener	container
ST1	3.4	0.7 g/l	sucrose	PP can + aluminum lid
ST2	3.4	0.7 g/l	stevia	PP can + aluminum lid
ST3	5.0	1.4 g/l	sucrose	PP tube + PP cap
ST4	5.0	1.4 g/l	stevia	PP tube + PP cap

4.5 *In vitro* intestinal lipid digestion

In study **III**, an *in vitro* intestinal lipid digestion was performed as described by Marze et al. (2012) using only the intestinal step as the interest was to understand the role of lipase and bile salt during digestion. The digestion medium was a 130 mmol/l NaH_2PO_4 buffer (pH 7.5) with 20 mg/ml sodium glycodeoxycholate (a bile salt) and 1 mg/ml pancreatic lipase was used to mimic the intestinal step of the gastrointestinal tract. Equal volumes of freshly prepared double emulsion and digestion medium were mixed together and the digestion was performed at 37 °C under magnetic stirring. Samples were taken with a micropipette for measurements of droplet size (LD) and encapsulated betanin (spectrophotometry) at 0, 30, 60, 90, 120, and 180 minutes of digestion using the same methods as described in section 4.3.2. Microscopic investigation was also done at 0, 10, 20, 50, 80, 110, and 170 minutes of digestion. A control experiment was done in the same conditions, but without the bile salt and lipase. The control experiment was measured only at 0 and 180 minutes.

4.6 Sensory evaluation

Sensory evaluation of model juices colored with spray dried betanin was carried out in study **IV**. Similarly to storage test juices in this study, the base mixture of each model juice was a solution of 9 g/l citric acid and 0.3 g/l ascorbic acid and all of the juices were sweetened with 10 g/l sucrose. The pH of the base mixture was adjusted to either 3.4 or 5.0 with 1 mol/l NaOH solution and colorants were added to produce either a ‘weak’ or a ‘strong’ color. Three different colorants were used in the study: spray dried betanin powder (same as in storage test juices), beet extract (extracted from cut whole beets as described in section 4.2), and a concentrated anthocyanin extract (from grapes, provided by Eckes-Granini Finland Oy Ab). The aim of using different colorants was to compare the consumer acceptance of betanin color with anthocyanin color and to see if there is any difference between spray dried betanin and beet extract used as such, mainly for possible off-odors and off-flavors derived from beet. The weak and strong colors were adjusted to

correspond to the maximum UV absorbance of 0.7 g/l and 1.4 g/l spray dried betanin solutions, respectively. A total of six different model juices were used in the study and the compositions of these juices are shown in table 3. Two of the sensory evaluation juices are the same as juices used in the storage test except that the sensory evaluation juices were not pasteurized as the sensory evaluations were carried out within 2 days of model juice preparation (SE1 is ST1 and SE3 is ST3).

Table 3 Summary of sensory evaluation juices in study **IV**.

Juice	pH	colorant	color
SE1	3.4	spray dried betanin 0.7 g/l	weak
SE2	3.4	spray dried betanin 1.4 g/l	strong
SE3	5.0	spray dried betanin 1.4 g/l	strong
SE4	3.4	beet extract 1:50 dilution	strong
SE5	3.4	anthocyanin 1:2500 dilution	weak
SE6	3.4	anthocyanin 1:1250 dilution	strong

70 voluntary subjects (age 19–65; 43 females; 27 males) were recruited to the consumer acceptance test. They rated the pleasantness of appearance, odor, and taste of the model juices on a 7-point balanced hedonic scales (from 1=extremely unpleasant to 7=extremely pleasant). Subjects were also asked to give free-form comments on appearance, odor, and taste of the samples and they rated also the question: 'How important it is to you whether added colors in the juice are natural?' on a scale from 1 (not important) to 5 (extremely important). The samples were presented in randomized order and data was collected using the Compusense-*five* software. Tests were carried out in controlled sensory laboratory conditions (ISO8589).

4.7 Statistical analysis

All analyses were done either in duplicate or in triplicate. Statistically significant differences between storage test samples in studies **II** and **IV** and between sensory evaluation sample ratings in study **IV** were analyzed with one-way analysis of variance (ANOVA) together with Tukey's test using IBM® SPSS® Statistics version 21 software (IBM Corporation, Armonk, NY, United States). The external free lutein standard curve in study **II** was made with eight concentration points ranging from 0.58 to 13.1 µg/ml and each point was calculated as a mean value of five replicate analyses. Encapsulation efficiency standard curve in study **III** was made with six points and each point was calculated as a mean value of four replicate measurements. In study **IV**,

Principal component analysis (PCA) was used to study the individual liking ratings (X) for six sensory samples. Principal component regression (PCR) and partial least squares regression (PLS) was used to study the interactions between the consumer comment frequencies or instrumental measurements (X-data in PCR and PLS, respectively) and averaged liking ratings (Y). Cross validation was used to estimate the number of components for a statistically reliable model. Multivariate models were conducted using Unscrambler 10.3 (Camo Process AS, Oslo, Norway).

5 RESULTS AND DISCUSSION

5.1 Composition of oat polar lipid emulsifier

The composition of the oat polar lipid fraction used as an o/w emulsifier was analyzed in study **I**. On the two fractionation steps, some minor peaks were not collected in any of the fractionations 1–3 (step 1) or subfractions 1a–1d (step 2) and some compounds may have been left uneluted. Thus, the mass proportions of the weighed fractions represent only crude estimates on the relative proportions of the identified compounds on the original sample. Based on co-injections, fraction 1b contained MGDG and SG, fraction 1d contained DGDG, and fraction 2 contained PC. Compounds in fractions 1a, 1c, and 3 could not be identified with the reference compounds used, and fractions 1b, 1d, and 2 also contained some unidentified compounds. Based on retention times, fraction 1 may also contain PI but the solvent system used in the second fractionation step did not include water and therefore could not elute PI into any of the subfractions 1a–1d. The effect of water on elution of PI was realized only after most of the fractionation work was already done and the presence of PI in the original sample was not verified with co-injection. Crude mass proportions of the fractions and subfractions are shown in figure 12, and based on these

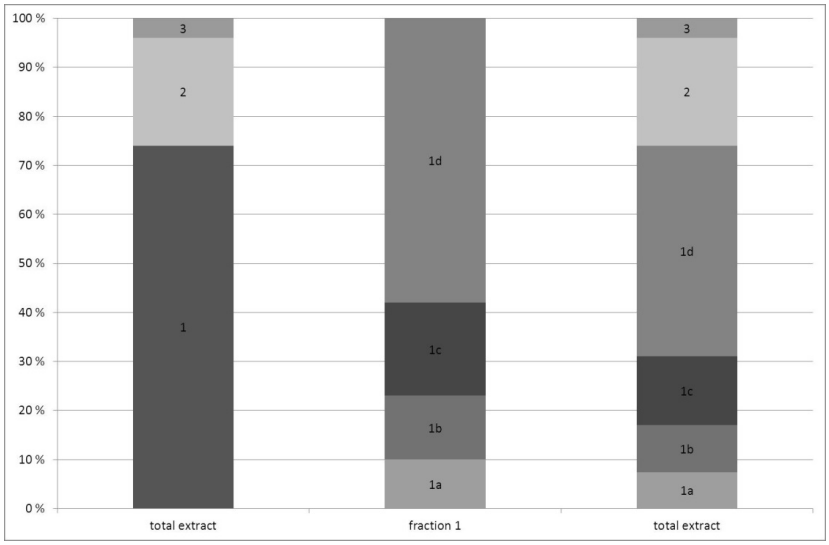


Fig. 12 Crude proportions of HPLC fractions 1–3 on the total extract (left), subfractions 1a–1d on the fraction 1 (middle), and fractions 1a–1d, 2, and 3 on the total extract (right). As some peaks were not collected, the percentages are crude estimates. Reprinted from Kaimainen et al. 2012 with permission. Copyright 2012 Springer Science+Business Media.

proportions, the identified compounds (MGDG, DGDG, SG, and PC) represent the majority of the original sample with DGDG and PC as the main compound classes. Polar lipids have been reported to account for 10–30 of total lipids in oat, and the majority of polar lipids are phospholipids (mainly PC and PE) and glycolipids (mainly DGDG and MGDG) (Sahasrabudhe 1979, Banas et al. 2007, Leonova et al. 2008, Doehlert et al. 2010). Our results are well in line with the previous research on oat polar lipids despite the different extraction process of using supercritical carbon dioxide instead of organic solvents.

5.2 Extraction of pigments

Enzyme-assisted oil extraction of lutein from marigold flowers in study **II** was successful. The lutein contents of oil extracts (present as lutein esters but calculated as free lutein) 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 1.1 mg/g of marigold flowers (fresh weight) and this was quite similar to what we extracted from the same marigold flowers using acetone – 0.9 mg/g of marigold flowers (fresh weight). The lutein yield was difficult to compare with reported research results as most reported lutein contents of marigold flowers are presented for dry weight. 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 five- to six-fold difference to content per dry weight. The range of lutein yields is quite wide depending on the variety. We did not have the variety information but compared to reported lutein contents and our own extraction with acetone, the enzyme-assisted oil extraction seems to be a good way to extract lutein from marigold flowers without the use of organic solvents.

Water extraction of betanin from red beet was done in study **III**, and the method was further optimized for study **IV**. In study **III**, the extract was not studied further other than for the UV spectrum, which showed a characteristic peak profile of betanin and measurement of osmolarity for preparation of the double w/o/w emulsion. In study **IV**, the effects of temperature (40 or 80 °C), ratio of beet:water (1:1 or 1:2), and extraction time (5, 15, 30 or 60 min) on the yield of betanin were studied. Especially with longer extraction times, high temperature induced degradation of betanin. Extraction time had otherwise no effect on the yield of betanin provided that the beet was in small enough pieces to allow the fast extraction of betanin into the water phase. Extraction with ratio of beet:water 1:2 had a betanin yield of 1.28 mg/g beet, which was 18

percent higher than the yield of 1.08 mg/g beet with ratio of 1:1, but as there was more extract, the concentration of betanin was 32 percent smaller (550 µg/ml and 804 µg/ml). For maximum betanin yield, the extraction with ratio of beet:water of 1:2 was better, and the extraction could even be repeated as a lot of color was left on the solid matter. However, if the process involved concentration or drying of the extract, a ratio of 1:1 could also be considered for lower energy consumption as less water needs to be evaporated.

5.3 Encapsulation

5.3.1 Preparation of o/w emulsions

The effects of emulsion composition (amount of oil and emulsifier) and creaming on droplet size were assessed in study **I**. Mean droplet sizes for o/w emulsions with different compositions are shown in table 4. Measurements with DLS and LD gave very different results as DLS detected only a small amount of droplets in the one to six µm range (upper limit of the instrument) whereas LD detected a population of droplets in the 0.5 to two µm range (lower limit of the HELOS/KR instrument). As the lower limit of detection in the LD measurement was 0.5 µm, the whole droplet size distribution of the droplets could not be measured and the mean diameters for LD measurements presented in table 4 were slightly larger than the actual sizes. Reasons for the contradicting results of DLS and LD measurement could be that DLS works better for submicron droplets with a narrow size distribution and there could also be so much more submicron droplets than larger ones that DLS only detects the submicron droplets. Emulsions also needed to be diluted for LD measurement, and this may also have affected the results. We tested that the dilution of emulsions did not affect the DLS measurement, but this may not be the case for LD measurement. Microscopic investigation, however, verified the presence of larger droplets in the range of a few µm as can be seen in figure 13.

The mean droplet sizes of o/w emulsions seemed to decrease with increasing emulsifier concentration and diminishing oil content except that the droplet sizes of all emulsions containing 10 mg/ml oil or emulsion containing 20 mg/ml oil and 10 mg/ml emulsifier are quite similar when measured with LD. Also, the droplet size distributions of these emulsions are quite similar when measured with LD. For emulsions containing 20 mg/ml oil and 2.5 mg/ml emulsifier or 50 mg/ml oil and 10 mg/ml emulsifier, LD revealed yet another population of droplets around 5 to 10 µm, which suggests that for these emulsion compositions there might not be enough emulsifier to produce smaller droplets.

