LUND UNIVERSITY

Development of bixin formulations. Extraction, microencapsulation, dispersibility and photostability

Curi, Cecilia

2021

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Curi, C. (2021). *Development of bixin formulations. Extraction, microencapsulation, dispersibility and photostability*. Lund University.

Total number of authors:
1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Development of bixin formulations

Extraction, microencapsulation, dispersibility and photo-stability

CECILIA KARINA CURI BORDA
DEPARTMENT OF FOOD TECHNOLOGY, ENGINEERING AND NUTRITION | LUND UNIVERSITY

The first memory I recall from my PhD studies was working to obtain the solid colorants needed for doing my experiments. This happened during the worst drought in the last twenty-five years of the history of my hometown city, La Paz, Bolivia. The water stopped running through the pipes. Official sources say it was because of climate change, and some others suggested problems during the administration too. The point was that we had very limited access to water for drinking or working for almost 6 months. I was using an equipment that needed running water to dry the solvents from the colorant extract. So, you ask me – was I able to finish my work on time to go back to Sweden? Luckily, the building where I used to work had a water storage container that made our access to water easier. Then, with the help of my friends and colleagues, we managed to put a container on top of a laboratory shelf to feed water to the equipment. Moreover, we put in the freezer water bottles every night to cool down the water that we manually recirculated. This way, we managed to operate the equipment and obtain sufficient solid colorant for my future experiments in Sweden. My PhD journey was not always easy, but certainly, the support and collaboration of my family, friends, and colleagues encouraged me to endure the process. One day, the results from these studies came out together and feelings of satisfaction and happiness arrived… Why did I keep spending these years doing applied science? Because during this process I learned that we have important challenges that need to be addressed for our loved ones to come; anything is possible if we have purpose and a little of creativity; life is beautiful through the eyes of love and gratitude; willingness is necessary to find solutions; and that we all can have a bit of luck if we learn to recognize and take the opportunities that may arise.

Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Department of Food Technology
Engineering and Nutrition
Faculty of Engineering
Lund University

ISBN 978-91-7895-751-4

Development of bixin formulations

Extraction, microencapsulation, dispersibility and photo-stability

Cecilia Karina Curi Borda

2021

LUND UNIVERSITY

DOCTORAL DISSERTATION

Which, by due permission of the Faculty of Engineering, Lund University, Sweden, will be publicly defended at the Center of Chemistry and Chemical Engineering, Naturvetarvägen 14, Lund, on Friday, March 19th, 2021 at 14:00 in Lecture Hall D, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent
Prof. Hanne Hjorth Tønnesen
Department of Pharmacy, University of Oslo, Oslo, Norway

Organization
LUND UNIVERSITY
Department of Food Technology, Engineering and Nutrition
Faculty of Engineering
P.O. Box 124, SE-221 00 Lund, Sweden

Author Cecilia Karina Curi Borda

Document name
DOCTORAL DISSERTATION

Date of issue
2021-03-19

Sponsoring organization:
Swedish International Development Agency

Title and subtitle
Development of bixin formulations: Extraction, microencapsulation, dispersibility and photo-stability

Abstract

The aril that covers the seeds of the plant species Bixa orellana is composed of the natural colorants bixin and norbixin, phenolic components and native carbohydrates composed of hemicellulose, starch, and a small fraction of proteins. Bixin is a hydrophobic carotenoid that is water insoluble and is mostly used for coloring oil-based formulations. This thesis presents the development of different bixin formulations to be used as coloring agents in aqueous continuous-based products.

Any colorant formulation needs the colorant to be available and extraction feasible. The first stage of the thesis consisted of the morphological and photochemical characterization of Bixa orellana fruits that were collected from Los Yungas of La Paz, Bolivia at different altitudes. The water content, seed weight, and the amount of bixin, norbixin and native carbohydrates were quantified. The aril of the seeds was extracted using a mild alkaline extraction method to obtain an aqueous-dispersible extract that was directly analyzed by meas of solvent dissolution and further injection into a HPLC or by means of analysis using spectroscopic techniques.

In a second stage, the use of the mild alkaline base was used as a method to obtain aqueous dispersions that were mixed with an encapsulating agent. This mixture was spray dried to obtain colorant microcapsules. Microencapsulation was applied to obtain bixin aqueous-dispersible formulations and offer protection to the colorant against light degradation. The impact of the native carbohydrates was evaluated as an encapsulating agent and as protection against bixin light degradation. The encapsulation efficiency of bixin by different wall materials was compared.

Light stability experiments were performed on Bixin microcapsules (powders) and unprotected colorants. The results show that lower sensitivity towards light was obtained for microencapsulated samples. Moreover, light stability of annatto extracts applied to gummy candies was evaluated. The results show faster color decay for samples containing lower colorant concentrations for the same degradation rates. Sensorial analysis showed good acceptability of the color of the candies presented as pictures to the panelists..

Key words: Bixin, annatto, natural colorants, ligth stability in solid multiphase systems, shelf life extension, auqeous physical stability, encapsulation efficiency, spray drying, natural colored candies

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language: English

ISSN and key title

ISBN (print) 978-91-7895-751-4
ISBN (Digital) 978-91-7895-752-1

Recipient's notes

Number of pages 95

Price

Security classification

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature

Date 2021-02-08

Development of bixin formulations

Extraction, microencapsulation, dispersibility and photo-stability

Cecilia Karina Curi Borda

2021

Department of Food Technology, Engineering and Nutrition
Faculty of Engineering
Lund University, Sweden

Cover photographs by Carol Vidaurre

Copyright © Main Thesis by Cecilia Karina Curi Borda

Paper I © Cecilia K. Curi-Borda, Luis Marconi, Álvaro Salinas, Marco Quino, Juan-Antonio Alvarado, Björn Bergenståhl (Manuscript)

Paper II © Multidisciplinary Digital Publishing Institute, MDPI, Basel, Switzerland.

Paper III © Cecilia K. Curi-Borda, Vandana Tannira, Niko Gentile, Juan-Antonio Alvarado, Björn Bergenståhl (Manuscript)

Paper IV © Cecilia K. Curi-Borda, Mina Hajian, Niko Gentile, Juan Antonio Alvarado, Björn Bergenståhl (Manuscript)

Department of Food Engineering, Technology and Nutrition
Faculty of Engineering, Lund University

ISBN (print) 978-91-7895-751-4
ISBN (Digital) 978-91-7895-752-1

Printed in Sweden by Media-Tryck, Lund University
Lund 2021

Media-Tryck is a Nordic Swan Ecolabel certified provider of printed material. Read more about our environmental work at www.mediatryck.lu.se

MADE IN SWEDEN

To those who are passioned about life and science

Popular Scientific Summary

From the coat of the seeds of a tree named “achiote” or *Bixa orellana,* it is possible to obtain the natural-colored paste “annatto” with the pigment “bixin”. These extracts may be composed of different proportions of bixin, norbixin, native carbohydrates, phenolic compounds and other minority compounds present in the seed’s coat. In this thesis, I extracted different pigments from the achiote seed for study. The pigments used were Bixin, and an annatto extract composed of bixin and the native carbohydrates of the seeds’ coat called “Carbohydrate rich bixin”.

In some cases, I used solvents to extract and purify bixin and the native carbohydrate rich bixin. Then, I developed an alternative solvent-free extraction method using a weak alkaline soda compound and water. This method was used to extract all the coat from the seed (annatto extract) and create an aqueous suspension of the pigments. These aqueous suspensions allow the achiote extract to be analyzed directly. It also enables microencapsulation of the pigment right after extraction through use of spray drying.

The mild alkaline extraction process made it possible to extract the seeds of 27 samples of fruits from *Bixa orellana* collected from different municipalities in Los Yungas in La Paz, Bolivia. The components of the seeds were quantified as the water content, the weight of the colorless seed, the total colorant content, and the amount of native carbohydrates contained in the achiote seeds. Moreover, characterization of the shapes and colors of the collected fruit samples was performed. An analysis was carried out with this data to determine the shape characteristics, such as fruit size, that predicted higher colorant contents in different fruit morphotypes (varieties). Larger fruits were found to contain higher colorant content.

Furthermore, I made microencapsulated pure bixin and microcapsules with carbohydrate-rich annatto extract and carried out studies on their stability against light degradation. The microencapsulation of the pigments was performed using spray drying. Encapsulating materials such as maltodextrin, whey, and sucrose were used. Microencapsulation was also achieved by adding gelling agents such as gum arabic, pectin, or carboxymethylcellulose. The encapsulating properties of the different mixtures were compared to determine the best encapsulating systems. The protection of dye inside the microcapsules for each system was evaluated. The results showed that the bixin pigment has better protection when the native

carbohydrate fraction is present in the microcapsule. In addition, CMC proved to be an excellent encapsulant agent for its film-forming properties.

Once the microcapsules were obtained, they were dispersed in water. Using these aqueous dispersions, physical stability studies of pigment particles were conducted over time. It was determined that suspensions containing particles of bixin with the native carbohydrates kept their color for at least 40 days. Instead, pure bixin particles settled on the first day.

We studied the pigment stability of the microencapsulated powders against exposure to visible light. For this, microencapsulated powders were irradiated with a visible light lamp, and the pigment content was measured after different days, for 30 days. The results show that microencapsulated dyes are 30 to 100 times more stable against light than pure unprotected pigments.

Finally, colored gummy candies were prepared with annatto and annatto microcapsules. Color stability studies were conducted in the candies. It was determined that the candy’s components protect the colorants to some extent. It was determined that the candy's components protect the colorants to some extent. Sensory studies showed that participants liked the orange color of the candy.

All results aim to enhance our capacity to develop formulations with the natural colorants from achiote for its application in aqueous rich products. Thus, this may allow for expanding its use in food and cosmetics. In addition, the microencapsulation of the colorants enables them to extend their stability against light degradation.

Resumen Científico Popular

A partir de la capa que recubre las semillas de un arbusto llamado "achiote" o *Bixa orellana*, es posible obtener la pasta de color natural "annatto" con el pigmento "bixina". Estos extractos pueden estar compuestos de diferentes proporciones de bixina, norbixina, carbohidratos nativos, compuestos fenólicos y otros compuestos minoritarios presentes en la capa externa de la semilla. En esta tesis, extraje diferentes pigmentos de la semilla de achiote para su estudio. Los pigmentos utilizados fueron Bixina, y un extracto de achiote compuesto de bixina y los carbohidratos nativos de la capa de las semillas. Este extracto se denominó "Bixina enriquecida con carbohidratos".

En algunos casos, utilicé disolventes para extraer y purificar la bixina, y la bixina rica en carbohidratos nativos. Luego, desarrollé un método alternativo de extracción libre de disolventes utilizando un compuesto de soda alcalina débil y agua. Este método se utilizó para extraer toda la capa de la semilla (extracto de achiote) y crear una suspensión acuosa de los pigmentos. La suspensión acuosa del extracto de achiote puede ser analizado directamente. También permite la microencapsulación del pigmento justo después de la extracción a través del uso de secado por aspersión.