Table 4 Average droplet sizes of o/w emulsions in study **I**.

Emulsion composition	mean diameter	
	DLS (nm)	LD (μm)
10 mg/ml oil, 2.5 mg/ml emulsifier	198	1.06
10 mg/ml oil, 5 mg/ml emulsifier	166	1.05
10 mg/ml oil, 10 mg/ml emulsifier	151	1.06
20 mg/ml oil, 2.5 mg/ml emulsifier	230	2.13
20 mg/ml oil, 5 mg/ml emulsifier	209	1.47
20 mg/ml oil, 10 mg/ml emulsifier	152	1.04
50 mg/ml oil, 10 mg/ml emulsifier	191	2.73

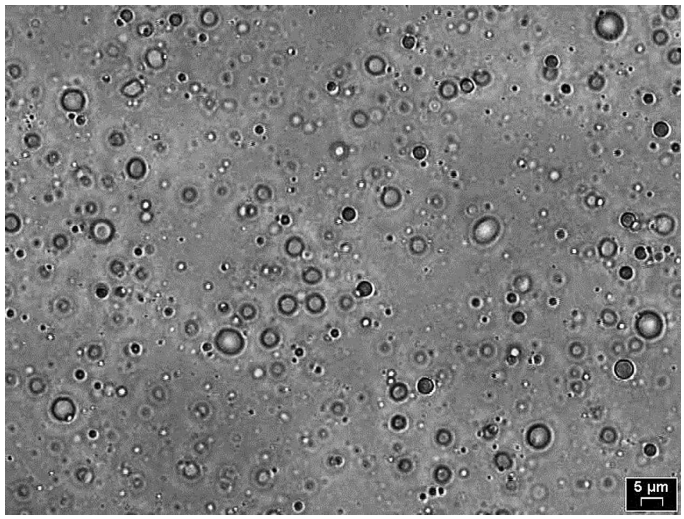


Fig. 13 Microscopic image of emulsion containing 10 mg/ml oil and 5 mg/ml emulsifier clearly showing droplets with the size of 1–5 μm.

The o/w emulsions in study **I** creamed quite rapidly, and we analyzed the droplet sizes of the upper and lower phases of emulsions after three days from preparation. The two phases could not be separated from all emulsions, and when collecting the upper and lower phases of emulsions, the upper phases contained also some lower phase. For this reason, DLS measurements were only made on the collected lower phases, as the detection of any submicron droplets would not have proved the presence of small droplets in the actual upper phase. Mean droplet sizes of upper and lower phase of creamed emulsions are shown in table 5. The mean droplet sizes of emulsion lower phases were smaller than those of freshly prepared emulsions whereas the mean droplet sizes of emulsion upper phases were considerably larger. For emulsion upper phases, LD revealed a population of very large droplets (5–30 μm or

even larger) but based on microscopic observation, this increase in droplet size seems to be an effect of aggregation. This implies that these emulsions may be stable against coalescence, which is usually more difficult to prevent or reverse than creaming or aggregation, which were a problem in these emulsions. Gentle agitation of the emulsion usually disrupts the cream layer and reverses its effects, whereas aggregation may be reversed or accelerated by agitation depending on the strength and type of interactions in the aggregates (Walstra 2003).

Table 5 Average droplet sizes of emulsion upper and lower phases after 3 days from preparation in study **I**.

Emulsion composition	mean diameter	
	DLS (nm)	LD (μm)
20 mg/ml oil, 2.5 mg/ml emulsifier, lower phase	203	nm
20 mg/ml oil, 5 mg/ml emulsifier, upper phase	nm	4.48
20 mg/ml oil, 5 mg/ml emulsifier, lower phase	239	1.05
50 mg/ml oil, 10 mg/ml emulsifier, upper phase	nm	14.90
50 mg/ml oil, 10 mg/ml emulsifier, lower phase	164	1.26

nm = not measured

In study **II**, the droplet sizes of emulsions were not measured as we had no access to suitable equipment during that study. The addition of small amounts of different long chain polysaccharides to emulsions effectively prevented creaming probably due to increased viscosity as most of the emulsion samples showed no creaming during the 10-week storage test. However, it would be interesting to see whether the addition of stabilizing polysaccharides has an effect on the droplet size and also how the droplet sizes of these stabilized emulsions develop during storage.

5.3.2 Preparation of w/o/w emulsions

Double $w_1/o/w_2$ emulsions with betanin in the inner water w_1 phase were prepared in study **III**. Formation of double emulsions was successful, which was confirmed by microscopic investigation, as primary w_1/o droplets could be seen inside the larger o/w_2 droplets as shown in figure 14. Encapsulation efficiency of betanin was 89.1 percent, which was quite high, but encapsulation efficiencies of up to 95 percent or even 99 percent have been reported for $w_1/o/w_2$ double emulsions (Hasegawa et al. 2001, Benichou et al. 2007, Mun et al. 2010, Sapei et al. 2012). However, also lower encapsulation efficiencies of less than 20 percent have been reported, and the encapsulation efficiency

probably depends on the composition of the whole system (Fechner et al. 2007, O'Regan & Mulvihill 2010).

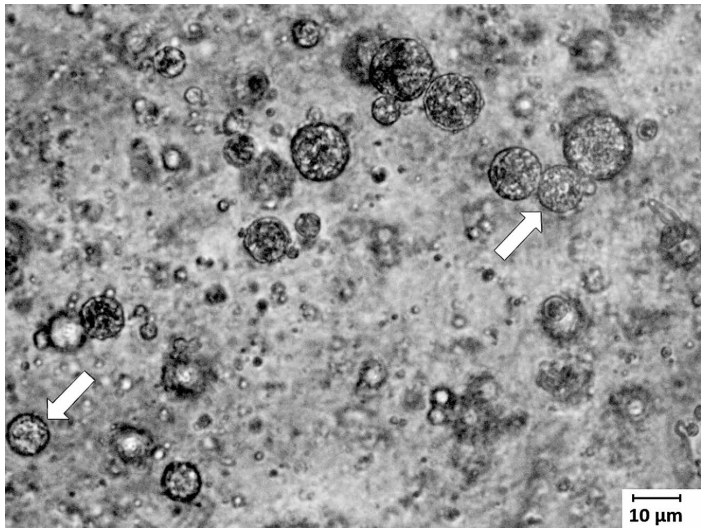


Fig. 14 Microscopic image of a double $w_1/o/w_2$ emulsion in study **III**. White arrows point out examples of $w_1/o/w_2$ droplets. Reprinted from Kaimainen et al. 2015 with permission. Copyright 2014 Elsevier Limited.

Droplet size analysis of the $w_1/o/w_2$ double emulsion with LD revealed two populations of droplets: a smaller droplet population accounting for 20 percent of the total droplet volume centered on a peak at 313 nm, and a larger droplet population accounting for 80 percent of the total droplet volume centered on a peak at 6.6 µm. As the primary w_1/o emulsion droplets could be observed with microscope, they can likely cause laser diffraction and thus be detected in the LD measurement. DLS measurement of the primary w_1/o emulsion showed a peak centered at 343 nm with a range similar to the smaller droplet population in the LD measurement, so that the population corresponded to the primary w_1/o emulsion droplets. We observed only a very little creaming of $w_1/o/w_2$ double emulsions during a few days although the droplet size was much larger than in study **I**. Similarly to o/w emulsions in study **II**, the $w_1/o/w_2$ double emulsions in study **III** contained stabilizing polysaccharides, and this could have led to a slow creaming rate. Another reason for slow creaming could be the smaller density difference of the oil and outer w_2 phase due to the presence of inner water phase w_1 droplets.

5.3.3 Spray drying

Spray drying was used in study **II** to dry o/w emulsions and in study **IV** to dry beetroot extract. In both studies, the yield of spray drying was not very high at 57 percent and 62 percent of theoretical maximum yield in study **II**, and 44 percent in study **IV**, as a large amount of powder was tightly adhered to the walls of the spraying chamber. Yields of the spray drying processes in small pilot-scale devices are usually rather low but careful process optimization can improve the yield to some extent (Langrish et al. 2007, Goula and Adamopoulos 2008). Particle sizes of powders were not measured in either study due to technical reasons but according to the instrument manual, the powder size is usually between one and 25 μm. This means that the powder size may be quite close to the emulsion droplet size in study **II**, which could affect encapsulation efficiency and stability. The moisture contents of powders were determined by oven drying and they were 60 mg/g in study **II** and 40 mg/g in study **IV**.

5.4 Storage tests

The stability of lutein during storage was assessed in study **II** and changes in various properties of model juices during storage were monitored in study **IV**. In study **II**, the visual appearances of the samples did not change during storage other than the emulsion with GG + XG 3:1 + MD (sample E) had slight creaming after five 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 pilot scale equipment. HPLC analysis of the samples revealed peaks for free lutein and lutein esters in all of the samples and time points. The amount of free lutein was 0.5 to 1.0 percent of the amount of lutein esters, and the ratio did not change markedly during the storage period. Due to the different sample matrices (wet emulsion, spray dried emulsion, or plain oil), the quantitative amounts of lutein varied between samples so the amount of lutein at each time point was compared to the initial amount of each for easier comparison of lutein stability between samples. Relative amounts of lutein (free and esterified, calculated as free lutein) left in the samples during storage with statistically significant differences between samples and time points are shown in table 6. The amount of lutein decreased in all of the samples during storage, and only in sample B the difference between initial amount and amount after 10 weeks was not statistically significant. Even in that sample, the amount after five weeks was significantly lower than the initial amount. The retention of lutein during 10 weeks varied between 67 and 91

percent, and it was lowest in the spray dried samples, 75 percent for sample G and 67 percent for sample H, although sample G did not differ significantly from the majority of the samples. Possible reasons for this are the high temperature in the spray drying process, although the contact time is short and most of the energy is used to evaporate water (Gharsallaoui et al. 2007), and the differences in the emulsion and powder structure on the proximity of oxygen species (from air) and lutein.

Carotenoids are easily oxidized by, for example, oxygen, light, and heat (Boon et al. 2010). Our samples were stored in the dark at 20 to 22 °C so the loss of lutein is probably not caused by light and heat and the atmosphere of the sample tubes was not controlled, so air and oxygen were present in the storage conditions. The effect of water activity (a_w) on carotenoid stability has been studied for different sample matrices with varying results (Rodriguez-Huezo et al. 2004, Lavelli et al. 2007, Oh et al. 2013). Rodriquez-Huezo et al. (2004) reported maximum carotenoid degradation at a_w 0.628 with lower and almost constant degradation at higher or lower a_w values, whereas Lavelli et al. (2007) reported maximum carotenoid stability at a_w 0.31–0.54, and Oh et al. (2013) reported increased carotenoid degradation with increasing a_w. It seems that the effect of a_w on carotenoid stability is greatly dependent on the sample material but it could be one reason for the different stability of lutein in wet and dried emulsions in our study. We did not measure the a_w of our samples and did not control the a_w of storage conditions (other than that samples were in closed containers), but it seems reasonable to assume low a_w values for spray dried emulsions and oil and very high a_w values for wet emulsions.

Changes in pH, °brix, UV spectrum (350–600 nm) and betacyanin content (total amount of betanin and isobetanin) of model juices during storage were monitored in study **IV**. The pH of model juices varied between 3.02 and 3.33 or 4.97 and 5.08 for model juices with initial pH 3.4 or 5.0, respectively, but there was no clear trend. Dry matter content as measured by °brix varied between 1.4 and 1.7 or 10.4 and 11.2 for model juices sweetened with steviol glycosides or sucrose, respectively, with no clear trend in storage at -20, 4, or 20°C. In storage at 60°C, °brix showed a slight increase during storage, probably due to concentration by evaporation of water (observed by weight loss of samples). The UV spectra of model juices stored at 4, 20, or 60°C showed a decrease of absorbance in a peak at 535 nm (betanin) and a development of a new peak at 430 nm and an increase of absorbance near 350 nm during storage with faster changes with increasing storage temperature. Interestingly, the UV spectra of model juices stored at -20°C showed only minimal changes during storage, although the HPLC analysis revealed a decrease in betanin content during storage also in these samples.