Este proceso de extracción alcalina hizo posible extraer el colorante de las semillas de 27 muestras de frutas de *Bixa orellana* colectadas de diferentes municipios de Los Yungas en La Paz, Bolivia. Se determinaron los porcentajes de agua, de semilla sin colorante, de colorantes y de carbohidratos nativos contenidos en las semillas de achiote. Por otro lado, se realizó la caracterización de las formas y colores de frutos de dichas muestras colectadas. Con estos datos se realizó un análisis para determinar que las características de forma como el tamaño de los frutos permite predecir mayores contenidos de colorante. Se determinó que frutos más grandes contienen mayores contenidos de colorante.

Más adelante, obtuve microcápsulas de bixina pura y microcápsulas con extracto de achiote rico en carbohidratos. Luego, llevé a cabo estudios de estabilidad frente a la degradación debido a su exposición a la luz. La microencapsulación de los pigmentos se realizó mediante secado por aspersión. Se utilizaron materiales encapsulantes como maltodextrina, suero de leche y sacarosa. La microencapsulación también se logró mediante la adición de agentes gelificantes como la goma arábiga, pectina, o carboximetilcelulosa, CMC. Las propiedades de encapsulación de las diferentes mezclas se compararon para determinar los mejores sistemas de encapsulación. Se evaluó la protección del colorante dentro de las microcápsulas para cada sistema. Los resultados mostraron que el pigmento de la bixina tiene una mejor protección cuando la fracción nativa de carbohidratos está presente en la microcápsula. Además, el CMC demostró ser un excelente agente encapsulante por sus propiedades de formación de películas.

Una vez obtenidas las microcápsulas, se dispersaron en agua. Usando estas dispersiones acuosas, los estudios de estabilidad física de partículas de pigmento se llevaron a cabo durante 40 días. Se determinó que las suspensiones que contenían partículas de bixina con los carbohidratos nativos mantuvieron su color durante al menos 40 días. En su lugar, las partículas de bixina pura se asentaron el primer día.

Estudiamos la estabilidad del pigmento de los polvos microencapsulados frente a la exposición a la luz visible. Para esto, polvos microencapsulados fueron irradiados con una lámpara de luz visible, y el contenido de pigmento se midió después de diferentes días, durante 30 días. Los resultados muestran que los pigmentos microencapsulados son 30 a 100 veces más estables frente a la luz que los pigmentos puros sin protección.

Finalmente, se prepararon dulces coloreados con annatto y con microcápsulas de annatto. Se realizaron estudios de estabilidad del color en los caramelos. Se determinó que los componentes del caramelo protegen los colorantes en cierto grado. Los estudios sensoriales mostraron que a los participantes les gustó el color naranja de los dulces.

Todos los resultados tienen como objetivo mejorar nuestra capacidad de desarrollar formulaciones con los colorantes naturales del achiote para su aplicación en productos en base a agua. Por lo tanto, esto puede permitir ampliar su uso en alimentos y cosméticos. Además, la microencapsulación de los colorantes les permite extender su estabilidad frente a la degradación por la luz.

List of Papers

The present thesis was written based on the following papers that are appended at the end of the thesis:

Paper I. Cecilia K. Curi-Borda, Luis Marconi, Álvaro Salinas, Marco Quino, Juan-Antonio Alvarado, Björn Bergenståhl, Morphological and phytochemical characterization of annatto samples (Bixa orellana) from Los Yungas of La Paz, Bolivia. (Manuscript)

Paper II. Curi-Borda, C.K.; Linares-Pastén, J.A.; Tat, T.; Tarqui-Dueñas, R.; Chino-Flores, N.; Alvarado, J.-A.; Bergenstahl, B. Multilayer Bixin Microcapsules: The Impact of Native Carbohydrates on the Microencapsulation Efficiency and Dispersion Stability. Foods 2019, 8, 108. https://doi.org/10.3390/foods8030108

Paper III. Cecilia K. Curi-Borda, Vandana Tannira, Niko Gentile, Juan-Antonio Alvarado, Björn Bergenståhl, Model for measuring light stability of photolabile substances in powder beds using spray dried bixin microcapsules. (Submitted Manuscript)

Paper IV. Cecilia K. Curi-Borda, Mina Hajian, Niko Gentile, Juan Antonio Alvarado, Björn Bergenståhl, Annatto applied to gummy candies: A light stability and colorimetry study. (Manuscript)

The Author's contributions to the Papers

Paper I. The author contributed to the conceptualization and methodology design of the study together with co-authors, performed the fruits collection together with co-authors, conducted the experimental investigation of the extraction and analysis of the samples by spectroscopy techniques, conducted the formal analysis of the results together with co-authors, the morphological analysis of the samples performed by a co-author was coordinated with the author, the author wrote the first draft of the article together with co-authors.

Paper II. The author contributed with the conceptualization and methodology for the study together with co-authors, conducted the experimental work of the extraction, spray drying, and physical stability experiments together with co-authors, the chemical analysis of the native carbohydrates performed by a co-author was coordinated with the author, the author analyzed the results together with co-authors, wrote the original draft.

Paper III. The author contributed with the conceptualization and methodology design of the study together with co-authors, conducted the experimental analysis by HPLC, performed the calibration curves for analysis, supervised the experimental investigation of the irradiation and analysis of samples performed by a co-author, conducted the formal analysis of the results together with co-authors, wrote the initial draft together with co-authors.

Paper IV. The author contributed to the conceptualization and methodology design of the study together with co-authors, conducted the initial analytical methodologies for analyzing the candies and the sensory analysis, supervised the experimental investigation of irradiation and analysis of samples performed by a co-author, conducted the formal analysis of the results together with co-authors, wrote the first draft of the article together with co-authors.

Related papers not included in the thesis:

Mendoza Sillerico, Evelyn V., Curi Borda, Cecilia K., Rojas Mercado, Virginia J., & Alvarado Kirigin, Juan A. (2016). Encapsulation, characterization, and thermal stability of anthocyanins from Zea Mays L. (Purple Corn). Revista Boliviana de Química, 33(5), 183-189. Recuperado en 31 de enero de 2021.

http://www.scielo.org.bo/scielo.php?script=sci_arttext&pid=S0250-54602016000500005&lng=es&tlng=es.

Conferences

Curi-Borda, C.K.; Linares-Pastén, J.A.; Tat, T.; Tarqui-Dueñas, R.; Chino-Flores, N.; Alvarado, J.-A.; Bergenstahl, B. *Multilayer Bixin Microcapsules: The Impact of Native Carbohydrates on the Microencapsulation Efficiency and Dispersion Stability.* Poster presented at the *17th Food Colloids Conference: Application of Soft Matter Concepts at the University of Leeds*., 8 – 11th April 2018, Leeds, United Kingdom.

Cecilia K. Curi-Borda, Vandana Tannira, Niko Gentile, Juan-Antonio Alvarado, Björn Bergenståhl, *Model for measuring light stability of photolabile substances in powder beds using spray dried bixin microcapsules.* Poster accepted to be presented at the 18th edition of the *Food Colloids conference: Structure, dynamics and function*, 19th – 22nd April 2020, Lund, Sweden

Cecilia K. Curi-Borda, Luis Marconi, Álvaro Salinas, Marco Quino, Juan-Antonio Alvarado, Björn Bergenståhl, *Morphological and phytochemical characterization of annatto samples (Bixa orellana) from Los Yungas of La Paz, Bolivia*. Poster presented at *2° Congreso Internacional de Biotecnología*, Bolivia INNOVA, 21st – 30th August 2020, Santa Cruz, Bolivia.

Abbreviations

B	Bixin
CRB	Carbohydrate rich bixin
MD	Maltodextrin
CMC	Carboxymethylcelullose
GA	Gum arabic
EE	Encapsulation efficiency
T_B	Total colorant content of the microcapsule
S_B	Surface colorant content of the microcapsule
X, Y, Z	Tristimulus functions
$\bar{x}, \bar{y}, \bar{z}$	Imaginary color matching functions
$P(\lambda)$	Spectral Power Distribution of a light source
$\rho(\lambda)$	Radiation Density
Xn, Yn, Zn	Perfect white tristimulus values
x, y, z	Chromaticity cartesian coordinates
$L^* a^* b^*$	CIELAB homogeneous chromaticity coordinates
ΔE	Difference in color in a system
$C_{a,b}$	Difference in chroma in a system
$K(\lambda)$	Absorption of a solid system according to the Kubelka Munk Theory as a function of wavelength
$S(\lambda)$	Scattering of a solid system according to the Kubelka Munk Theory as a function of wavelength
$R(\lambda)$	Reflectance of an opaque or translucent system according to the Kubelka Munk theory
C_{Bixin}	Concentration of Bixin

ε	Absorptivity of the absorbing substance
A	Absorbance of the system
b	$b = 1\ cm$ is the path length of light under standard conditions.
c	Concentration of the analyte
z	Path length of the incident light through the system
$D_{4,3}$	Particle average diameter
TSI	Turbiscan Stability Index
$\epsilon(\lambda)$	Absorptivity of the colorant as a function of wavelength
S	Scattering of the powder bed per length unit $[m^{-1}]$
$\hat{S}$	Scattering of the total powder bed $[unitless]$
$b(t,z)$	Local bixin load through the thickness Z of the powder bed
$A(\lambda,t)$	Light absorption through a solid multiphase system as a function of wavelength
I_T, I_a, I_A, I_S, I_q	Transmitted, attenuated, absorbed, scattered and incident light intensity.
$B(t)$	Colorant powder load
Z	Thickness of the powder bed
$Q(t)$	Absorbed accumulated light dose per area unit
Y	Quantum yield, reaction rate
$t_{1/2}$	Half-life of a reaction

Table of Contents

1 Introduction

Color is one of the most important organoleptic characteristics in food products, cosmetics, and other products [1]. Colorants are responsible for imparting color and enhancing the products' appeal or attractiveness. These colorants may have synthetic or natural origin. For the last 20 years, there has been an increasing consumer interest in colorants of natural origin [2]. Moreover, this demand is forecast to increase dramatically over the coming years, since it is expected to reach USD 3.2 billion by 2027 [3]. Today, some of the most valued natural colorants are carotenoids. Among them, bixin and norbixin, which are contained in the pigment "annatto", have drawn great attention and demand. Annatto extracts are obtained from the waxy aril of the seeds of the vegetal species *Bixa orellana*. This plant has the common names of achiote (Spanish), annatto (English) and urucum (Portuguese) [4].

Bixa orellana is a shrub (or tree) that presents morphological diversity in its flowers and fruits where the seeds are enclosed. The seeds are surrounded by a colored aril that contains native carbohydrates, the pigments, and minor phenolic components [5-9]. The colorant content in the seeds varies from one variety to another. Consequently, global markets are interested in annatto seeds with a colorant content above 2.5% [10]. For instance, Brazil has set a minimum content of 4% for export [11, 12].

Annatto has been commercialized all around the world, especially in the United States (Annatto), Western Europe (Additive E160), and Japan [4, 13-15]. Producer countries include Mexico, Brazil, Bolivia, Colombia and Peru in Latin America; India in Asia; and Kenya in Africa among others [7, 13, 15]. Bolivia has a history of using annatto in traditional dishes. Furthermore, "achiote" or "urucú" is considered among the most important crops that are produced and exported in Bolivia. The main Bolivian markets for annatto seeds exports are Ecuador, Peru, Argentina, and the United States. The weight of exported seeds has decreased from 72,600 kg per year (USD 100,000) in 2010 to 23,600 kg per year (USD 5,000) in 2018. In contrast, the amount of annatto powder has increased from 454 kg (USD 350) in 2010 to 143,300 kg (USD 51,000 USD) in 2018 (Data taken from INE Bolivia) [16]. This shows a development in this sector.

The FDA requires certification for certain colorants to prove that the batch does not contain heavy metals or other possible harmful components every time a new batch is produced. The annatto extracts are exempt from requiring safety certification.

Nevertheless, as for all colorants, the annatto colorants must comply with the identity of the component, purity specifications and product usage limitations [2, 17]. The FDA set the acceptable ADI intake of bixin (92% purity) to 12 mg/kg of body weight per day, while norbixin (91% purity) intake is 0.6 mg/kg of body weight per day [18, 19]. The differences in ADI may be due to the difference in human metabolic absorbance of bixin and norbixin [18].

Annatto's importance is related not only with its coloring properties, but also to its health-promoting benefits. Annatto has a long tradition of use for producing orange to red colorations in food and textiles. Moreover, annatto extracts have antioxidant and antimicrobial properties. Different annatto extracts containing mainly bixin, norbixin, or mixtures of these have demonstrated their antioxidant activities [20-22].

Colorants may be classified as dyes or pigments. Depending on their water solubility, dyes will dissolve in water, while the pigments will not [23]. Bixin and norbixin are practically insoluble in water, while norbixin salts are water dispersible [24, 25]. Depending on the product's application, several extraction methods have been applied to obtain annatto extracts with different compositions of bixin and norbixin [7, 15, 24, 26]. Annatto extracts containing mainly bixin are commonly used to color oil-based products. The main products include cheeses, emulsions, snacks, bakery products, margarines, spreads, fillings, and liqueurs among others [7, 19, 27-30]. On the other hand, norbixin salts, which are aqueous dispersible colorants, are mainly used in cheeses, meat and fish products [27].

The formulation of a coloring agent requires considering several aspects such as the accessibility of the raw material, the extraction of the colorant, the processing, the miscibility of the colorant formulation with the product to be applied to, the colorant concentration and quantification in the product, the perception of the color in the product, the useful life of the product, and its packaging requirements. These considerations may be needed to extend the shelf life during storage and under the conditions of product usage.

Since the annatto pigment is obtained from natural sources (i.e. from *Bixa orellana* vegetal species), the availability of the annatto pigment depends on the management of its cultivation, the yield of the seed's pigment production, the breeding programs for genetical improvement of the cultivars, and the logistics of transporting the materials to the processing facilities. There is research that aims to identify genetic and morphological characteristics related to the seeds' colorant production yield. This is very important to stablish breeding programs and increase the production yield.

Once the seeds are harvested, any subsequent processing of the pigment starts with its extraction. The aqueous insolubility of the seeds' pigment, with bixin as the major component, presents a challenge for solvent-free extraction processes. Furthermore, the pigment's aqueous insolubility limits its application in aqueous products such as sodas and juices. Moreover, it also limits the pigment's application in aqueous continuous-based products like jellies and candies. Consequently, this research is aimed at addressing this issue from a colloidal chemistry and technological perspective.

Annatto has great potential to be applied in aqueous continuous-based products like foods, cosmetics, and pharmaceuticals. The colorant formulation may be solid powders that could be used as eye shadows for example or be applied as powders to produce other products like foundation, blush, and lipsticks. In the pharmaceutical industry may be used to coat capsules or be compressed as powder to produce colored tablets. When doing so, it is important to evaluate the light stability of the colorant in relation to the intended use of the colorant. Then, the aim of the present research contemplated the evaluation of the photostability of solid (powdered) colorant formulations that could increase the pigment stability against light degradation. In this sense, it would be possible to attain longer storage lives as well as an estimation of the lifetime of the colored powders under light exposure.

The colorant influences the human perception of the product. The color, and the intensity of that color depend on the concentration of pigment added and the stability of the color through time. Changes in colorant concentration may produce differences in the color of a product, which may be visually perceived and will determine the consumers acceptance of that product. Thus, an assessment of the product's color degradation caused by light gives important information regarding the shelf life and usage life of a product as well as the packaging requirements.

Given the global importance of annatto as a natural colorant, it is of great interest to expand its applicability in an eco-friendly and sustainable way. Consequently, there is a great need to advance the research, development, and innovation in relation to the whole chain of production for this valuable product. Thus, the importance of the present thesis that looked at addressing some of these challenges. To do this, annatto seeds samples studied in the present thesis were collected or purchased from the geographical area of Bolivia, a South American country (western-central) that is briefly described below.

Bolivia's territorial administrative division is set in 9 departments. Where the capital of Bolivia is Sucre, while the seat of government is found in La Paz. Bolivia is one of the countries with the highest biodiversity, both for vegetation and geological formations. The high geographical diversity is due to the presence of mountains up to 7000 m.a.s.l. Then, the Andean highlands (~4000 m a.s.l.) with the region around Uyuni's Salar Dessert as one of the most important ecosystems. Decreasing in altitude (2000 – 300 m a.s.l.), the Valley region located at the center and south part of the country is characterized by moderate climate and farming activities. Finally, the Llanos region or lowlands, with altitudes below 300 m a.s.l., has one of the most humid areas on Earth called "Chapare" in Cochabamba. Equally important, the

Amazonas rain forest extends through the departments of Santa Cruz, Beni, Pando and the northern of La Paz [31].

Bolivia's high biodiversity comprises more than 23,700 species of plants, from which around 980 vegetal species have been domesticated due to their nutritional, medicinal or other economic importance [32]. Among these species, *Bixa orellana* seeds are produced, harvested, and processed for export in municipalities in La Paz, Cochabamba, and Santa Cruz. Inside La Paz, there is also great variation in altitudes, climate conditions, temperatures, and ecosystems. The southern part of La Paz comprises part of the Andean highlands. However, municipalities in the middle part belong to the Valley region. They are characterized as having tropical climate conditions with temperatures varying between 20 and 32°C, annual precipitation around 600 to 2000 mm and accidented geography with altitudes varying from 300 to 1700 m.a.s.l. Certainly, *Bixa orellana* was reported to grow in those conditions [33, 34].

2 Aim

The aim of this study was to develop comprehensive knowledge of *Bixa orellana* seeds' pigment for addressing some important challenges in its production chain. This aims at generating social, economic, and scientific impact around this natural product. A further aim was to take a step forward in satisfying the markets needs for natural coloring agents for foods with information required to comply with safety and quality standards.

In this sense, the present study seeks to assess the content of annatto in Bolivian samples that can be found in the regions in which this vegetal species is found in the department of La Paz. Moreover, it seeks to map the fruit morphological traits associated with these higher annatto contents. These studies are important to create a basis for the establishment of breeding programs and develop the research in the agronomical field.

Likewise, this thesis seeks to study the technological approaches needed for attaining aqueous dispersible formulations of annatto pigments to expand its applicability into aqueous continuous-based products. Also, annatto colorant powder formulations as an ended product or for application in other products, require assessment of the pigment's light stability in solid-state and its relation to color, concentration of pigment and color perception of the product.

The present thesis seeks to employ existing scientific knowledge on the background of emulsions and colloids, light-pigment interactions, light scattering phenomena, color quantification, color perception, and analytical techniques. Consequently, the study's accumulated results seek to expand and generate new understandings.

3 Background

This chapter introduces a review of the present scientific knowledge to give context to this study. This knowledge makes it possible to stablish the state of art of research regarding the topics contemplated in the thesis to be able to define our study's objectives.

Bixa orellana characteristics

Bixa orellana is a shrub that is naturally fertilized by allogamy, a cross-fertilization mechanism. This generates high genetic variation which results in high morphological diversity in its aerial parts [35, 36]. White, pink, or purple flowers have been observed. After maturation of the flowers, dehiscent or indehiscent pods are formed with different shapes and colors. The most common pod shapes include oval, lanceolate, conical, hemispherical, and heart shaped. Moreover, the pods' color can vary from red coloration, red-greenish colorations, to green coloration [35, 37, 38]. The pods contain around 40 seeds, each of which is covered with a reddish aril.

The aril surrounding the seeds is composed of hemicellulose, starch and a small amount of protein and ash [6]. The phytochemical profile shows the presence of the major pigments bixin followed by cis-norbixin. Several minor components are other carotenoids, phenolic compounds, terpenoids, and volatile compounds [5, 7-9]. It has been reported bixin contents between 1 and 5% of the seeds' total solids [39]. Studies have found that carotenoids present in the aril comprise approximately 80% Bixin and 20% Norbixin [27].

Several studies have shown that the phytochemical profile of Bixa orellana seeds varies from one variety to another, with contrasting results. Mantovani et al. observed higher bixin content in annatto plants with some specific genotypes, like red pods with lanceolate shape [35]. Likewise, ovate red colored pods had the highest colorant contents in samples analyzed by Akshatha [38]. It has been shown that the gene related to the biosynthesis of β-Xanthophills is expressed in pink petals as well. This may explain why pink flower varieties appear to have higher concentration of bixin in their seeds than white flower varieties [40]. Another study showed that dehiscent reddish pods had higher bixin concentration than indehiscent greenish pods [41]. Indehiscence might have been used by *Bixa urucuana* (*Bixa orellana*'s ancestor) to disperse seeds inside the pods by the water (hydrochloric character) [42]. This characteristic might have been lost during domestication of the plant, since indehiscent fruits are desired in agricultural process for protection of the seeds from environmental damages (i.e. rain, pests, etc.). In Bolivia, a study showed that varieties with green pods and brownish pods had the highest colorant content among the collected varieties [37]. A recent study showed that heterosis (cross-breeding benefits) in fruits may produce higher colorant contents [43]. Thus, further research is needed in this regard.

Environmental factors affecting phytochemical profile

Environmental factors like altitude, soil, precipitation, and geographical position can affect some phenotypes in plants, the yield of macromolecules' production and its phytochemical composition [44-49]. The environmental changes directly related to altitude are differences in temperature, atmospheric pressure (O_2 and CO_2 pressure), and clear-sky turbidity. The higher the altitude, the lower the temperature (0.55 °C decrease per 100 meters of altitude). Then, temperature affects evaporative-transpiration process in plants, although these depend also in the type of plant. It has been shown that differences in oxygen pressure does not significantly affect the plants' metabolism. At higher altitudes, there is an increase in solar radiation dose and the UV-B component. It is very difficult to separate the effect of altitude (represented by temperature and O_2) from the effects caused by differences in regional characteristics such as differences in precipitation, wind velocity, and soil. Therefore, the results from altitudinal observations will always reflect the combined effect of the altitude and regional characteristics [45, 49].