Table 6 Retention of lutein during the 10-week storage period with standard deviations of triplicate measurements presented as % lutein left compared to initial amount in study **II**. Time points not sharing at least one same superscript letter (a-f) are statistically different from each other for each sample ($p \leqslant 0.05$, 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 ($p \leqslant 0.05$, comparison by columns).

Sample	Stabilizer composition	Storage time				
		0 weeks	1 week	2 weeks	5 weeks	10 weeks
A	GG + XG 3:1	100 ± 1 %^{a,b}	99 ± 3 %^{a,1,2}	97 ± 2 %^{b,1}	83 ± 4 %^{c,1,2,3}	77 ± 4 %^{d,1,2}
B	GG + XG 1:3	100 ± 5 %^{a,b}	112 ± 10 %^{c,d,4}	99 ± 9 %^{a,c,e,1}	90 ± 8 %^{e,f,3,4}	91 ± 11 %^{b,d,f,3}
C	LBG + XG 3:1	100 ± 2 %^{a}	101 ± 5 %^{b,1,2,3}	97 ± 4 %^{a,b,1}	87 ± 4 %^{c,2,3,4}	82 ± 4 %^{d,1,2}
D	LBG + XG 1:3	100 ± 3 %^{a,b}	109 ± 4 %^{a,3,4}	94 ± 4 %^{b,1,2}	83 ± 4 %^{c,1,2}	77 ± 4 %^{d,1}
E	GG + XG 3:1 + MD	100 ± 2 %^{a}	98 ± 4 %^{a,1,2}	91 ± 3 %^{b,1,2}	81 ± 5 %^{c,1,2}	80 ± 3 %^{c,1,2}
F	LBG + XG 1:3 + MD	100 ± 3 %^{a}	98 ± 6 %^{a,b,1}	97 ± 4 %^{a,1}	81 ± 5 %^{c,1,2}	82 ± 8 %^{b,c,1,2}
G	GG + XG 3:1 + MD, spray dried	100 ± 5 %^{a,b}	106 ± 9 %^{a,2,3,4}	93 ± 11 %^{b,1,2}	79 ± 11 %^{b,c,1,2}	75 ± 8 %^{c,1,4}
H	LBG + XG 1:3 + MD, spray dried	100 ± 1 %^{a}	99 ± 5 %^{a,1,2}	88 ± 6 %^{b,2}	79 ± 2 %^{b,1}	67 ± 5 %^{c,4}
oil		100 ± 1 %^{a}	101 ± 3 %^{a,1,2,3}	96 ± 3 %^{b,1}	91 ± 2 %^{c,4}	85 ± 2 %^{c,2,3}

HPLC analysis of model juices revealed a decrease of betacyanin content in all of the samples during storage with faster degradation with increasing storage temperature and decreasing pH. As the initial amounts of betacyanins were different in various model juices, betacyanin contents at time points were compared to the initial amount in the same model juice for easier comparison between samples. Relative amounts of betacyanin left in samples during storage at different temperatures are shown in tables 7, 8, and 9. In model juices, stored at 60 °C, there was not even a trace of betacyanins left even at the first time point, one week for ST1 and ST2, or three days for ST3 and ST4. The reason for different storage times for cold storage is that the test began with only model juices ST1 and ST2 and only for storage at 20 and 60 °C, but as it was quickly revealed that the stability of betacyanins was not good in these samples, two more model juices with more betacyanins and higher pH were prepared. At the same time, we also decided to prepare some samples of model juices ST3 and ST4 for cold storage as temperature seemed to have a great effect on betacyanin stability, and we only had a small amount of the first two model juices in cold storage for a limited number of time points. The pH 5.0 was in the optimum range for betacyanin stability, but as model juices with pH 5.0 contained twice the amount of betacyanins than model juices with pH 3.4, the better stability cannot be issued to pH alone as the higher betacyanin concentration could also have had an effect (Herbach et al. 2006). At pH 3.4, sucrose seemed to accelerate betacyanin degradation compared to steviol glycosides, but at pH 5.0, there was no big difference caused by the sweetener (or dry matter content which was modelled by the sweetener). The mechanism is not known but Cai & Corke (1999) reported a somewhat similar effect of sucrose above a concentration of 13 percent that increased Amaranth betacyanin degradation in a model beverage system. However, their model beverage had a pH of 5.6, whereas in our system, the effect was only seen at pH 3.4 and not at pH 5.0.

Table 7 Relative amounts of betacyanins presented as % betacyanins left as compared to the initial amount in model juices stored at 20 °C in study **IV**.

Model juice	Storage time				
	1 week	2 weeks	3 weeks	4 weeks	2 months
ST1	19 %	nd	nd	nd	nm
ST2	38 %	tr	nd	nd	nm
ST3	85 %	47 %	20 %	7 %	tr
ST4	76 %	48 %	24 %	9 %	tr

nd = not detected, nm = not measured, tr = trace

Table 8 Relative amounts betacyanins presented as % betacyanins left as compared to the initial amount in model juices stored at 4 °C in study **IV**.

Model juice	Storage time		
	2 months	3 months	4 months
ST1	5 %	nm	nd
ST2	40 %	nm	nd
ST3	77 %	52 %	34 %
ST4	68 %	50 %	35 %

nd = not detected, nm = not measured

Table 9 Relative amounts of betacyanins presented as % betacyanins left as compared to the initial amount in model juices stored at -20 °C in study **IV**.

Model juice	Storage time		
	3 months	4 months	5.4 months
ST1	nm	nm	18 %
ST2	nm	nm	17 %
ST3	83 %	67 %	nm
ST4	82 %	71 %	nm

nm = not measured

The stability of betacyanins in the spray dried powder was much better than in the model juices. After six months of storage at 20 °C, the amount of betacyanins was virtually unchanged, and after four or six months of storage at 60 °C, the relative amount of betacyanins was 80 or 60 percent of the initial amount, respectively. This is in accordance with previous reports describing that low water activity greatly enhances betanin stability (Herbach et al. 2006, Pasch & Von Elbe, 1975). The effect of different wall materials on the stability of betalains from beetroot or Amaranth has been studied, and the mixture of maltodextrins with DE25 and 10 was better than either maltodextrin alone (Cai & Corke, 2000). Only little differences were found between Arabic gum and maltodextrin (DE 11) (Janiszewska, 2014). There seemed to be quite high variation in the amount of betacyanins in parallel samples at the same time point, which was noticed only after six months of storage. There were not many parallel samples done from the initial samples or after storage for four months, so the actual values for these time points may be different. As the initial amount may be slightly higher than measured, the relative values after storage could be slightly smaller than reported.

The ratio of betanin:isobetanin changed during storage, and the change was different for various samples and storage temperatures. In model juices ST1and ST2 (pH 3.4), the ratio decreased during storage at 20 or 4 °C. During storage at -20 °C, the ratio increased in sample ST1 and decreased in sample ST2. In

model juices ST3 and ST4 (pH 5.0), the ratio increased during storage at 20 or 4 °C. During storage at -20 °C, the ratio remained relatively constant in sample ST3 and decreased in sample ST4. In the spray dried powder, the ratio remained constant during storage at 20 °C and decreased markedly during storage at 60 °C. The big decrease in the ratio of betanin:isobetanin during storage at 60 °C could be an effect of different degradation rates of betanin and isobetanin, or more probably due to isomerization of betanin to isobetanin, which is known to occur at elevated temperatures (Herbach et al. 2004). Overall, the effect of temperature on betacyanin stability would suggest using them as colorants for products that are stored cold. Indeed, the use of betalains from Malabar spinach (*Basella rubra*) for coloring ice cream has been studied with good results (Kumar et al. 2014). Betacyanins from Amaranth were used to color jelly, ice cream, and beverage with good color stability at low temperatures after 12 or 18 weeks, but with inferior stability at room temperature (Cai & Corke, 1999). Commercial beetroot red colorant (E162) can also be found in chilled dairy products; e.g., fruit yogurts.

5.5 *In vitro* intestinal lipid digestion

In vitro intestinal lipid digestion of a $w_1/o/w_2$ double emulsion was done in study **III** to see the effects of lipase and bile salts on the structure of double emulsion and on the retention of betanin in the inner w_1 phase during digestion. Multimodal droplet size distributions were present during the whole digestion, and the smallest sized droplet population likely corresponded to the primary w_1/o emulsion droplets. We calculated the surface mean diameter D3,2, which represents the primary w_1/o emulsion droplets and the volume mean diameter D4,3, which represents the $w_1/o/w_2$ double emulsion droplets. The D3,2 and D4,3 for the digestion and control experiments are shown in table 10. As the droplet size distributions were multimodal, the larger sized droplet population slightly increased the D3,2, whereas the effect of the smaller sized droplet population on the D4,3 was negligible. Both the D3,2 and D4,3 increased quickly in the *in vitro* intestinal lipid digestion experiment, whereas both remained relatively unchanged in the control experiment. The increases in the digestion experiment reflected coalescence of the inner w_1/o droplets and aggregation of the outer o/w_2 droplets, the latter process characterized by the formation of larger sized droplets (30–100 μm) from 30 minutes onwards. Both the coalescence and aggregation processes could be verified under microscope as can be seen in figure 15.

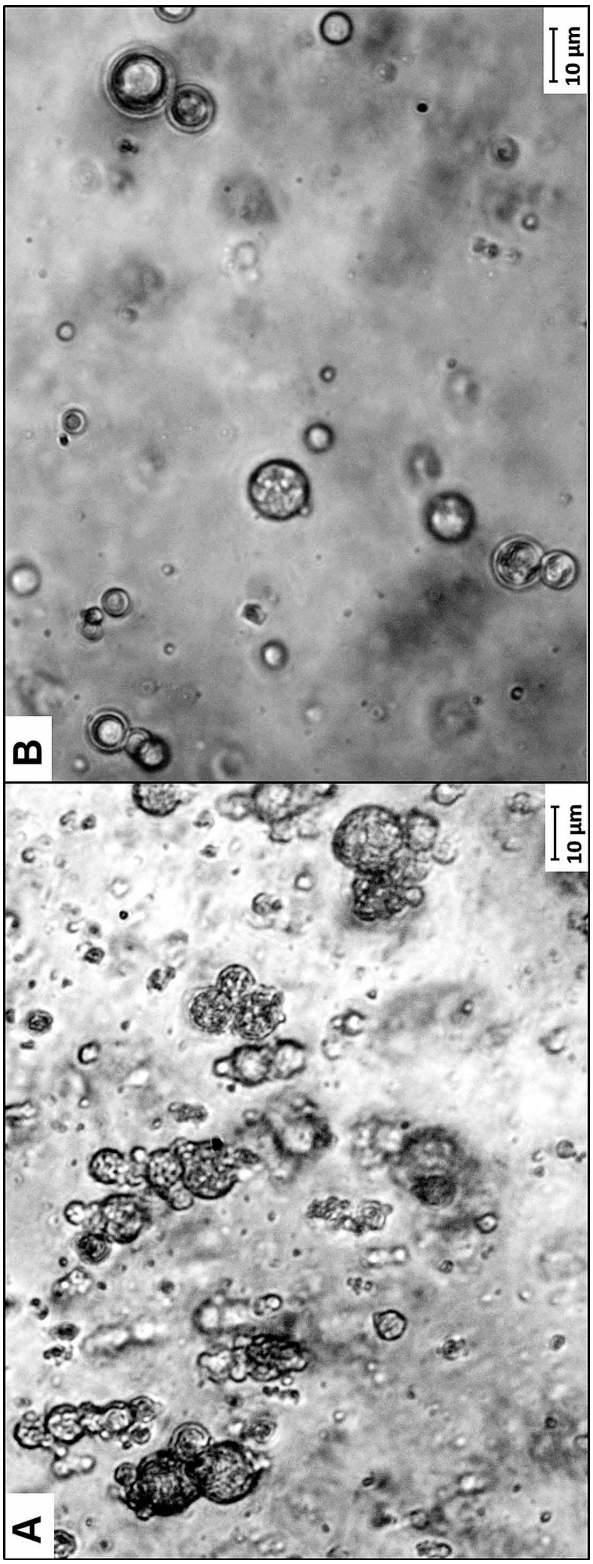


Fig. 15 Microscopic images of the double emulsion during *in vitro* intestinal lipid digestion showing the aggregation of the $w_1/o/w_2$ droplets after 20 minutes (A) and the complete coalescence of some inner w_1/o emulsion droplets after 110 minutes (B). Reprinted from Kaimainen et al. 2015 with permission. Copyright 2014 Elsevier Limited.