There are several examples that illustrate the effect of altitude in the phytochemical composition of important commercial plants. Billberry leaves presented higher phenolic composition and antioxidant capacity at higher altitudes [50]. Similarly, in Bolivia, studies on wines cultivated at high altitude valleys presented higher production of resveratrol and phenolic components [51-53]. Conversely, grape stems and strawberries presented lower phenolic content and lower antioxidant capacity at higher altitudes [54].

Bixin

As in many other vegetal species, carotenoids are the result of the secondary metabolism of the plant. Bixin is biosynthesized from the isoprenoid biosynthetic pathway that produces lycopene which in turn transforms to norbixin (9-cis-norbixin) and then bixin (9-cis-bixin) [40, 55]. Bixin is the monomethyl ester of the dicarboxylic acid norbixin. It can be chemically hydrolyzed to norbixin salts under alkaline conditions [27].

Bixin and norbixin are hydrophobic colorants, practically insoluble in water, and somewhat soluble in methanol and other solvents of intermediate polarity like dichloromethane, ethyl acetate, acetone and acetonitrile [26, 27, 56]. These salts remain water soluble at alkali pH but will precipitate under acidic pH as the dicarboxylic acid is formed.

Bixin has a conjugated system of 10 double bonds that produce absorbance bands between 400 to 550 nm. These fall within the visible light spectrum [27]. The light absorbance translates into red to orange colorations. The carotenoid presents instability to processing, solvent of extraction, temperature, light exposure due to the reactivity of its conjugated system [57], presence of oxygen, pro-oxidants and hydrolysis reactions [27, 58]. In dark conditions, oxidation of carotenoids occurs when exposed to oxygen resulting in the formation of radicals and their reaction products [59].

Scotter et al. collected annatto industrial food coloring formulations with different production histories and different storage times (2 to 10 years old) to analyze their colorant content and degradation products [60]. The products included dry powders, encapsulated powders, oil suspensions, and granules. Several degradation products like norbixin trans-, mono-cis-, di-cis-isomers and trans-monomethyl ester of 4,8-dimethyl-hexa-tetradecaene-dioic acid were reported. Other studies have shown that carotenoids photodegradation products may include low molecular weight components (7-oxo-4-methyl-(2,4)-di-heptenoic acid and 8-oxo-4-methyl-(2,4,6)-tri-nonenoic acid) [59] and/or isomerization products [27, 61, 62].

Studies in solid-phase over 360 days have shown that annatto dye powder and oleoresin are much less stable when exposed to light compared to when stored in darkness. For both storage conditions, it was shown that the lower the temperature, the higher the stability of the dyes. It is important to mention that the degradation rate in each condition was higher for the dye powder compared to the oleoresin [63].

Colorant extraction

There are mainly three different industrial processes for pigment extraction – extraction into oil, extraction with solvents and extraction into aqueous alkali. Depending on the extraction procedure, different annatto formulations can be used for different applications. Commercial water-soluble annatto formulations with 1.4, 2.8, or 3.8% norbixin are available as well as 15% norbixin powder. Oil soluble annatto formulations contain from 0.2 to 5% bixin and higher concentrations are available as emulsions or suspension colors [27, 28]. Examples of the extraction processes are given in Table 1.

The extraction of annatto into oil is beneficial to color oil-based products, however, it is not useful for aqueous based products. For this group, extraction into solvents is an alternative, although higher costs, safety as well as environmental considerations may be encountered. The process of abrasion into cold, mildly alkaline solutions is an attractive option for formulating coloring agents for aqueous based products. Nevertheless, the costs for abrasion, followed by drying and milling may be considered. Inside this alternative, there is room for improvement to facilitate the extraction of annatto, and design aqueous dispersible formulations that could be attained by a continuous, straightforward processing from the extraction of the annatto pigment.

Table 1: *Extraction methods of bixin and annatto extracts. The table shows the type of extract that can be obtained, and the product to which this formulation may be applied.*

Extraction method	**Formulation**	**Application**	**Ref.**
1. Mechanical abrasion of seeds with vegetable oil, below 70°C, followed by filtration of unwanted solids.	cis-bixin rich oil suspensions	Margarine, fats, and oils	[7, 15]
2. Mechanical abrasion of seeds with vegetable oil, below 130°C under vacuum, followed by filtration of unwanted solids.	trans-bixin oil solutions	Salad oils, margarines, fats	[7]
3. Solvent extraction of seeds with acetone, methanol, hexane, ethanol, and ethyl acetate	Annatto extract	Highly pure oil- and water-soluble formulations	[7]
4. Seed extraction with medium polar solvents such as acetone or ethyl acetate. Followed by washing steps to remove impurities	Bixin enriched annatto extracts	Highly pure oil- and water-soluble formulations	[7, 24, 27]
5. Strong, hot, alkali solutions for seed coating removal to hydrolyze bixin and precipitate it with or without acid addition. Followed by filtration, drying and milling.	Granular powder of norbixin or norbixin salts	Dry mixes, soups, drinks cakes	[7, 24, 27]
6. Seed abrasion with cold, mildly alkaline solutions, precipitation by acidifying the suspension and subsequent filtration, drying and milling.	Powder with high bixin content.	Drinks, cheeses, cakes, cereal, icecream, tablets	[24]

Physical Stability of aqueous dispersions

An aqueous dispersion can be described as hydrophobic particles being dispersed in water. The hydrophobic particles constitute the dispersed phase and water is the continuous phase. The physicochemical characteristics of the dispersion will be given by several parameters. Examples include the particle size distribution, the rheology of the system, and the repulsive or attractive interactions between particles. These parameters will determine whether the particles remain dispersed or follow a destabilization mechanism that leads to phase separation [64].

For instance, when the forces of gravity or attraction between particles are not prevented, several instability mechanisms arise. The most common ones for solid particles are sedimentation or creaming, and flocculation [65]. The movement of the particles driven by Brownian motions may generate flocculation. That is two or more particles will aggregate into each other generating a bigger particle but keeping the individual's particle shape. Then, sedimentation will occur when particles are denser than the continuous phase and thus travel downwards due to gravity. In contrast, creaming will occur when particles are less dense than the continuous media and travel upwards in the dispersion container.

Dispersions may be kept stable using different mechanisms. For example, for particle sizes that are below 1 μm, Brownian motions may prevent particle sedimentation. However, the most developed techniques to keep a stable dispersion include the use of stabilizers. Stabilizers are classified in emulsifiers or texture modifiers. Emulsifiers can be surface-active molecules, such as surfactants or ions, that will adsorb at the particles surface and may create repulsive interactions between particles, keeping them apart from each other. Texture modifiers are generally macromolecules that increases the viscosity of the continuous phase or create a gel. This increase of viscosity or the network will decrease the movement of particles due to gravity or Brownian motion. In this sense, the particles will be kept separate from each other and the dispersion will remain stable [65].

The surface-active molecules will adsorb at the surface of the particle, creating a surface charge density. This charged density will attract a layer of counter-ions from the continuous phase, that is, a counter-ion charge density. This so-called "double layer" will keep particles apart by means of osmotic repulsion. The range of electrostatic interaction is referred to as the Debye layer. Another important parameter is the Z-potential, which gives insight into the interaction between particles. It has been shown that zeta-potential values above $\pm$ 30 mV are characteristic of stable dispersions [66].

As previously mentioned, another way of achieving aqueous dispersibility and physical stability is through Brownian motion of particles in an aqueous medium. There is a relationship between the particle size of the dispersant and the viscosity of the continuous phase where the particles are dispersed. This relationship is given by Stoke's law [65]. In this matter, a previous study produced bixin nanoparticles through nanoencapsulation by interfacial deposition of preformed polymers with mixtures of polymers as poly-ε-caprolacton\e (PCL), capric/caprylic triglyceride, and sorbitan monostearate [66]. In this case, solvents were used for dissolving bixin before the encapsulation step. Then, the obtained capsules were suspended in water. The physical stability of bixin nanoparticles was monitored over time, showing stable colorant particle suspensions for 119 days.

Another way of stabilizing a dispersion may be as described by Zhang et al. They produced aqueous dispersions by obtaining bixin microcapsules. Bixin was spray dried using sodium caseinate as encapsulant [67]. A binding was produced between the sodium caseinate and the bixin in the microcapsules. When redispersed in water, it was found that caseinates stabilized the colorant-bound particles at different pH away from the PI of sodium caseinate.

Microencapsulation

Encapsulation is a process in which solid particles, liquid drops or gaseous bubbles are entrapped or surrounded by a solid wall material (Figure 1). The entrapped particles are generally called the core material. There may be a desire to protect the core from external factors such as light and oxygen. There may also be a need to change the core's physical characteristics as well as to increase its aqueous dispersibility [67]. Wall materials are generally macromolecules such as polysaccharides, gums and proteins [68]. Hemicelluloses may be interesting wall materials because of their thickening and stabilizing properties [69, 70]. The properties that are important for microencapsulation include film formation capacity and emulsifying properties [71, 72]. Wall materials can also be mixtures of different components. Examples include macromolecules such as maltodextrins, gums or proteins, and low molecular weight compounds, such as sucrose and other saccharides [73].

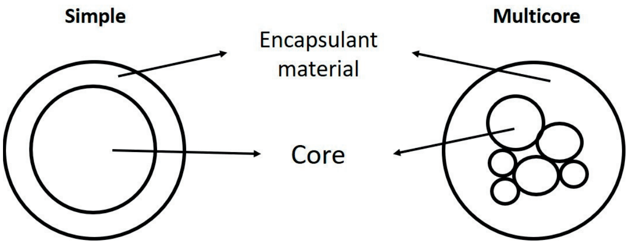


***Figure 1**: Graphical representation of a simple and a multicore microcapsule. Representations taken from Gharsallaoui et al. [68]*

There are several techniques intended to microencapsulate colorants, hydrophobic compounds, vitamins, and other sensitive compounds. The appropriate ones (due to physicochemical limitations) for colorants are spray drying, freeze drying, coacervation, and molecular inclusion complexation. Their advantages and disadvantages are described below (Table 2) to enable the selection of the most suitable technique for encapsulating *Bixa orellana* colorants [68].

The microencapsulation of bixin or norbixin has been performed using spray-drying, freeze-drying and complexation techniques, among others. Complexation of bixin with β-cyclodextrin by means of co-precipitation produced powders with 1.9 times higher light stability than unprotected bixin under natural light storage conditions [74]. On the other hand, potassium norbixinate was microencapsulated with maltodextrin through freeze-Drying. Under light exposure, microcapsules had higher stability under vacuum storage conditions than under atmospheric air [75]. In all cases, encapsulated systems were more stable when stored at dark conditions.

Table 2: *Differet techniques applied for microencapsulation of natural active compounds for food applications. The advantages, disadvantages and applications are presented for each.*

Encapsulation method	**Advantage**	**Disadvantage**	**Application**
Spray Drying [76-78]	Rapid, continuous, straightforward, cost affordable, reproducible, easy to scale up, well established. Low moisture content, efficient, safe, higher chemical, physical and microbiological quality.	Loss of product particles in the walls of drying vessels, especially sugar. Uncontrolled broad particle size distribution. Low solubilities of some wall materials (some polysaccharides and proteins)	Flavors, colors, vitamins, milk, antioxidants.
Freeze Drying [76, 77]	Low operating temperatures. Absence of air. Prevents deterioration caused by oxidation or chemical modification.	Long process time, high initial capital and operating costs. Porous structure due to ice sublimation that results in low flowability powders and exposure to atmosphere.	Suitable for sensitive colors
Coacervation [76]	High loading capacity, low temperature, reduced evaporation losses, reduced thermal degradation, Specific equipment not necessary. Use of non-toxic solvents	High costs for particle isolation procedure. Complexity of the technique The crosslinking includes Glutaraldehyde which is a toxic compound.	β-carotene, broccoli particles rich in chlorophyl, anthocyanins
Molecular Inclusion Complexation [76]	Protection against oxidation, and light-induced decomposition, controlled release. Uses cyclodextrins, Flavor burst through unique release characteristics Thermal stability up to 200°C	Only three cyclodextrins are recognized as safe by the FDA. Uses solvent during the process Low entrapment Segregation during encapsulation process High costs of cyclodextrins	Volatile organic compounds, masking odor, flavors and preservation of aromas

Another study has proven that bixin encapsulated with sodium caseinate had a higher thermal stability than non-protected bixin (20). In addition, bixin microcapsules obtained by spray drying were dispersed in water. Light exposure produced two sequential first-order decays [57].

Because spray drying has affordable costs, is a solvent-free and toxic component-free process, is easy to scale up, and produces higher encapsulation efficiencies [78], the spray-drying technique was selected for microencapsulating bixin and annatto extracts in the present thesis. Consequently, the spray-drying process for encapsulation can be described as follows. First, a dispersion of the target substance (core) is dispersed in a solution of low molecular weight molecules or polymers (wall material). Then, the dispersion (feed) is pumped into the spray-dryer. The feed is atomized through a nozzle into a chamber and is heated by a co-current flow of hot air. In less than a second, the atomized droplets are dried by the hot air flow sprayed through the nozzle inside the chamber. While the water evaporates, the wall material builds up a capsule enclosing the core [68].

The selection of the wall material is critical for achieving good protection of the active core. A way of determining the level of protection is by measuring the encapsulation efficiency. This parameter may be understood as the percentage of active core that is unavailable to be “washed” or extracted from the surface of the capsule with a certain solvent for a short period of time. The higher this value, the higher the entrapment of the core inside the capsule. Wall materials like maltodextrin and sucrose showed to produce microcapsules with increased light stability [57, 75].

The visual perception of color

The visual appearance of a product is determined by three factors – the characteristics of the material, the nature of the light source, and the observer or detector of light stimuli that reflects from the material [79]. The main visual characteristic covered in this thesis is color. Thus, this section will cover the properties of the materials that affect its color perception; light and matter interactions; and how it is possible to detect and quantify the color of a material.

The sun or lamps that resemble sunlight emit visible radiation that reaches objects. The incident light is absorbed, reflected and/or transmitted by the material. The visible radiation that is reflected from an object can be perceived by the human eye. A colorant will absorb certain wavelengths of the incident light and will reflect the remaining spectrum of light. The reflected remaining wavelengths, together, are perceived as a particular color (Chroma). For example, a colorant may absorb wavelengths corresponding to yellow-to-indigo light. Thus, all the reflected wavelengths will account as red light (complementary color). White objects reflect all the wavelengths of an incident light, while black objects absorb all wavelengths of the incident light. To be able to see color, the human eye or a detector detects the incoming radiation (reflected from the object). The reflected radiation will also depend on the physicochemical properties of the material containing the colorant [23].

According to the physicochemical properties of food products and cosmetics, they can be classified between opaque and transparent systems. Opaque systems will reflect or absorb all the incident light intensity. Colored opaque systems will absorb all the incident photons at a specific range of wavelengths. Still, note that they will reflect all the photons with wavelengths of the complementary color. Some examples of opaque materials are powders, granulates, fruits, and vegetables. Transparent materials will transmit most of the incident light intensity, even though, the absorbance may be higher in certain wavelength ranges. Common examples are transparent liquid beverages [79, 80]. Translucent systems are materials that fall in between transparent and opaque systems, such as candies, slurries, pastes, and some emulsions. They absorb light in the direction of the light beam, and some of this light is diffused within the sample and then scattered in different directions [79-81]. This phenomenon happens for any wavelength to a higher or lower degree depending on the color of the system.

There are different techniques to measure the color or reflected light that comes from an object. The reflectance of the solid system in the range of absorbed wavelengths is inversely proportional to the colorant concentration. The higher the colorant concentration, the higher the absorbance, and consequently, the lower the reflected light in that range. The measurement of the color may be achieved by different approaches. The first one is by measuring the colorant concentration of a system by means of e.g. spectrophotometry. The second one is by the reflection curves that are given by the Kubelka Munk Theory. The third one is by colorimetry using the color space functions XYZ, a*b*L*, or RGB. And the fourth one is by human visual perception (sensory analysis). These approaches are described below [81-83].

Color measurement by Colorimetry:

Any color is defined by three parameters: hue, chroma, and lightness. Hue represents the identification of a specific color as in red, blue, yellow. This parameter is related to a specific range of wavelengths that reach the observer or detector. Chroma is defined as the saturation or intensity of that color; and lightness is perceived as the luminosity of a surface [80, 81]. These parameters are perceived by the human eye by cones and rode cells that detect the reflected light from an object (range of wavelengths). As previously mentioned, the perceived color depends on the incident light on the surface, the reflected light from that surface, and the observer.

Experiments were conducted to define color matching functions (chromaticity values). These functions were obtained using an observer who had to combine red, green, and blue light to achieve or match a reference color [83]. Since the color matching functions were required to have negative values at certain wavelengths to match certain colors, a transformation to the imaginary color matching functions $\bar{x}, \bar{y}, \bar{z}$ was made. These functions have positive values for all wavelengths.

Then, the tristimulus functions XYZ were defined to produce any color (Hue). These XYZ tristimulus functions (Equation 1,2 & 3) are constructed by the reflectance curve $\rho(\lambda)$ of the viewed object (for opaque objects), the imaginary color matching functions $\bar{x}, \bar{y}, \bar{z}$ of a 10° standard observer, the spectral power distribution $P(\lambda)$ of a standard illuminant at a range of wavelengths, all divided by the luminance of the incoming light $\sum P_i(\lambda)\bar{y}(\lambda)$. The reflectance curve $\rho(\lambda)$ of the viewed object is also referred to as the "radiation density", i.e., the intensity of the reflected light relative to the intensity of the exposing light. The color matching functions, and the standard illuminant are standardized by the International Commission d'Eclairage, CIE. On the other hand, the standard illuminant corresponds to spectral power distributions of light that resembles average daylight excluding sunrise and sunset. The D_{65} illuminant was constructed considering measurements in several countries. It corresponds to a color temperature between 6000 and 7000K (Light emission spectrum of a black body at that temperature) [79-81, 84].

$$X = \frac{\sum \rho(\lambda_i) P(\lambda_i) \bar{x}(\lambda_i)}{\sum P(\lambda_i) \bar{y}(\lambda_i))} \quad (1)$$

$$Y = \frac{\sum \rho(\lambda_i) P(\lambda_i) \bar{y}(\lambda_i)}{\sum P(\lambda_i) \bar{y}(\lambda_i))} \quad (2)$$

$$Z = \frac{\sum \rho(\lambda_i) P(\lambda_i) \bar{z}(\lambda_i)}{\sum P(\lambda_i) \bar{y}(\lambda_i))} \quad (3)$$

The tristimulus functions XYZ are used for calculating the chromaticity coordinates that are unitless. And the $XnYnZn$ represent the perfect white tristimulus values and are equal to $X_n = 100 \frac{\sum P(\lambda) \bar{x}(\lambda)}{\sum P_i(\lambda) \bar{y}(\lambda))}$, $Z_n = 100 \frac{\sum P(\lambda) \bar{z}(\lambda)}{\sum P_i(\lambda) \bar{y}(\lambda))}$, $Y_n = 100$.

From the tristimulus functions, xyz chromaticity cartesian coordinates can be obtained in order to define any color. These xyz coordinates are given by the equations:

$$x = \frac{X}{X+Y+Z} \quad (4)$$

$$y = \frac{Y}{X+Y+Z} \quad (5)$$

$$z = \frac{Z}{X+Y+Z} \quad (6)$$

These xyz are not linearly independent of each other. They are a plane described by $x + y + z = 1$. The graph of y as a function of x is known as the CIE chromaticity diagram, where all the spectrally pure colors are represented inside a horseshoe-like curve. The CIELAB chromaticity coordinates $L^* a^* b^*$ are homogeneous color space coordinates that resembles the human visual perception. They are calculated by the tristimulus values, by means of the following formulas [83]:

$$L^* = f_{L*}\left(\frac{Y}{Y_n}\right) \quad \text{where } f_{L*}(s) \rightarrow \begin{cases} s^{\frac{1}{3}} & s > 0.008856 \\ 903.3 \cdot s & s < 0.008856 \end{cases} \quad (7)$$

$$a^* = 500\left[f_{a*}\left(\frac{X}{X_n}\right) - f_{a*}\left(\frac{Y}{Y_n}\right)\right] \quad \text{where } f_{a*}(s) \rightarrow \begin{cases} s^{\frac{1}{3}} & s > 0.008856 \\ 7.787 \cdot s + \frac{16}{116} & s < 0.008856 \end{cases} \quad (8)$$

$$b^* = 200\left[f_{b*}\left(\frac{Y}{Y_n}\right) - f_{b*}\left(\frac{Z}{Z_n}\right)\right] \quad \text{where } f_{b*}(s) \rightarrow \begin{cases} s^{\frac{1}{3}} & s > 0.008856 \\ 7.787 \cdot s + \frac{16}{116} & s < 0.008856 \end{cases} \quad (9)$$

The resulting CIELAB parameters L*a*b* are a function of the tristimulus values and were adopted by CIE in 1976. The L* parameter denotes the lightness of the system, positive values of a* denote red colorations and negative values denote green colorations. The b* parameter denotes the amount of yellow in the negative axis (-b*) or blue in the positive axis (+b*) of the color perceived by humans [83]. Note that the extremes do not represent spectrally pure colors. These parameters denote colors very proximate to the ones perceived by the human eye. Other color space is the RGB (Red, Green, Blue) color space. This color space is usually used in photography [85, 86].