Table 10 Droplet size calculations, during the *in vitro* intestinal lipid digestion in study **III**, showing the calculated surface means D3,2 and volume means D4,3. The control experiment was done in the same conditions but without the bile salt and lipase.

Digestion time	D3,2 (μm)	D4,3 (μm)
Control 0 min	0.81 ± 0.15	5.49 ± 0.42
Control 180 min	0.75 ± 0.07	5.34 ± 0.67
0 min	1.04 ± 0.09	6.28 ± 0.25
30 min	1.66 ± 0.13	11.95 ± 1.75
60 min	2.02 ± 0.37	26.74 ± 1.60
90 min	2.55 ± 0.18	35.30 ± 0.62
120 min	2.98 ± 0.27	38.82 ± 6.58
180 min	2.74 ± 0.39	43.63 ± 3.40

Retention or release of betanin in the inner w_1 phase of the double emulsion during *in vitro* intestinal lipid digestion was studied by measuring the encapsulation efficiency during the digestion experiment. The release of betanin was quite fast in the beginning but it slowed down as digestion processed and during the last hour (from 120 to 180 minutes), no more release was detected, and the total release was 35 percent. This relatively low release can be explained by the fact that double emulsion structure was retained throughout the digestion, even though part of the inner w_1/o emulsion droplets coalesced. In the control experiment, the release of betanin was very small during the 180 minute experiment confirming that the release of betanin is an effect of the bile salt and/or lipase and not the environmental conditions (pH, osmolarity, temperature).

Several studies on lipid digestion of simple o/w emulsions have reported an increase in droplet size during digestion, usually due to aggregation of the oil droplets (Mun et al. 2007, Nik et al. 2011, Salvia-Trujillo et al. 2013). The type and amount of emulsifier (Mun et al. 2007, Nik et al. 2011, Yao et al. 2013) and the initial droplet size of the emulsion (Troncoso et al. 2012, Salvia-Trujillo et al. 2013 affected the lipase efficiency. Only a few studies on lipid digestion of w_1/o/w_2 double emulsions have been made. Shima et al. (2004) showed that small outer droplets released a more hydrophilic marker from the inner w_1 water phase than large ones because the latter were not hydrolyzed by lipase. Our results are in agreement with this as the aggregation of outer o/w_2 droplets seemed to limit the release of betanin from the inner w_1 water phase in our system.

5.6 Sensory evaluation

Sensory evaluation of model juices colored with betanin or anthocyanins were done in study **IV** to study consumer acceptance of model juices colored with spray dried betanin compared to model juices colored with anthocyanins or beetroot extract. The appearances of model juices SE4 and SE6 (i.e., colored with beet extract or anthocyanin, both 'strong' colors) were the most liked. Mean scores with standard deviations and statistically significant differences between model juices ($p<0.05$) for pleasantness of appearance are shown in figure 16. Overall, the 'strong' colored samples were preferred over 'weak' colors made with the same colorant. This is in accordance with a previous finding of more intense color of a juice against other juices resulting in increased liking of appearance of anthocyanin-rich berry juices (Laaksonen et al. 2013). There were no big differences between pleasantness of odor of the samples (mean scores: 3.66–4.31). The only statistically significant difference was that sample SE6 (anthocyanin 'strong') was more pleasant than samples SE1, SE2, and SE3 (spray dried betanin samples). Similarly, the pleasantness of taste was very similar in all of the samples (mean scores 3.01–4.51), and the only statistically significant difference was that sample SE3 (only one with pH 5.0) was less liked than other samples, except that difference to sample SE2 was not statistically significant. These small differences in odor and taste could be explained by the very similar juice compositions – possible odors originated from the colorants, and the higher pH of model juice SE3 affected the taste of that model juice.

The internal preference map (principal component analysis, PCA) revealed a segment of 18 subjects with different preferences in liking of appearance than the main group. The main group preferred the sample SE6 (anthocyanin 'strong') more than the segment, whereas the segment preferred samples SE1, SE2, and SE3 (spray dried betanin samples) more than the main group (t-test, $p<0.05$). In general, there was no difference in liking of sample SE4 (beet extract) between the segment and the main group. Classification of subjects into two groups correlated with the importance of natural colorants (Pearson's correlation) with the main group rating higher importance of natural colorants than the segment with group means 3.9 and 3.1, respectively (between classes 3=moderately important and 4=very important). The segment classification of subjects was not dependent on age or gender.

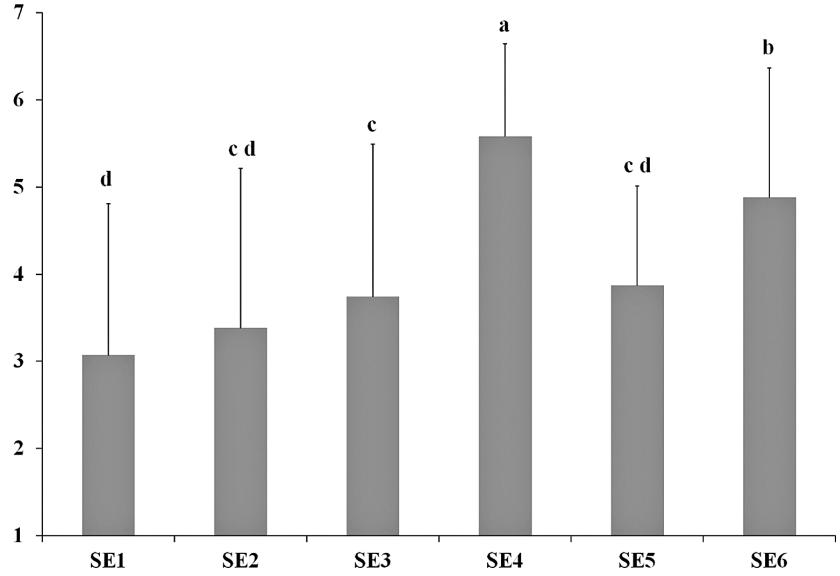


Fig. 16 Mean scores of pleasantness of appearance of the model juices in study **IV**. Samples not sharing a letter (a-d) are statistically different ($p<0.05$).

Free-form descriptions of samples were analyzed with principal component regression (PCR) that correlated sample descriptions with liking separately for appearance, odor, and taste. PCR analysis of appearance correlated descriptions such as 'artificial', 'not real juice', 'unnatural', and 'chemical colorant' with disliking and these were associated with samples SE1, SE2, and SE3 (spray dried betanin samples). Descriptions such as 'natural', 'berry', 'tempting', and 'pleasant' were correlated with liking of samples and associated with samples SE4 (beet extract) and SE6 (anthocyanin 'strong'). The observations made during the appearance ratings in this study may have affected the ratings for odor and flavor because food colours may generate specific expectations regarding the other sensory aspects of the food (Spence et al. 2010). For example, red colored drinks may be often linked to strawberry and other berries (Zampini et al. 2007). The most interesting result of PCR analysis of odor was that descriptions 'beetroot' and 'earthy' were associated with sample SE4 (beet extract) but not with the other samples, and this was also observed in PCR analysis of taste. This result was actually partly expected, as during the concentration step (with rotary evaporator) of spray dried betanin preparation, the characteristic beetroot smell was removed from the colored extract into the condensed water phase. This means that off-odors and off-flavors derived from beet seem to be removed from the spray dried betanin, most probably in the rotary evaporation step. PCR analysis of taste also correlated descriptions such as 'artificial', 'acidless', 'sweet', 'unpleasant', and 'sugar dilution' with disliking and these were associated with sample SE3 (the pH 5.0 sample).

Overall, the results of the sensory evaluation study would suggest not to use spray dried betanin as a colorant for juices. However, the model juice colored with beet extract was the most liked sample for appearance, and the beet material was different for that extract than the extract used in preparation of spray dried betanin. The situation could also be different when the colorants are used in real berry juices with natural anthocyanins from the berries present, as the mixture of anthocyanins and betanin could produce a more acceptable color than betanin alone. In fact, mixtures of anthocyanins and betalains can produce color hues that are impossible to produce with either pigment class alone, but no consumer preference tests were done in that study (Stintzing et al. 2006). The consumer preference of color hue could also be different for food products other than juices, so in some products, the pink color produced by spray dried beetroot powder could be favorable. Most importantly, the spray dried betanin did not impart any beet-derived off-odors or off-flavors into the model juice.

6 SUMMARY AND CONCLUSION

In this thesis work, extraction procedures without organic solvents and encapsulation of lutein and betanin and the stability of encapsulated pigments during storage were studied. Furthermore, preliminary studies on the bioavailability and consumer acceptance of encapsulated pigments were conducted.

The composition of oat polar lipid fraction was studied and it was found to comprise mainly of glycolipids (DGDG, MGDG and SG) and phospholipids (PC). Preliminary studies showed that polar lipids from oat could be used as o/w emulsifier with potentially reduced coalescence tendency of the emulsion droplets. The emulsions were prone to creaming but it was possible to retard this in later parts of the thesis work.

An enzyme-assisted oil extraction procedure of lutein from marigold flowers was developed and the yield was comparable to solvent extraction. This method can be used to produce vegetable oil containing up to 0.50 mg/ml lutein without the need to remove extraction solvents afterwards. This oil can be used for food coloring either as such for oil-based products or in an o/w emulsion for aqueous products. Similar stability of lutein in oil and in o/w emulsion was observed during storage in the dark at room temperature.

Optimal conditions for extraction of betanin form industrial red beet peeling by-product with water were described. This process can give more value to industrial red beet peeling by-product, as the extract could potentially be used for food coloring purposes. Although the stability of spray dried betanin was not good in model juices stored at room temperature, stability in model juices at cold storage and in the dry pigment powder was better. Thus, betanin could be added into foods that are stored cold and the spray dried powder can be stored for longer times.

Betanin was incorporated in the inner w_1 water phase of a $w_1/o/w_2$ double emulsion and preliminary *in vitro* intestinal lipid digestion of a $w_1/o/w_2$ double emulsion was performed. Encapsulation efficiency of betanin was quite high, 89 percent, and the double emulsion showed good stability with only a slight creaming during storage for a few days. The double emulsion structure was retained throughout the three-hour experiment, and also majority of betanin was retained in the inner w_1 phase with a 35 percent release. The control experiment without bile salt and lipase confirmed that virtually no changes were due to osmolarity and pH of the digestion medium and this indicated good stability of the double emulsion. As the experiment only included the intestinal step of the gastrointestinal tract, further studies with gastric step included in the model should be conducted.

Consumer acceptance of model juices colored with spray dried betanin was not as good as similar model juices colored with anthocyanins, although a small segment of consumers preferred the betanin-colored model juices. However, the color produced by a mixture of added betanin and naturally occurring anthocyanins could be more acceptable for consumers. Also as the stability of betanin suggests use in cold products, such as ice cream, the pink color produced by spray dried betanin could be more acceptable in these kinds of products but further studies are needed on this subject.