Color measurement in terms of reflectance of the system

The reflectance $R(\lambda)$ of an opaque or translucent system will be affected by the relationship between the absorption $K(\lambda)$ and scattering $S(\lambda)$ coefficients of the material as explained by the Kubelka-Munk (KM) theory [81]. The reflectance R_∞ at infinite thickness of the sample is affected by the ratio of K/S. This ratio depends on the concentration of the colorant present in the material, its refractive index, the light scattering properties of the material and its thickness [84]. The Kubelka-Munk model is [81, 87]:

$$R(\lambda) = 1 + \frac{K(\lambda)}{S} - \sqrt{\left(\frac{K(\lambda)}{S}\right)^2 + \frac{2K(\lambda)}{S}} \qquad (10)$$

This equation can be used for systems where the thickness is wide enough to not allow for color changes (constant optical thickness). This means that the equation assumes no translucence [88]. Note that $K = \varepsilon C_{Bixin}$ and S equals the scattering. This model assumes as well that the colorant concentration is constant over the thickness of the material. That is, that K and S are constant.

Human visual perception

When selecting a product, appearance plays a major role when evaluating a product's characteristics. Among the appearance attributes, color is the main factor observed by consumers. It gives information regarding the safety of a food product and the expectations towards it. In this sense, sensory analysis evaluations are of use for evaluating the perception of a consumer with respect to a product. Moreover, discrimination tests make it possible to determine the perception of a difference between two or more colored objects. The detection of possible differences helps the standardization of technological process for quality controls, and the acceptability of color intensity of a product. Thresholds in differences in color perception may be assessed. These color perceptions are assessed by panelists who participate in identifying differences in color among samples. In addition, an acceptability test may be performed to assess the satisfaction of a consumer towards the color of a product [81, 89, 90].

Light stability

Organic molecules absorb light of different energy based on the chromophores they have. When exposed to light, the π electrons in the double bonds of carotenoids get excited to π^* states by absorbing different radiation of the visible spectrum. Once they are in excited states, they undergo vibrionic relaxation processes, whereby the electrons return to ground state. However, degradation reactions may occur when molecules in exited states do not return to ground state. Degradation products may be produced by light-induced carotenoid radicals and their reaction with singlet oxygen. On the other hand, indirect light-induced reactions occur when triplet oxygen is produced and subsequently reacts with the carotenoids. In addition, carotenoids may undergo degradation reactions due to their light absorbance [91].

Moreover, there is a wide spectrum of colorants used in food and cosmetics as well as photolabile drugs used in oral, topical, or parenteral formulations [92, 93]. In order to apply these molecules in different products, photostability evaluations are considered [94]. The information obtained is used to determine the shelf life of the molecule of interest, along with its lifetime in retail and during consumption.

There are guidance and standards for the evaluation of these substances like "Q1B Photostability testing Guidance of new drugs substances and products" [95] and the ISO 18909:2006 standard, which state different irradiation sources (UV – Vis) and different conditions for the experiments. Initially, forced degradation testing (stress testing) is performed, followed by confirmatory testing. The stress tests may be performed in liquid or in solid state (tablets and powder beds) depending on the formulation. Then, the irradiation sources are selected based on the intended application and usage conditions. The confirmatory testing is performed to obtain information regarding the appropriate package that should be selected for the drug [96].

Light stability of colorants in single phase systems

Single phase systems may be described as non-turbid systems, solutions with low analyte concentration and with insignificant light scattering. These systems are studied as typical analytical solutions of different compounds. Photostability tests of molecules in solution are usually performed under standardized conditions. This means using a spectrophotometer. In this equipment, light traverses the pathlength of a cuvette of $z \equiv b = 1\ cm$ of diameter following the Beer-Lambert law:

$$Log\left(\frac{I_q}{I_T}\right) = A = \varepsilon\, b\, c \qquad (11)$$

Where A is the absorbance, ε is the extinction coefficient (an intrinsic property of the molecule), I_q and I_T are the intensities of the incident and transmitted light respectively. Then, c is the concentration of the analyte in solution. The absorbance under the Beer-Lambert law will be a linear function of the concentration of the colorant for absorbances below < 2. Under these conditions, light decreases exponentially through the pathlength of the cuvette. Important to note that the linearity of the Beer-Lambert law is lost at higher molecule concentrations [94].

Under standard conditions, molecules are analyzed in a solvent or aqueous solution at very diluted concentrations. Very diluted concentrations of colorant (Absorbance < 1) lead to a regime where the light decreases exponentially over the path length. No significant light scattering is produced by the liquid under these conditions. Moreover, substances degrade following apparent first, second or subsequent orders of reaction. The final degradation curve may be influenced during the different elementary photoreaction steps by the absorption of degradation products for example [94].

The photodegradation rate is defined experimentally even though the reaction order of a molecular reaction is given theoretically by the stoichiometric relation between reactants and the rate-determining step [97]. Moreover, for any system, the degradation rate of the photodegradation is defined as the quantum yield. A previous study on light stability of lutein and β-carotene solubilized in aqueous solutions showed that the photoreaction quantum yield increased proportionally to the squared root of the oxygen vapor pressure [91]. The study showed that oxygen is necessary for β-carotene photodegradation.

Light stability of colorants in multiphase systems

Technical (real) systems are multiphase systems. These systems may be liquids or solutions with very high concentration of analytes, aqueous dispersions, or emulsions. Multiphase systems may also include opaque or translucent solids, powder inclusions as microcapsules, and systems with a gradient concentration of colorant through the thickness of the system.

The light stability of substances in multiphase systems depends on the interactions between the light and the system containing the absorbing molecule. The total incident light in such a system, is the sum of the absorbed, reflected, scattered and transmitted light. The attenuation of the incident light (a) through the system is the sum of the absorption (A) and scattering (S) of the particles. The absorption depends on the concentration (c) and absorption coefficient (ε) of the chromophores present in the solid for example plus the scattering, multiplied by the length of the solid (z) as shown below [98]:

$$a = (\varepsilon\, c + S)z \qquad (12)$$

The transmission will be the non-absorbed and non-scattered light that is detected in the forward direction. It will depend on the path length. Notice that many substances as encapsulant materials do not absorb visible light. However, the light scattering of these materials will depend on their refractive indexes, which depend on their amorphous chemical structure.

The reflected light depends on the roughness of the surface of a powder or a system. When the roughness is high because of the particle sizes of the system, the reflected light emerges from the powder as diffuse light [99]. This also happens for the backscattered light given the dependent multiple light scatterings of the particles of the powder [100].

Then, the light stability of the coloring agents in a system will depend on all of the described parameters, and how these vary through time or irradiation dose. Systems can be composed of particles. These particles can be in the nanometer range of sizes or be in the micrometer range of sizes (powders and granulates). Yet, a particle in a broader sense, can be on the centimeter range of sizes as the case of candies, and other pieces. When the particles scattering cross section is much bigger than the wavelength of the incident light ($a >> \lambda$, where a is the particle radius [101]), and the refractive index increases, the electromagnetic approach for describing particulate systems is no longer valid [98, 100, 102]. Then, the optical geometry is used for measuring the radiative properties of micron-sized particles and of continuous surfaces [99, 101]. This is because, for a 90° incidence angle, the light will penetrate the particle until it is scattered by any point in the amorphous or crystalline structure of the particle that produces this effect [103].

The absorption of the colorant will decrease with irradiation dose as well as the concentration of the colorant. Thus, this influences the radiative properties of the system through time, generating color changes. Consequently, the degradation of the colorant and the color change in a system can be measured by the different approaches, already mentioned in previous sections, through time. The variation in concentration of the colorant may be measured by spectrophotometry. The color change may be measured by ΔE and $\Delta Chroma\ C_{a,b}$ using the $L^{*}a^{*}b^{*}$ colorimetric parameters. The variation in the radiative properties of the system may be measured by the reflected, scattered and absorbed light using the Kubelka Munk theory [81-83, 99, 104]. These parameters may be measured after certain periods of irradiation to account for the changes through time and dose.

When concentrated solutions (with Absorbance >>1 under standard conditions) are irradiated, all the incident light is absorbed by the liquid. Under this condition, the variation of concentration of the reactive substance may be so small that approximations to an apparent zero order kinetics are observed. This means that the degradation of the molecules will not depend on the initial concentration of the molecule, but rather on its extinction coefficient. In other words, the molecule will degrade at a constant rate [94].

Very little has been studied regarding the kinetics of degradation of colorants or photolabile substances in solid state, which highlights the importance of the present thesis in this regard. In general, the rate of photodegradation of a substance describes the level of sensitivity of a substance towards light-induced reactions like photodegradation or oxidation. This rate of photodegradation, identified as the Quantum Yield, is defined by the "number of degraded molecules in relation to the number of absorbed photons". A study done by Petersen et al. on light sensitivity of annatto in cheddar cheese reported an annatto's quantum yield in solution at 436 nm of 190 μmol bixin/mol photons [105].

Carvalho et al. [106] developed a kinetic model describing the solid state photodegradation of pharmaceutical powders in different conditions. The study used the formalism of the kinetics of degradation of substances in solution phase, assuming first order degradation kinetics. These formalisms were extended to the solid state. This was done under the principle that when photons interact with a molecule, this will react regardless of its location (liquid or solid matrix). The attenuation of light was stated to decrease exponentially through the powder bed following the Beer-Lambert law. Then, the degradation of a substance through time will be proportional to the light intensity, an effective quantum yield and the concentration of the substance. Their results presented photodegradation profiles of a photo-degradant for different exposure times of white light. They used 3mm and 5mm powder beds with different powder densities. Moreover, they showed that the transmittance of an incident white light (30 W/m^2) is negligible for powder beds thicker than 500 μm. In their study, the concentration of the degradant was assumed constant. Moreover, the substrate and the degradant substance were assumed to have the same absorptivity over the irradiated wavelengths. Their analytical solution followed those assumptions [106].

4 Objectives

The aim of the thesis is to generate comprehensive knowledge that makes it possible to expand the applicability of the natural colorant bixin in aqueous continuous-based products and solid products by using technological approaches that makes it possible to satisfy main product requisites during the intended use of the colorant. Based on the reviewed scientific knowledge, the following objectives were stated:

1. Map the morphological diversity of fruits of *Bixa orellana* in La Paz, Bolivia and find specific traits that correlate with higher colorant content in the seeds of samples.

2. Evaluate the encapsulation efficiency of different encapsulants for microencapsulating bixin and annatto aqueous dispersions by spray drying.

3. Evaluate the physical stability of aqueous dispersible bixin and annatto colorant formulations that were obtained through microencapsulation by spray drying.

4. Evaluate how the encapsulation efficiency of the microcapsules affects the light stability of the colorants' microcapsules in solid powdered form and determine the degradation rate of the evaluated systems under artificial visible irradiation conditions.

5. Evaluate the color stability of a product (aqueous continuous solid matrix) containing annatto microencapsulated formulations and understand how this color change relates to the decrease in the colorant concentration in the matrix.

5 Approach and Methods

Morphological characteristics of *Bixa orellana* fruits

Two collecting trips were planned for collecting fruits of *Bixa orellana* from different geographical positions of Los Yungas of La Paz, Bolivia. The visited municipalities were selected based on previous knowledge and experience of places where achiote was cultivated. After the panicles (fruits) were collected, the seeds were threshed and saved in paper bags at ambient temperature (4 – 20 °C). The analysis of the samples was performed one year after sample collection. This is important to mention since degradation of the colorant may be expected. The same degradation through time was assumed for all samples. The same maturity of the seeds was also assumed.

Shape, size and color descriptors were measured in both the frontal plane and lateral plane of the collected fruits (Figure 2). These parameters were used to classify the samples and compare morphological traits with colorant contents in the seeds.

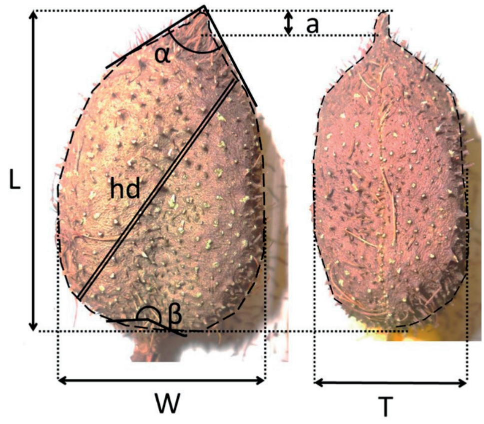


Figure 2: *Morphometric fruit measurements, in frontal (left) and lateral (right) plane of fruits of* Bixa orellana. *L: Length, W: Width, T: Thickness, --- Dashed line denotes the surface of the fruit that was used to calculate its area and perimeter. Hd: Bristle density as the number of bristles intercepting a diagonal line. Alfa (α_a) and beta (β_a) are angles. a: apex length. Note that β_a angles higher than 180° correspond to cordate fruits. In contrast, angles lower than 180° correspond to truncate or attenuate fruit base shape, among others.*

Colorant extraction

During the present study, several extracts were obtained from the annatto seeds to run the experiments with. The first was the extraction and purification of bixin crystals (Paper II and III). The second was a bixin rich extract denominated "Carbohydrate rich bixin, CRB" (Paper II). This extract was composed of bixin and the seed's native carbohydrates (starch and arabinogalactans). CRB was named as "annatto extract" in Paper III. The third one was an annatto extract containing all the components from the seed's aril (Paper I, II and IV).

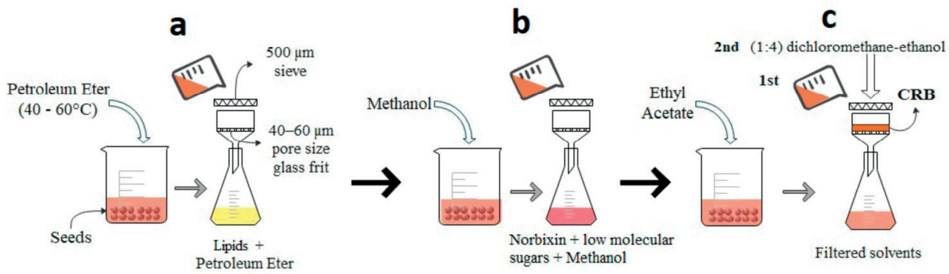


Figure 3: *Extraction process of Carbohydrate rich bixin extract (CRB) using solvents for extraction and further purification.*

The extraction procedure used for obtaining CRB and bixin crystals used a modified version of the method developed by Rios & Mercadante [107] that is based on solvent extraction (Methods 3 and 4 from Table 2). The schematic procedure is presented in Figure 3 and Figure 4, respectively.

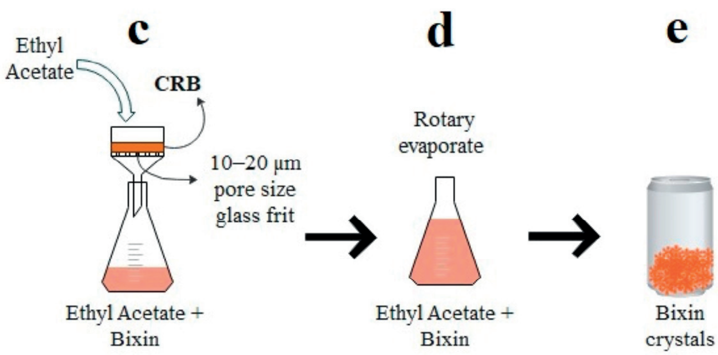


Figure 4: *Schematic extraction process of Carbohydrate rich bixin, CRB and bixin crystals.*

The second extraction procedure presented in this thesis, which uses sodium carbonate to extract the pigment was developed in the present thesis (Paper II) [73] based on extraction with cold mild alkaline solutions (method 6) [24]. Even though it is a result from Paper II, it was presented as a methodology procedure in the same paper. A schematic procedure is presented below (Figure 5):

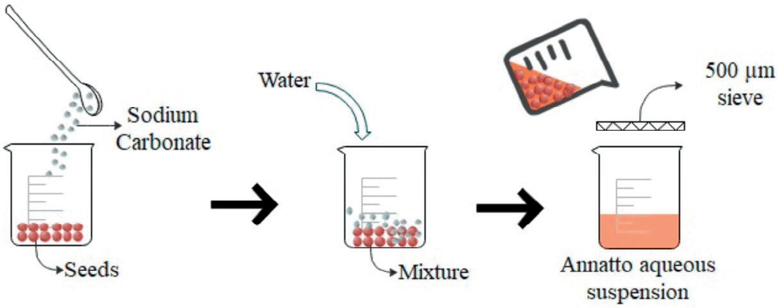


Figure 5: *Annatto mild alkaline aqueous extraction*

Once the annatto aqueous suspensions are obtained, they can be sedimented by adjusting the pH to 3 by adding a few drops of HCl 0.2 N. Alternatively, this suspension can be spray dried directly. The extraction method using mild alkaline conditions makes it possible to obtain an extract with bixin contents around 80% with respect to norbixin. Furthermore, the use of sodium carbonate under the same procedure as shown in Figure 5 allows bixin crystals, CRB and the annatto extracts to be water dispersible. Thus, it is possible to spray dry these dispersions to obtain colorant microcapsules as product formulations that are readily water dispersible.

A Brazilian study [108] showed by HPLC that there was not significant saponification of bixin when extracted with solutions of low concentrations of KOH, <1%, and low temperature. The time did not increase the saponification either (up to 3 hours). On the contrary, at higher concentration of KOH solutions and temperatures above 40°C, the concentration of norbixin increased. In the same way, in a short communication written by the same authors, no norbixin was detected when 0.6% of KOH was used [108, 109]. Therefore, no saponification was expected from the extraction method developed in the present thesis. This was supported in Paper II by taking an HPLC chromatogram of bixin and CRB extracts before and after dispersion with sodium carbonate and microencapsulation of each system.

The CRB or annatto extract used in Paper II and III was selected to study the effect of the native carbohydrates on the protection of bixin and to evaluate their effect on the aqueous dispersibility. Note that this extract contains only bixin as colorant. On the other hand, the annatto extract obtained by mild alkaline extraction for Papers I and IV contained norbixin, the minor phenolic compounds and the native carbohydrates. This extract was selected to see the possibility of spray drying the extract directly obtained from the seeds by aqueous suspension and the feasibility of using this extract on candy formulations.

Microencapsulation by spray drying

The technique selected for the thesis was spray drying. The reason for selecting this technique was the affordability, the low operating costs compared to freeze drying, and higher encapsulation efficiencies reported for this technique. Spray drying uses water as the transporter and is flexible in terms of the variety of wall materials that are possible to use. All the materials used are food grade in comparison to other techniques, such as coacervation and molecular inclusion complexation that use solvents during the process.

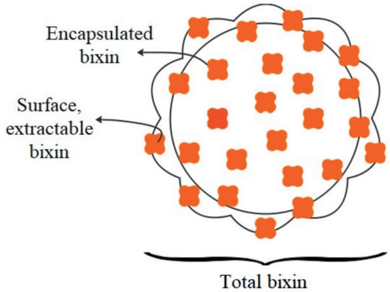
All of the wall materials for spray drying were selected based on the market availability of different substances and the physicochemical properties of these materials. Maltodextrin was selected as an encapsulant because it gives low viscosity solutions at high concentrations. Carboxymethylcelullose (CMC), and gum arabic were selected as encapsulant enhancers due to their film-forming properties. Alternatively, whey was selected because it has been reported to bind strongly with bixin [68, 78]. Sucrose was selected for its light reflecting properties [102].

Two sets of suspensions were used for the encapsulation experiments: Pure Bixin (PB) or Carbohydrate Rich Bixin (CRB) extract. The suspensions were obtained by adding sodium carbonate to the colorants and then water. The suspensions were homogenized for 3 minutes at 15,000 rpm. Then, they were dispersed in a solution containing an encapsulating agent. The encapsulating agents were sucrose and maltodextrin. The enhancing agents were gum arabic, carboxymethylcellulose (CMC), and pectin. The dispersion contained 10% (m/V) of total solids. The final suspension was homogenized for 2 minutes at 15,000 rpm.

Two spray dryers were used for the experiments. The spray-drying experiments were conducted in La Paz for Papers II and III, and in Lund for paper IV. Both equipment used a co-current mode with two-fluid nozzle for atomization. In La Paz, the spray drying inlet temperature was set to 120°C and the outlet temperature stood between 54 and 59°C. In contrast, in Lund, the spray drying inlet temperature was set to 140°C and the outlet temperature was around 90-100°C. The higher difference between inlet and outlet temperature ΔT in La Paz compared to Lund may be due to the lower water boiling temperature in La Paz (86 °C) due to the higher altitude (3600 m a.s.l.). The inlet temperatures were assessed based on previous trials where no water droplets were observed in the chambers prior to spray drying at these temperatures and the spray drier's characteristics. Likewise, these temperatures did not produce caramelization of sugars during spray drying.

Encapsulation Efficiency

The encapsulation efficiency EE (Equation 13) of an encapsulated powder is defined by the fraction of colorant that is covered or protected by the wall material in a capsule with respect to the total amount. The surface colorant S_B is the solvent extractable amount present in the outer layer of the capsule (Figure 6). If the total amount of the colorant T_B is measured, as well as the surface colorant, then the encapsulation efficiency can be calculated using the following equation [57]:



$$EE = \frac{(T_B - S_B)}{T_B} \quad (13)$$

Figure 6: *Schematic representation of Bixin or CRB microcapsule. The orange particles represent the colorant particles encapsulated with a wall material. The solvent extractable bixin is present on the particle's surface.*

The measurement of the total and surface colorant was obtained by spectrophotometry. The solvent used for the extraction of the surface bixin was dichloromethane. The solubility of bixin in dichloromethane was high enough to extract all of the surface colorant present without reaching saturation. Colorant concentrations in the extraction solvent varied from approximately 60 to 800 mg/l. Surface bixin concentrations in the extraction solvent were around 60–400 mg/l.