ACKNOWLEDGEMENTS

This thesis work was carried out mainly at the Department of Biochemistry, University of Turku, Turku, Finland with parts of the research done at MTT Agrifood Research Finland, Jokioinen, Finland and the French National Institute for Agricultural Research (INRA), Nantes, France. Close collaboration was also done with the Department of Chemistry, University of Turku and Department of Physics and Astronomy, University of Turku. This thesis belongs to the University of Turku Graduate School (UTUGS) and the Finnish Graduate School on Applied Bioscience: Bioengineering, Food & Nutrition, Environment (ABS).

I am deeply grateful for the financial support provided by the Raisio Plc Research Foundation, the Doctoral Programme in Molecular Life Sciences (DPMLS) of UTUGS and Eeva-Liisa Hirvisaari Fund of the KAUTE Foundation. I also want to thank the Turku University Foundation, ABS, The Finnish Chromatography Society, DPLMS of UTUGS and the Bioencapsulation Research Group (BRG) for travel grants which allowed me to participate in numerous International Conferences on Bioencapsulation across Europe and also to make a research visit to INRA, Nantes, France as part of my thesis work.

The official reviewers, Professor Stephan Drusch and Docent Kirsi Jouppila are thanked for their constructive criticism and valuable suggestions. I also want to thank Robert M. Badeau, Ph.D. of Aura Professional English Consulting, LTD. (www.auraenglish.com) for language checking this thesis.

I want to thank my supervisors, Professor Rainer Huopalahti and Doctor Eila Järvenpää for their guidance and support during this thesis work and my master and doctoral studies. They always rejoiced with me when things were going smoothly and encouraged me through difficulties, as well.

My sincere thanks go to Professor Marc Anton, who let me come to his laboratory at INRA for a research visit as part of my thesis work. Thanks also to Doctor Sébastien Marze for guiding me with the experiments. I really enjoyed my time at Nantes and everyone there were very friendly. Thanks to Dominique, Hernan, Bérénice and everyone else there for good company, enjoyable conversations, and nice memories. Merci á vous tous!

Thanks to all my co-authors who have helped to write and finalize the original research articles that make up this thesis work. Especially, I want to thank Oskar Laaksonen, Mari Sandell, and Martti Kaasalainen for their contribution to parts of the manuscripts that were not my areas of expertise.

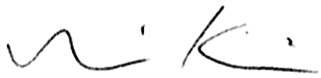
I have enjoyed my time at the food sciences group at University of Turku. I have worked with so many nice people there over the years and I want to thank all of them for creating a pleasant working atmosphere. Thanks to Professors

Heikki Kallio and Baoru Yang for their encouragement to do good science. I want to express my gratitude to Kaisa Linderborg for her help and co-operation during the times when I worked as part of the Food Chemistry teaching staff. I have shared my office with Jukka-Pekka Suomela and Leeni Mäkilä, and I want to thank them for their company and joyful conversations. Thanks to fellow doctoral candidates Marika Kalpio, Maaria Kortesniemi, Tuuli Puolimatka, Anu Nuora, and our foreign friends Wei Yang, Xueying Ma, Liz Gutierrez and Tian Ye for sharing the joys and sorrows of doctoral studies. Almost the same goes also for recently graduated doctors Marko Tarvainen, Jie Zheng, and Pengzhan Liu. Thanks to Anssi Vuorinen and Antti Knaapila for company during lunches and the conversations. I want to thank also Heikki Aisala, Johanna Paasio, and Aleksi Uusitupa for doing some of their laboratory work courses as part of my thesis work. My gratitude goes to the technical staff of our department, namely to Anu Hirvensalo and Jani Sointusalo for keeping the reagents available and the instruments working.

I give my gratitude to my family for their support. My parents, Lasse and Soile, have always believed in me as have my brothers Marko and Matti. Thank you for everything.

Finally, thanks to my dear wife Heidi. You have supported me during this whole thesis work and you have never grown tired of me doing some work at home. You have given me great happiness and joy in my life, and also a son, Aleksi. You two are my everything.

Raisio, November 2014



REFERENCES

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

Abdel-Aal, E.M., Young, J.C., Akhtar, H. & Rabalski, I. 2010, Stability of Lutein in Wholegrain Bakery Products Naturally High in Lutein or Fortified with Free Lutein. *Journal of Agricultural and Food Chemistry,* vol. 58, no. 18, pp. 10109–10117.

Ahmed, J., Shivhare, U.S. & Raghavan, G.S.V. 2004, Thermal degradation kinetics of anthocyanin and visual colour of plum puree, *European Food Research and Technology,* vol. 218, no. 6, pp. 525–528.

Alves-Rodrigues, A. & Shao, A. 2004, The science behind lutein. *Toxicology letters,* vol. 150, no. 1, pp. 57–83.

Amiot-Carlin, M.J., Babot-Laurent, C. & Tourniaire, F. 2008, Plant pigments as bioactive substances in *Food Colorants: Chemical and Functional Properties*, ed. C. Socaciu, Taylor & Francis Group, LLC, Boca Raton, FL, pp. 127–146.

Andersen, Ø.M. & Jordheim, M. 2006, The Anthocyanins in *Flavonoids - Chemistry, Biochemistry and Applications*, eds. Ø.M. Andersen & K.R. Markham, Taylor & Francis Group, LLC, Boca Raton,FL, pp. 471–551.

Aro, H., Aro, T., Huopalahti, R., Järvenpää, E. & Rostiala, N. 2007a, Luontaisten väripigmenttien rikastaminen kotimaisesta kasviraaka-aineesta, *Final report, Tekes project 2005–2007*, pp. 17

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

Ashraf, M. & Harris, P.J.C. 2013, Photosynthesis under stressful environments: An overview, *Photosynthetica,* vol. 51, no. 2, pp. 163–190.

Awika, J.M., Rooney, L.W. & Waniska, R.D. 2004, Properties of 3-Deoxyanthocyanins from Sorghum, *Journal of Agricultural and Food Chemistry,* vol. 52, no. 14, pp. 4388–4394.

Bacon, M.F. & Holden, M. 1967, Changes in chlorophylls resulting from various chemical and physical treatments of leaves and leaf extracts, *Phytochemistry,* vol. 6, no. 2, pp. 193–210.

Banas, A., Debski, H., Banas, W., Heneen, W.K., Dahlqvist, A., et al 2007, Lipids in grain tissues of oat (*Avena sativa*): differences in content, time of deposition, and fatty acid composition, *Journal of Experimental Botany,* vol. 58, no. 10, pp. 2463–2470.

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

Bauernfeind, J.C. 1972, Carotenoid vitamin A precursors and analogs in foods and feeds. *Journal of Agricultural and Food Chemistry,* vol. 20, no. 3, pp. 456–473.

Benichou, A., Aserin, A. & Garti, N. 2007, W/O/W double emulsions stabilized with WPI-polysaccharide complexes, *Colloids and Surfaces A,* vol. 294, pp. 20–32.

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

Blum, C.A., Xu, M., Orner, G.A., Dario Diaz, G., Li, Q., et al 2003, Promotion versus suppression of rat colon carcinogenesis by chlorophyllin and chlorophyll: modulation of apoptosis, cell proliferation, and β-catenin/Tcf signaling, *Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis,* vol. 523–524, pp. 217–223.

Boon, C.S., McClements, D.J., Weiss, J. & 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.

Britton, G. 1996, Carotenoids in *Natural Food Colorants*, eds. G.A.F. Hendry & J.D. Houghton, second edition, Blackie Academic & Professional, London, UK, pp. 197–243.

Brouillard, R. 1982, Chemical structure of anthocyanins in *Anthocyanins as Food Colors*, ed. P. Markakis, Academic Press, Inc., New York, NY, pp. 1–40.

Cai, Y.Z. & Corke, H. 2001, Effect of postharvest treatments on *Amaranthus* betacyanin degradation evaluated by visible/near-infrared spectroscopy, *Journal of Food Science,* vol. 66, no. 8, pp. 1112–1118.

Cai, Y.Z. & Corke, H. 2000, Production and properties of spray-dried *Amaranthus* betacyanin pigments, *Journal of Food Science,* vol. 65, no. 7, pp. 1248–1252.

Cai, Y. & Corke, H. 1999, *Amaranthus* betacyanin pigments applied in model food systems, *Journal of Food Science,* vol. 64, no. 5, pp. 869–873.

Cai, Y., Sun, M., Schliemann, W. & Corke, H. 2001, Chemical stability and colorant properties of betaxanthin pigments from *Celosia argentea*, *Journal of Agricultural and Food Chemistry,* vol. 49, no. 9, pp. 4429–4435.

Cai, Y., Sun, M. & Corke, H. 2003, Antioxidant Activity of Betalains from Plants of the Amaranthaceae, *Journal of Agricultural and Food Chemistry,* vol. 51, pp. 2288–2294.

Cai, Y., Sun, M. & Corke, H. 1998, Colorant Properties and Stability of *Amaranthus* Betacyanin Pigments, *Journal of Agricultural and Food Chemistry,* vol. 46, pp. 4491–4495.

Cano, M.P. 1991, HPLC Separation of Chlorophyll and Carotenoid Pigments of Four Kiwi Fruit Cultivars, *Journal of Agricultural and Food Chemistry,* vol. 39, pp. 1786–1791.

Cerón-García, M.d.C., Campos-Pérez, I., Macías-Sánchez, M.D., Bermejo-Román, R., Fernández-Sevilla, J.M., et al 2010, Stability of Carotenoids in Scenedesmus almeriensis Biomass and Extracts under Various Storage Conditions. *Journal of Agricultural and Food Chemistry,* vol. 58, no. 11, pp. 6944–6950.

Cervantes-Paz, B., Yahia, E.M., de Jesus Ornelas-Paz, J., Victoria-Campos, C.I., Ibarra-Junquera, V., et al 2014, Antioxidant activity and content of chlorophylls and carotenoids in raw and heat-processed Jalapeno peppers at intermediate stages of ripening, *Food Chemistry,* vol. 146, pp. 188–196.

Chernomorsky, S., Segelman, A. & Poretz, R.D. 1999, Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth, *Teratogenesis, carcinogenesis, and mutagenesis,* vol. 19, no. 5, pp. 313–322.

Christ, B. & Hortensteiner, S. 2014, Mechanism and Significance of Chlorophyll Breakdown, *Journal of plant growth regulation,* vol. 33, no. 1, pp. 4–20.

Clifford, M.N. 2000, Anthocyanins - nature, occurrence and dietary burden, *Journal of the science of food and agriculture,* vol. 80, no. 7, pp. 1063–1072.

Clifford, M. & Brown, J.E. 2006, Dietary Flavonoids and Health – Broadening the Perspective in *Flavonoids – Chemistry, Biochemistry and Applications*, eds. Ø.M. Andersen & K.R. Markham, Taylor & Francis Group, LLC, Boca Raton, FL, pp. 319–370.

Craft, N. E. & 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, pp. 431–434.

Curran, P.J., Dungan, J.L. & Gholz, H.L. 1990, Exploring the relationship between reflectance red edge and chlorophyll content in slash pine, *Tree physiology,* vol. 7, no. 1–4, pp. 33–48.

Deineka, V.I., Sorokopudov, V.N., Deineka, L.A. & Tret'yakov, M.Y. 2007, Flowers of marigold (*Tagetes*) species as a source of xanthophylls, *Pharmaceutical Chemistry Journal,* vol. 41, no. 10, pp. 540–542.

Demmig-Adams, B., Gilmore, A.M. & Adams, W.W.,III 1996, *In vivo* functions of carotenoids in higher plants, *FASEB Journal,* vol. 10, no. 4, pp. 403–412.

Desai, K.G.H. & Park, H.J. 2005, Recent Developments in Microencapsulation of Food Ingredients, *Drying Technology,* vol. 23, no. 7, pp. 1361–1394.

Dewettinck, K. & Huyghebaert, A. 1999, Fluidized bed coating in food technology, *Trends in Food Science & Technology,* vol. 10, no. 4–5, pp. 163–168.

Doehlert, D.C., Moreau, R.A., Welti, R., Roth, M.R. & McMullen, M.S. 2010, Polar Lipids from Oat Kernels, *Cereal Chemistry,* vol. 87, no. 5, pp. 467–474.

Donhowe, E.G. & Kong, F. 2014, Beta-carotene: Digestion, Microencapsulation, and *In Vitro* Bioavailability, *Food and Bioprocess Technology,* vol. 7, no. 2, pp. 338–354.

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

von Elbe, J.H., Maing, I. & Amundson, C.H. 1974, Color stability of betanin, *Journal of Food Science,* vol. 39, no. 2, pp. 334–337.

Estevinho, B.N., Rocha, F., Santos, L. & Alves, A. 2013, Microencapsulation with chitosan by spray drying for industry applications – A review, *Trends in Food Science & Technology,* vol. 31, no. 2, pp. 138–155.

Ezhilarasi, P.N., Karthik, P., Chhanwal, N. & Anandharamakrishnan, C. 2013, Nanoencapsulation Techniques for Food Bioactive Components: A Review, *Food and Bioprocess Technology,* vol. 6, no. 3, pp. 628–647.

Fahey, J.W., Stephenson, K.K., Dinkova-Kostova, A.T., Egner, P.A., Kensler, T.W., et al 2005, Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes, *Carcinogenesis,* vol. 26, no. 7, pp. 1247–1255.

Fechner, A., Knoth, A., Scherze, I. & Muschiolik, G. 2007, Stability and release properties of double-emulsions stabilised by caseinate-dextran conjugates, *Food Hydrocolloids,* vol. 21, no. 5–6, pp. 943-952.

Ferruzzi, M.G., Böhm, V., Courtney, P.D. & Schwartz, S.J. 2002, Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays, *Journal of Food Science,* vol. 67, no. 7, pp. 2589–2595.

Ferruzzi, M.G. & Blakeslee, J. 2007, Digestion, absorption, and cancer preventative activity of dietary chlorophyll derivatives, *Nutrition Research (New York, NY, United States),* vol. 27, no. 1, pp. 1–12.

Fossen, T., Cabrita, L. & Andersen, O.M. 1998, Color and stability of pure anthocyanins influenced by pH including the alkaline region, *Food Chemistry,* vol. 63, no. 4, pp. 435–440.

Frank, H.A. & Cogdell, R.J. 1996, Carotenoids in photosynthesis. *Photochemistry and photobiology,* vol. 63, no. 3, pp. 257–264.

Galka, P., Santabarbara, S., Khuong, T.T.H., Degand, H., Morsomme, P., et al 2012, Functional analyses of the plant photosystem I-light-harvesting complex II supercomplex reveal that light-harvesting complex II loosely bound to photosystem II is a very efficient antenna for photosystem I in state II, *Plant Cell,* vol. 24, no. 7, pp. 2963–2978.

Gandía-Herrero, F., Cabanes, J., Escribano, J., Garcí-a-Carmona, F. & Jiménez-Atiénzar, M. 2013, Encapsulation of the Most Potent Antioxidant Betalains in Edible Matrixes as Powders of Different Colors, *Journal of Agricultural and Food Chemistry,* vol. 61, no. 18, pp. 4294–4302.

Gandía-Herrero, F., Jiménez-Atiénzar, M., Cabanes, J., García-Carmona, F. & Escribano, J. 2010, Stabilization of the Bioactive Pigment of Opuntia Fruits through Maltodextrin Encapsulation, *Journal of Agricultural and Food Chemistry,* vol. 58, no. 19, pp. 10646–10652.

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

Gibbs, B.F., Kermasha, S., Alli, I. & Mulligan, C.N. 1999, Encapsulation in the food industry: a review, *International Journal of Food Sciences and Nutrition,* vol. 50, no. 3, pp. 213–224.

Gitelson, A.A., Gritz, Y. & Merzlyak, M.N. 2003, Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves, *Journal of Plant Physiology,* vol. 160, no. 3, pp. 271–282.

Glei, M., Rechkemmer, G., Spänkuch, B. & Pool-Zobel, B.L. 2000, All-*trans*-β-Carotene Reduces Genetic Damage *In vitro* in Human Lymphocytes in *Dietary Anticarcinogens and Antimutagens, Chemical and Biological Aspects*, eds. I.T. Johnson & G.R. Fenwick, The Royal Society of Chemistry, Cambridge, UK, pp. 129–133.

Gómez-Cordovés, C., Bartolomé, B., Vieira, W. & Virador, V.M. 2000, Antioxidant Properties of Wine Phenolic Fractions in *Dietary Anticarcinogens and Antimutagens, Chemical and Biological Aspects*, eds. I.T. Johnson & G.R. Fenwick, The Royal Society of Chemistry, Cambridge, UK, pp. 238–241.

Gorusupudi, A. & Vallikannan, B. 2012, Glycolipids improve lutein bioavailability and accumulation in eyes in mice, *European Journal of Lipid Science and Technology,* vol. 114, pp. 710–717.

Gouin, S. 2004, Microencapsulation: industrial appraisal of existing technologies and trends, *Trends in Food Science & Technology,* vol. 15, pp. 330–347.

Goula, A.M. & Adamopoulos, K.G. 2008, Effect of Maltodextrin Addition during Spray Drying of Tomato Pulp in Dehumidified Air: I. Drying Kinetics and Product Recovery, *Drying Technology*, vol. 26, no. 6, pp. 714–725.

Gould, K.S. & Lister, C. 2006, Flavonoid Functions in Plants in *Flavonoids – Chemistry, Biochemistry and Applications*, eds. Ø.M. Andersen & K.R. Markham, Taylor & Francis Group, LLC, Boca Raton, FL, pp. 397–441.

Guignon, B., Duquenoy, A. & Dumoulin, E. 2002, Fluid Bed Encapsulation of Particles: Principles and Practice, *Drying Technology,* vol. 20, no. 2, pp. 419–447.

Guo, H., Huang, Y., Qian, J., Gong, Q. & Tang, Y. 2014, Optimization of technological parameters for preparation of lycopene microcapsules, *Journal of Food Science and Technology,* vol. 51, no. 7, pp. 1318–1325.

Han, D., Kim, S.J., Kim, S.H. & Kim, D.M. 1998, Repeated regeneration of degraded red beet juice pigments in the presence of antioxidants, *Journal of Food Science,* vol. 63, no. 1, pp. 69–72.

Harttig, U. & Bailey, G.S. 1998, Chemoprotection by natural chlorophylls *in vivo*: inhibition of dibenzo[*a,l*]pyrene-DNA adducts in rainbow trout liver, *Carcinogenesis,* vol. 19, no. 7, pp. 1323–1326.

Hasegawa, Y., Imaoka, H., Adachi, S. & Matsuno, R. 2001, Preparation of W/O/W Emulsions at Low Emulsifier Concentrations, *Food Science and Technology Research,* vol. 7, no. 4, pp. 300–302.

Hasenhuettl, G.L. 2008, Overview of Foof Emulsifiers in *Food Emulsifiers and Their Applications*, eds. G.L. Hasenhuettl & R.W. Hartel, 2nd edn, Springer Science+Business Media, LCC, New York, USA, pp. 1–9.

Havaux, M. 2013, Carotenoid oxidation products as stress signals in plants, *The Plant Journal: for cell and molecular biology,* vol 79, no. 4, pp. 597–606.

Hellström, J., Mattila, P. & Karjalainen, R. 2013, Stability of anthocyanins in berry juices stored at different temperatures, *Journal of Food Composition and Analysis,* vol. 31, no. 1, pp. 12–19.

Hendry, G.A.F. 1996, Chlorophylls and chlorophyll derivatives in *Natural Food Colorants*, eds. G.A.F. Hendry & J.D. Houghton, second edition edn, Blackie Academic & Professional, London, UK, pp. 131–156.

Hendry, G.A.F., Houghton, J.D. & Brown, S.B. 1987, Tansley review no. 11. The degradation of chlorophyll - a biological enigma, *New Phytologist,* vol. 107, no. 2, pp. 255–302.

Henry, L.K., Catignani, G.L. & Schwartz, S.J. 1998, Oxidative degradation kinetics of lycopene, lutein, and 9-*cis* and all-*trans* β-carotene, *Journal of the American Oil Chemists' Society,* vol. 75, no. 7, pp. 823–829.

Herbach, K.M., Stintzing, F.M. & Carle, R. 2004, Impact of Thermal Treatment on Color and Pigment Pattern of Red Beet (*Beta vulgaris* L.) Preparations, *Journal of Food Science,* vol. 69, no. 6, pp. C491–C498.

Herbach, K., Stintzing, F. & Carle, R. 2006, Betalain Stability and Degradation–Structural and Chromatic Aspects, *Journal of Food Science,* vol. 71, no. 4, pp. R41–R50.

Howitt, C.A. & Pogson, B.J. 2006, Carotenoid accumulation and function in seeds and non-green tissues, *Plant, Cell and Environment,* vol. 29, no. 3, pp. 435–445.

IUPAC 1975, Nomenclature of carotenoids, *Pure and Applied Chemistry,* vol. 41, no. 3, pp. 407–431.

Jackman, R.L. & Smith, J.L. 1996, Anthocyanins and betalains in *Natural Food Colorants*, eds. G.A.F. Hendry & J.D. Houghton, second edition edn, Blackie Academic & Professional, London, UK, pp. 244–309.

Janiszewska, E. 2014, Microencapsulated beetroot juice as a potential source of betalain, *Powder Technology*, 264, 190–196.

Jiao, J. & Burgess, D. 2008, Multiple Emulsion Stability: Pressure Balance and Interfacial Film Strength in *Multiple Emulsions: Technology and Applications*, ed. A. Aserin, John Wiley & Sons. Inc., pp. 1–27.

Johnson, E.J. & Krinsky, N.I. 2009, Chapter 14: Carotenoids and Coronary Heart Disease in *Carotenoids, Volume 5: Nutrition and Health*, eds. G. Britton, S. Liaaen-Jensen & H. Pfander, Birkhäuser Verlag, Basel, Switzerland, pp. 287–300.

Jomova, K. & 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., et al 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.

Kaimainen, M., Marze, S., Järvenpää, E., Anton, M. & Huopalahti, R. 2015, Encapsulation of betalain into w/o/w double emulsion and release during *in vitro* intestinal lipid digestion, *LWT – Food Science and Technology,* vol. 60, pp. 899–904, doi 10.1016/j.lwt.2014.10.016.

Kandansamy, K. & Somasundaram, P.D. 2012, Microencapsulation of Colors by Spray Drying – A Review, *International Journal of Food Engineering,* vol. 8, no. 2, pp. Article 1.

Kazimierczak, R., Hallmann, E., Lipowski, J., Drela, N., Kowalik, A., et al 2014, Beetroot (*Beta vulgaris* L.) and naturally fermented beetroot juices from organic and conventional production: metabolomics, antioxidant levels and anticancer activity, *Journal of the science of food and agriculture,* vol 94, no. 13, pp. 2618–2629.

Khalil, M., Raila, J., Ali, M., Islam, K.M.S., Schenk, R., et al 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. & Gordon, M.H. 2004, Antioxidant Properties of Carotenoids *In Vitro* and *In Vivo*, *Food Reviews International,* vol. 20, no. 2, pp. 99–121.

Kirca, A., Özkan, M. & Cemeroglu, B. 2006, Stability of black carrot anthocyanins in various fruit juices and nectars, *Food Chemistry,* vol. 97, no. 4, pp. 598–605.

Konovalov, V.V. & Kispert, L.D. 1999, AM1, INDO/S and optical studies of carbocations of carotenoid molecules. Acid induced isomerization. *Journal of the Chemical Society, Perkin Transactions 2: Physical Organic Chemistry,* vol. 4, pp. 901–910.

Kovinich, N., Kayanja, G., Chanoca, A., Riedl , K., Otegui, M.S., et al 2014, Not all anthocyanins are born equal: distinct patterns induced by stress in Arabidopsis, *Planta,* Ahead of Print.

Kraeutler, B. 2014, Phyllobilins - the abundant bilin-type tetrapyrrolic catabolites of the green plant pigment chlorophyll, *Chemical Society Reviews,* vol. 43, no. 17, pp. 6227–6238.

Krinsky, N.I. 1989, Antioxidant functions of carotenoids, *Free radical biology & medicine,* vol. 7, no. 6, pp. 617–635.

Kumar, S.S., Manoj, P., Shetty, N.P., Prakash, M. & Giridhar, P. 2014, Characterization of major betalain pigments -gomphrenin, betanin and isobetanin from *Basella rubra* L. fruit and evaluation of efficacy as a natural colourant in product (ice cream) development, *Journal of Food Science and Technology,* Ahead of Print.

Kumon, K., Sasaki, J., Sejima, M., Takeuchi, Y. & Hayashi, Y. 1990, Betacyanin-decolorizing enzymes from *Phytolacca americana*, *Plant and Cell Physiology,* vol. 31, no. 2, pp. 233–240.

Kurvinen, J., Kuksis, A., Sinclair, A.J., Abedin, L. & Kallio, H. 2000, The effect of low α-linoleic acid diet on glycerophospholipid molecular species in guinea pig brain, *Lipids,* vol. 35, no. 9, pp. 1001–1009.

Laaksonen, O., Ahola, J., & Sandell, M. 2013, Explaining and predicting individually experienced liking of berry fractions by the *hTAS2R38* taste receptor genotype, *Appetite*, 61, 85–96.

van der Laan, L.N., de Ridder, D.T.D., Viergever, M.A. & Smeets, P.A.M. 2011, The first taste is always with the eyes: A meta-analysis on the neural correlates of processing visual food cues, *NeuroImage,* vol. 55, no. 1, pp. 296–303.

van der Laan, Laura N., Hooge, I.T.C., de Ridder, Denise T.D., Viergever, M.A. & Smeets, P.A.M. 2015, Do you like what you see? The role of first fixation and total fixation duration in consumer choice, *Food Quality and Preference,* vol. 39, pp. 46–55.

LaBorde, L.F. & von Elbe, J.H. 1994, Chlorophyll Degradation and Zinc Complex Formation with Chlorophyll Derivatives in Heated Green Vegetables, *Journal of Agricultural and Food Chemistry,* vol. 42, no. 5, pp. 1100–1103.

Langrish, T.A.G., Chan, W.C. & Kota, K. 2007, Comparison of maltodextrin and skim milk wall deposition rates in a pilot-scale spray dryer, *Powder Technology*, vol. 179, no. 1–2, pp. 84–89.

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

Leal-Calderon, F., Schmitt, V. & Bibette, J. 2007a, Double Emulsions in *Emulsion Science Basic Principles*, eds. F. Leal-Calderon, V. Schmitt & J. Bibette, Springer Science+Business Media, LLC, New York, USA, pp. 173–199.

Leal-Calderon, F., Schmitt, V. & Bibette, J. 2007b, Introduction in *Emulsion Science Basic Principles*, eds. F. Leal-Calderon, V. Schmitt & J. Bibette, Springer Science+Business Media, LLC, New York, USA, pp. 1–4.

Lehtonen, H., Rantala, M., Suomela, J., Viitanen, M. & Kallio, H. 2009, Urinary Excretion of the Main Anthocyanin in Lingonberry (*Vaccinium vitis-idaea*), Cyanidin 3-O-Galactoside, and Its Metabolites, *Journal of Agricultural and Food Chemistry,* vol. 57, no. 10, pp. 4447–4451.

Leonova, S., Shelenga, T., Hamberg, M., Konarev, A.V., Loskutov, I., et al 2008, Analysis of Oil Composition in Cultivars and Wild Species of Oat (*Avena* sp.), *Journal of Agricultural and Food Chemistry,* vol. 56, pp. 7983–7991.

Li, W., Gao, Y., Zhao, J. & 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.

Losso, J.N., Khachatryan, A., Ogawa, M., Godber, J.S. & Shih, F. 2005, Random centroid optimization of phosphatidylglycerol stabilized lutein-enriched oil-in-water emulsions at acidic pH, *Food Chemistry,* vol. 92, pp. 737–744.

Ma, L. & Lin, X. 2010, Effects of lutein and zeaxanthin on aspects of eye health, *Journal of the Science of Food and Agriculture,* vol. 90, no. 1, pp. 2–14.

Mahdavi, S.A., Jafari, S.M., Ghorbani, M. & Assadpoor, E. 2014, Spray-Drying Microencapsulation of Anthocyanins by Natural Biopolymers: A Review. *Drying Technology,* vol. 32, no. 5, pp. 509–518.

Markakis, P. 1982, Stability of anthocyanins in foods in *Anthocyanins as Food Colors*, ed. P. Markakis, Academic Press, Inc., New York, NY, pp. 163–180.

Marze, S., Choimet, M. & Foucat, L. 2012, *In vitro* digestion of emulsions: diffusion and particle size distribution using diffusing wave spectroscopy and diffusion using nuclear magnetic resonance, *Soft Matter,* vol. 8, no. 42, pp. 10994–11004.

McCann, D., Barrett, A., Cooper, A., Crumpler, D., Dalen, L., et al 2007, Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial, *The Lancet,* vol. 370, no. 9598, pp. 1560–1567.

Merzlyak, M.N. & Gitelson, A. 1995, Why and what for the leaves are yellow in autumn? On the interpretation of optical spectra of senescing leaves (Acer platanoides L.), *Journal of Plant Physiology,* vol. 145, no. 3, pp. 315–320.

Monreal, M., De Ancos, B. & 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.

Montoya-Olvera, R. & Torres-Quiroga, J. 2012, *Micro-micellar compositions and applications of carotenoids of improved absorption and bioavailability.*, Patent Application Country: US, Application: US, Priority Application Country: US.

Mun, S., Choi, Y., Rho, S., Kang, C., Park, C., et al 2010, Preparation and Characterization of Water/Oil/Water Emulsions Stabilized by Polyglycerol Polyricinoleate and Whey Protein Isolate, *Journal of Food Science,* vol. 75, no. 2, pp. E116–E125.

Mun, S., Decker, E.A. & McClements, D.J. 2007, Influence of emulsifier type on *in vitro* digestibility of lipid droplets by pancreatic lipase, *Food Research International,* vol. 40, pp. 770–781.

Nielsen, I.L.F., Haren, G.R., Magnussen, E.L., Dragsted, L.O. & Rasmussen, S.E. 2003, Quantification of Anthocyanins in Commercial Black Currant Juices by Simple High-Performance Liquid Chromatography. Investigation of Their pH Stability and Antioxidative Potency, *Journal of Agricultural and Food Chemistry,* vol. 51, no. 20, pp. 5861–5866.

Nigg, J., Lewis, K., Edinger, T. & Falk, M. 2012, Meta-Analysis of Attention-Deficit/Hyperactivity Disorder or Attention-Deficit/Hyperactivity Disorder Symptoms, Restriction Diet, and Synthetic Food Color Additives, *Journal of the American Academy of Child & Adolescent Psychiatry,* vol. 51, no. 1, pp. 86–97.

Nik, A.M., Wright, A.J. & Corredig, M. 2011, Impact of interfacial composition on emulsion digestion and rate of lipid hydrolysis using different *in vitro* digestion models, *Colloids and Surfaces B,* vol. 83, pp. 321–330.

Oh, S., Shin, M., Lee, K. & 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.

O'Regan, J. & Mulvihill, D.M. 2010, Sodium caseinate-maltodextrin conjugate stabilized double emulsions: Encapsulation and stability, *Food Research International,* vol. 43, pp. 224–231.

Ornelas Cravioto, A. 2007, *Trans-lutein xanthophyll ester of high purity and high bioavailability in micellar solution.*, Patent Application Country: US, Application: US, Priority Application Country: US.

Osawa, Y. 1982, Copigmentation of anthocyanins in *Anthocyanins as Food Colors*, ed. P. Markakis, Academic Press, Inc., New York, NY, pp. 41–68.

Pasch, J.H. & Von Elbe, J.H. 1975, Betanine degradation as influenced by water activity, *Journal of Food Science,* vol. 40, no. 6, pp. 1145–1146.

Patras, A., Brunton, N.P., O'Donnell, C. & Tiwari, B.K. 2010, Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation, *Trends in Food Science & Technology,* vol. 21, no. 1, pp. 3–11.

Pavokovic, D. & Krsnik-Rasol, M. 2011, Complex biochemistry and biotechnological production of betalains, *Food Technology and Biotechnology,* vol. 49, no. 2, pp. 145–155.

Piccaglia, R., Marotti, M. & 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.

Pintea, A., Diehl, H.A., Momeu, C., Aberle, L. & Socaciu, C. 2005, Incorporation of carotenoid esters into liposomes, *Biophysical chemistry,* vol. 118, no. 1, pp. 7–14.

Pitalua, E., Jimenez, M., Vernon-Carter, E.J. & Beristain, C.I. 2010, Antioxidative activity of microcapsules with beetroot juice using gum Arabic as wall material, *Food and Bioproducts Processing,* vol. 88, no. 2–3, pp. 253–258.

Pojer, E., Mattivi, F., Johnson, D. & Stockley, C.S. 2013, The case for anthocyanin consumption to promote human health: a review, *Comprehensive Reviews in Food Science and Food Safety,* vol. 12, no. 5, pp. 483–508.

Ratnoglik, S.L., Aoki, C., Sudarmono, P., Komoto, M., Deng, L., et al 2014, Antiviral activity of extracts from Morinda citrifolia leaves and chlorophyll catabolites, pheophorbide a and pyropheophorbide a, against hepatitis C virus, *Microbiology and immunology,* vol. 58, no. 3, pp. 188–194.

Rock, C.L. 2009, Chapter 13: Carotenoids and Cancer in *Carotenoids, Volume 5: Nutrition and Health*, eds. G. Britton, S. Liaaen-Jensen & H. Pfander, Birkhäuser Verlag, Basel, Switzerland, pp. 269–286.

Rodriguez-Huezo, M.E., Pedroza-Islas, R., Prado-Barragan, L.A., Beristain, C.I. & 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.

Rubio-Diaz, D.E., Santos, A., Francis, D.M. & Rodriguez-Saona, L.E. 2010, Carotenoid Stability during Production and Storage of Tomato Juice Made from Tomatoes with Diverse Pigment Profiles Measured by Infrared Spectroscopy. *Journal of Agricultural and Food Chemistry,* vol. 58, no. 15, pp. 8692–8698.

Rugina, D., Sconta, Z., Leopold, L., Pintea, A., Bunea, A., et al 2012, Antioxidant Activities of Chokeberry Extracts and the Cytotoxic Action of Their Anthocyanin Fraction on HeLa Human Cervical Tumor Cells, *Journal of Medicinal Food,* vol. 15, no. 8, pp. 700–706.

Sadilova, E., Stintzing, F.C. & Carle, R. 2006, Thermal degradation of acylated and nonacylated anthocyanins, *Journal of Food Science,* vol. 71, no. 8, pp. C504–C512.

Sadilova, E., Carle, R. & Stintzing, F.C. 2007, Thermal degradation of anthocyanins and its impact on color and *in vitro* antioxidant capacity, *Molecular Nutrition & Food Research,* vol. 51, no. 12, pp. 1461–1471.

Saénz, C., Tapia, S., Chávez, J. & Robert, P. 2009, Microencapsulation by spray drying of bioactive compounds from cactus pear (*Opuntia ficus-indica*), *Food Chemistry,* vol. 114, no. 2, pp. 616–622.

Sahasrabudhe, M.R. 1979, Lipid composition of oats (*Avena sativa* L.), *Journal of the American oil chemists' society,* vol. 56, pp. 80–84.

Salvia-Trujillo, L., Qian, C., Martín-Belloso, O. & McClements, D.J. 2013, Influence of Particle size on lipid digestion and β-carotene bioaccessibility in emulsions and nanoemulsions, *Food Chemistry,* vol. 141, pp. 1472–1480.

Sapei, L., Naqvi, M.A. & Rousseau, D. 2012, Stability and release properties of double emulsions for food applications, *Food Hydrocolloids,* vol. 27, pp. 316–323.

Sato, Y., Kobayashi, M., Itagaki, S., Hirano, T., Noda, T., et al 2011, Pharmacokinetic properties of lutein emulsion after oral administration to rats and effect of food intake on plasma concentration of lutein. *Biopharmaceutics & drug disposition,* vol. 32, no. 3, pp. 151–158.

Schaefer, H.M. & Rolshausen, G. 2006, Plants on red alert: do insects pay attention? *BioEssays : news and reviews in molecular, cellular and developmental biology,* vol. 28, no. 1, pp. 65–71.

Schalch, W., Landrum, J.T. & Bone, R.A. 2009, Chapter 15: The Eye in *Carotenoids, Volume 5: Nutrition and Health*, eds. G. Britton, S. Liaaen-Jensen & H. Pfander, Birkhäuser Verlag, Basel, Switzerland, pp. 301–334.

Schanderl, S.H., Chichester, C.O. & Marsh, G.L. 1962, Degradation of chlorophyll and several derivatives in acid solution, *Journal of Organic Chemistry,* vol. 27, pp. 3865–3868.

Scheer, H. 1991, Structure and occurrence of chlorophylls in *Chlorophylls*, ed. H. Scheer, CRC Press, Boca Raton, FL, pp. 3–30.

Schutyser, M.A.I., Perdana, J. & Boom, R.M. 2012, Single droplet drying for optimal spray drying of enzymes and probiotics, *Trends in Food Science & Technology,* vol. 27, no. 2, pp. 73–82.

Schwartz, S.J., von Elbe, J.H. & Giusti, M.M. 2008, Colorants in *Fennema's Food Chemistry (4th Edition)*, eds. S. Damodaran, K.L. Parkin & O.R. Fennema, Taylor & Francis Group, LLC, Boca Raton, FL, pp. 571–638.

Schwartz, S.J., Woo, S.L. & Von Elbe, J.H. 1981, High-performance liquid chromatography of chlorophylls and their derivatives in fresh and processed spinach, *Journal of Agricultural and Food Chemistry,* vol. 29, no. 3, pp. 533–535.

Serris, G.S. & Biliaderis, C.G. 2001, Degradation kinetics of beetroot pigment encapsulated in polymeric matrices, *Journal of the Science of Food and Agriculture,* vol. 81, no. 8, pp. 691–700.

Shima, M., Tanaka, M., Kimura, Y., Adachi, S. & Matsuno, R. 2004, Hydrolysis of the oil phase of a W/O/W emulsion by pancreatic lipase, *Journal of Controlled Release,* vol. 94, no. 1, pp. 53–61.

Socaciu, C., Jessel, R. & Diehl, H.A. 2000, Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy, *Chemistry and physics of lipids,* vol. 106, no. 1, pp. 79–88.

Socaciu, C., Bojarski, P., Aberle, L. & Diehl, H.A. 2002, Different ways to insert carotenoids into liposomes affect structure and dynamics of the bilayer differently, *Biophysical chemistry,* vol. 99, no. 1, pp. 1–15.

Spence, C., Levitan, C. A., Shankar, M. U., & Zampini, M. 2010, Does Food Color Influence Taste and Flavor Perception in Humans? *Chemosensory Perception*, 3(1), 68–84.

Stafford, H.A. 1994, Anthocyanins and betalains: evolution of the mutually exclusive pathways, *Plant Science,* vol. 101, no. 2, pp. 91–98.

Stintzing, F.C. & Carle, R. 2008, N-Heterocyclic Pigments: Betalains in *Food Colorants: Chemical and Functional Properties*, ed. C. Socaciu, Taylor & Francis Group, LLC, Boca Raton, FL, pp. 87–99.

Stintzing, F.C. & Carle, R. 2004, Functional properties of anthocyanins and betalains in plants, food, and in human nutrition, *Trends in Food Science & Technology,* vol. 15, no. 1, pp. 19–38.

Stintzing, F. C., Trichterborn, J., & Carle, R. 2006, Characterisation of anthocyanin-betalain mixtures for food colouring by chromatic and HPLC-DAD-MS analyses, *Food Chemistry*, 94(2), 296–309.

Strack, D., Vogt, T. & Schliemann, W. 2003, Recent advances in betalain research, *Phytochemistry,* vol. 62, no. 3, pp. 247–269.

Subagio, A., Wakaki, H. & Morita, N. 1999, Stability of Lutein and Its Myristate Esters, *Bioscience, biotechnology, and biochemistry,* vol. 63, no. 10, pp. 1784–1786.

Suganyadevi, P., Saravanakumar, M., Aravinthan , K.M., Arunkumar, A., Krishna, R.K., et al 2010, Extraction of betacyanin from red beet root (*Beta vulgaris* L.) and to evaluate its antioxidant potential, *Journal of Pharmacy Research,* vol. 3, no. 11, pp. 2693–2696.

Sytykiewicz, H., Sprawka, I., Czerniewicz, P., Sempruch, C., Leszczynski, B., et al 2013, Biochemical characterisation of chlorophyllase from leaves of selected Prunus species – a comparative study, *Acta Biochimica Polonica,* vol. 60, no. 3, pp. 457–465.

Sztatelman, O., Waloszek, A., Banas, A.K. & Gabrys, H. 2010, Photoprotective function of chloroplast avoidance movement: *In vivo* chlorophyll fluorescence study, *Journal of Plant Physiology,* vol. 167, no. 9, pp. 709–716.

Tadros, T.F. 2009, Emulsion Science and Technology: A General Introduction in *Emulsion Science and Technology*, ed. T.F. Tadros, Wiley-VCH Verlag GmbH & Co. KGa, Weinheim, Germany, pp. 1–56.

Takahashi, A., Takeda, K. & Ohnishi, T. 1991, Light-induced anthocyanin reduces the extent of damage to DNA in UV-irradiated *Centaurea cyanus* cells in culture, *Plant and Cell Physiology,* vol. 32, no. 4, pp. 541–547.

Tang, G. & Russell, R.M. 2009, Chapter 8: Carotenoids as Provitamin A in *Carotenoids, Volume 5: Nutrition and Health*, eds. G. Britton, S. Liaaen-Jensen & H. Pfander, Birkhäuser Verlag, Basel, Switzerland, pp. 149–172.

Troncoso, E., Aguilera, J.M. & McClements, D.J. 2012, Fabrication, characterization and lipase digestibility of food-grade nanoemulsions, *Food Hydrocolloids,* vol. 27, pp. 355–363.

Tsuda, T., Shiga, K., Ohshima, K., Kawakishi, S. & Osawa, T. 1996, Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Biochemical pharmacology,* vol. 52, no. 7, pp. 1033–1039.

Tsuda, T., Watanabe, M., Ohshima, K., Norinobu, S., Choi, S., et al 1994, Antioxidative Activity of the Anthocyanin Pigments Cyanidin 3-O-β-D-Glucoside and Cyanidin, *Journal of Agricultural and Food Chemistry,* vol. 42, no. 11, pp. 2407–2410.

Vergara, C., Saavedra, J., Sáenz, C., García, P. & Robert, P. 2014, Microencapsulation of pulp and ultrafiltered cactus pear (*Opuntia ficus-indica*) extracts and betanin stability during storage, *Food Chemistry,* vol. 157, pp. 246–251.

Vershinin, A. 1999, Biological functions of carotenoids - diversity and evolution, *BioFactors,* vol. 10, no. 2,3, pp. 99–104.

Viacava, G.E., Gonzalez-Aguilar, G. & Roura, S.I. 2014, Determination of Phytochemicals and Antioxidant Activity in Butterhead Lettuce Related to Leaf Age and Position, *Journal of Food Biochemistry,* vol. 38, no. 3, pp. 352–362.

Vishwanathan, R., Wilson, T.A. & 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.

de Vogel, J., Jonker-Termont, Denise S. M. L., van Lieshout, Esther M. M., Katan, M.B. & van der Meer, R. 2005a, Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon, *Carcinogenesis,* vol. 26, no. 2, pp. 387–393.

de Vogel, J., Jonker-Termont, Denise S .M. L., Katan, M.B. & van der Meer, R. 2005b, Natural chlorophyll but not chlorophyllin prevents heme-induced cytotoxic and hyperproliferative effects in rat colon, *Journal of Nutrition,* vol. 135, no. 8, pp. 1995–2000.

Vulic, J.J., Cebovic, T.N., Canadanovic-Brunet, J.M., Cetkovic, G.S., Canadanovic, V.M., et al 2014, *In vivo* and *in vitro* antioxidant effects of beetroot pomace extracts, *Journal of Functional Foods,* vol. 6, pp. 168–175.

Walstra, P. 2003, Changes in Dispersity in *Physical Chemistry of Foods*, ed. P. Walstra, Marcel Dekker Inc., New York, USA, pp. 494–565.

Warburg, O. 1958, Photosynthesis, *Science (New York, N.Y.),* vol. 128, no. 3315, pp. 68–73.

Wenzel, M., Seuss-Baum, I. & Schlich, E. 2010, Influence of Pasteurization, Spray- and Freeze-Drying, and Storage on the Carotenoid Content in Egg Yolk. *Journal of Agricultural and Food Chemistry,* vol. 58, no. 3, pp. 1726–1731.

Whittingham, C.P. 1970, Mechanism of photosynthesis and the structure of the chloroplast, *Progress in Biophysics & Molecular Biology,* vol. 21, pp. 125–141.

Wroblewska, M., Juskiewicz, J. & Wiczkowski, W. 2011, Physiological properties of beetroot crisps applied in standard and dyslipidaemic diets of rats, *Lipids in Health and Disease,* vol. 10, pp. 178.

Xiong, Y., Li, R. & Yue, Y. 2013, Quantitative estimation of photosynthetic pigments using new spectral indices, *Journal of Forestry Research (Harbin, China),* vol. 24, no. 3, pp. 477–483.

Yang, Z., Han, Y., Gu, Z., Fan, G. & Chen, Z. 2008, Thermal degradation kinetics of aqueous anthocyanins and visual color of purple corn (Zea mays L.) cob, *Innovative Food Science & Emerging Technologies,* vol. 9, no. 3, pp. 341–347.

Yao, X., Wang, N., Fang, Y., Phillips, G.O., Jiang, F., et al 2013, Impact of surfactants on the lipase digestibility of gum arabic-stabilized O/W emulsions, *Food Hydrocolloids,* vol. 33, pp. 393–401.

Yeum, K., Aldini, G., Russell, R.M. & Krinsky, N.I. 2009, Chapter 12: Antioxidant/Pro-oxidant Actions of Carotenoids in *Carotenoids, Volume 5: Nutrition and Health*, eds. G. Britton, S. Liaaen-Jensen & H. Pfander, Birkhäuser Verlag, Basel, Switzerland, pp. 235–268.

Zampini, M., Sanabria, D., Phillips, N., & Spence, C. 2007, The multisensory perception of flavor: Assessing the influence of color cues on flavor discrimination responses, *Food Quality and Preference*, 18(7), 975–984.

Zhao, C.L., Chen, Z.J., Bai, X.S., Ding, C., Long, T.J., et al 2014, Structure-activity relationships of anthocyanidin glycosylation, *Molecular diversity,* vol. 18, no. 3, pp. 687–700.

APPENDIX: ORIGINAL PUBLICATIONS

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II Submitted manuscript.

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61. **JIE ZHENG (2013)** Sugars, acids and phenolic compounds in currants and sea buckthorn in relation to the effects of environmental factors.
62. **SARI MÄKINEN (2014)** Production, isolation and characterization of bioactive peptides with antihypertensive properties from potato and rapeseed proteins.
63. **MIKA KAIMAINEN (2014)** Stability of natural colorants of plant origin.

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