The encapsulation efficiency depends on the molecular weight of the active component, its polarity, and its volatility. It has been shown that hydrophobic components will present higher encapsulation efficiency than hydrophilic ones. The reason is that polar compounds will diffuse easily in water and will be lost during the evaporation process [110]. In this case, bixin is a hydrophobic compound that was expected to have a relatively higher encapsulation efficiency with respect to polar active components. Then, the colorants were introduced in the spray dryer as solid particles that were denser than the aqueous carrier. Thus, it was expected to generate information regarding the encapsulation efficiency as a function of the particle size of the colorant.

A study on microencapsulation of poorly soluble drugs produced microcapsules of ibuprofen and curcumin using glucan porous particles. During spray drying, bigger atomization droplet sizes gave higher encapsulation efficiencies. The explanation was that bigger droplet sizes presented a higher probability of containing glucan particles [111]. Another study showed that smaller particle sizes of oil emulsions generated microcapsules with higher encapsulation efficiencies for non-volatile oils [112]. Nonetheless, variations in the results were found in literature in relation to particle size and encapsulation efficiency. These variations are attributed to relationships between volatile oil retention, particle size and initial concentration of the spray dryer feed [112].

Physical stability of aqueous dispersions

There are mainly two approaches for gaining an understanding of the stability behavior of aqueous dispersions. The first approach comprises the ageing tests that accelerate the destabilization process under different storage conditions. However, this approach uses equipment that requires very diluted concentrations of the dispersions. The second approach observes the real process of instability of aqueous dispersions as a function of time under desired ambient conditions. Although this might take a longer time, it offers a real measurement of the stability of emulsions and dispersions. This second approach is attained by light scattering measurements using the equipment Turbiscan LAB (Formulaction SAS, L'Union, France). The Turbiscan allows the measurement of the physical instability mechanisms of concentrated and diluted dispersions [113].

The equipment emits a light beam at 800 nm that scans the emulsion or dispersion through the 40 µm length of the sample container to measure the scattered or transmitted light through the sample. The sample container can be divided into three sections – the bottom of the container, the middle section, and the top of the container. In the bottom of the container, sedimentation of particles can be identified. In the middle section, flocculation phenomena can be detected, and in the top of the container it is possible to distinguish creaming processes. For example, the transmitted light over the bottom of the container will decrease when the particles of the dispersion settle. In contrast, light transmittance will increase at the top of the container since fewer particles that scatter light are present (User Guide) [114, 115].

Depending on the concentration of the sample, the equipment can be operated in two different modes: Backscattering and Transmission. Backscattering mode is used for measuring the light scattered by the sample from the incident beam at 180° and 45° from the light source. This mode is used for concentrated samples where the light transmittance is negligible. Transmission mode, on the other hand, is used for clear or turbid dispersions where the light traverses the sample to the detector. The backscattering or the transmittance signals are detected through the whole length (top to bottom) of the sample container every 40 µm (User Guide) [114, 115].

In the case of the samples used for the present study, concentrations of 0.3 mg/ml were prepared. The fast sedimentation of big particles at the beginning of the experiment was evaluated by light transmission mode between 0 and 3 mm. The same mode was then used to evaluate the flocculation that took place in the middle section of the container between 3 and 37 mm.

A decrease in light transmittance between 0 and 3 mm indicates that there is higher concentration of particles in this zone that prevents light from traversing the light path. Thus, less light is detected through the sample. An increase in the light transmittance between 3 and 37 mm indicates that the particle concentration in this zone is decreasing. This could have two meanings: the particles are settling and/or flocculating. Flocculation will produce bigger particles and decrease the probability of light encountering a particle. Thus, more photons reach the detector through the sample [116]. This is true for particle sizes bigger than the wavelength of the light, since the light scattering as a function of size reaches its maximum when the particle size equals the wavelength of the incident light [117].

The evaluation of the dispersion stability is important to determine lifespans of product formulations. In this case, it is important to evaluate the stability of the colorant dispersions at acid pH since most of the liquid products, such as sodas and juices, are formulated at acid pH. The acid pH is important for preservation purposes since it prevents bacterial growth. However, other components as preservatives may be required for the prevention of mold and yeast [118].

Light stability

Bixin visible absorption spectrum bands are between 400 and 550 nm. The most significant absorption is given in the visible range (maxima at 458 nm) where the most light-molecule interactions take place. The aim was to expand the use of the colorant in solid powdered products as cosmetics. Thus, visible light degradation experiments were performed in solid. The degradation of bixin (unprotected colorant) was evaluated as well as the protection given by the encapsulant materials (microcapsules). In addition, the stability of bixin inside a matrix like candies was evaluated. The irradiation experiments were performed using a Plasma Sulphur lamp that had a visible spectral emission of irradiation close to daylight. The samples were placed on a white table 5 meters below the lamp. The degradation experiments were performed under this condition to have the light intensity, temperature and humidity parameters controlled, and thereby, produce reproducible and comparable results.

Having a standardized light intensity (given by the lamp) is important to determine the irradiation dose (light intensity per unit of time) that each sample received. Note that is possible to obtain the same irradiation dose by irradiating a sample five days at certain light intensity or irradiating the sample one day at five times that light intensity. Thus, the lamp allowed for reproducible irradiation doses to the samples. The degradation of the colorant was calculated as a function of the visible range of wavelengths emitted to the samples. This means that contributions at each wavelength were considered instead of average values. Bixin was responsible for light absorption in the studied systems, since the encapsulants and excipients did not present visible light absorption.

The temperature recordings showed temperatures between 48 and 55 °C. However, no degradation due to heat was expected since previous reports have shown that bixin is stable until 70°C [119]. Thus, no cooling systems were employed. The results related to the stability of protected and unprotected colorants show the effects of light at a temperature as high as 55 °C. Thus, the results may be considered useful when applying the colorant in products to be consumed at ambient temperature.

Assessment of colorant in powders

The irradiated powders (for 30 days) were bixin, annatto, spray-dried annatto, bixin microcapsules and annatto microcapsules. Because of the morosity of bixin extraction process, we counted with a limited amount of powder per sample. Samples were placed in uncovered polystyrene flat-bottomed 96 wells plates, where each well had an area of 37.8 mm^2. The approximate sample quantities varied between 1.5 mg for bixin, 3 mg for bixin microcapsules and 4 mg for annatto microcapsules. The complete sample was withdrawn at each time position to be analyzed. This ensured a small margin of error in the analysis of the samples. The small amount of powder in the samples allowed for independent samples at each irradiation period. Moreover, it allowed for two replicates per each irradiation period. This gave a good estimation of the error.

The powders contained about 100 $mmol/m^2$ of bixin. Given the high colorant concentration in the powders, complete absorbance of the light was attained, which translates in the systems being opaque. The powder bed is about 20– 40 µm thick per mg of sample. Then, the total thickness of the powder is about 5– 20 times the average particle size of the microcapsules. This might contribute to the observed scattering of the data, since inhomogeneities in the powder layer may be expected. This is because of the polydispersity of the particle sizes that may produce a rather big variation of the data points. Even though we assume that the powder is statistically homogenous considering the scattering of light, the mentioned aspect limited the interpretability of the results somewhat.

Assessment of colorant in candies

Candies were selected as a translucent system that could contain low colorant concentration. Thus, light stability of annatto could be evaluated in an applied product under more real conditions. Moreover, this system would allow a deeper light penetration through the thickness of the candies. This thickness was about 0.6 cm and the surface area between 0.0004 and 0.0006 m^2. This accounted for the use of geometrical optics and the Radiative Transfer Theory for evaluation of color change in the candies [99].

Candies were prepared containing spray dried annatto, and annatto microcapsules using sucrose or maltodextrin as encapsulating agents. The candies were prepared with three different colorant loads: 3.7, 1.0, and 0.5 $mmol/m^2$ for candies containing A-encap, A-Sucrose and A-MD, respectively. Variation in annatto concentration made it possible to evaluate the effect of concentration with respect to the half-lives and quantum yield of the colorant.

The photolysis reaction of annatto extract in the candies was evaluated every 24 hours for a period of 8 days. The candies were placed in a desiccator to maintain a constant humidity. The desiccator was covered with a quartz glass plate with light transmittance from 225 to 2300 nm. This quartz glass allowed all the incident wavelengths from the lamp to reach the candies.

Having independent candies for each irradiation period made it possible to decrease the analysis error since there was no further manipulation of the candies during the extraction of the colorant. This also made it possible to have at least 7 independent measurements. Furthermore, the three sets of systems can be considered as 3 replicates given the same annatto extract used, and the dissolution of the encapsulants in the candy fudge. Smoother degradation curves were obtained with lower scattering of the data points contrary to the powders.

Analytical monitoring/determination of bixin

Bixin has an absorbance band between 400 and 560 nm. The band has three peaks. These peaks will present a bathochromic shift with respect to hexane (maximum peak at 408 nm) with the increase of solvent polarity. In addition, the extinction coefficient of bixin will vary in intensity. Higher values are obtained for aprotic polar solvents than in aprotic non-polar solvents [56]. The extinction coefficient of bixin measured by a calibration curve in dichloromethane is $\varepsilon = 0.2810\, L/mg \cdot cm^2$ at 468 nm. This value vas used in Paper II to calculate the encapsulation efficiencies.

In Papers I, III and IV, the colorant systems were analyzed in solvent mixtures of methanol: acetonitrile: water (2:2:1). Bixin has a fairly high solubility in medium polar solvents such as methanol. In addition, previous studies have reported HPLC methods for the analysis of annatto using acetonitrile. Thus, these solvents were selected to dissolve bixin. However, the extraction methods developed in this thesis include water. Consequently, the maximum amount of water that could be added to the mixture without producing turbidity in the sample was measured to be 10%.

In-house standards were prepared in this solvent mixture to obtain a calibration curve by UV spectroscopy and by HPLC. From this, an extinction coefficient was obtained. The value at 458 nm is 0.196 $L/mg \cdot cm$ which is equivalent to 7995.9 m^2/mol bixin by transforming the units. This value is basically the same for a solvent mixture of methanol: acetonitrile (1:1).

UV-vis Spectroscopy

The UV-vis spectrophotometry was used for measuring the absorbance spectra of bixin and annatto extracts. This was a straightforward technique for quantifying the colorant concentration and the variation of concentration due to light exposure. The quantification of bixin and annatto extracts was done employing the Beer-Lambert equation (Equation 11) using bixin’s extinction coefficient.

For Paper II, samples were dissolved in dichloromethane. For Papers I and III, the samples were dissolved in a mixture of methanol: acetonitrile: water (2:2:1). In the case of pure bixin, annatto and spray-dried annatto in Paper III, a mixture of methanol: acetonitrile (1:1) was employed. For Paper IV, the colorants were extracted from the candies by means of a solid-liquid extraction process using a mixture of water followed by ethanol. The water-ethanol extract was measured by UV-vis spectroscopy. Given the small colorant concentrations in the candies, the solubility of bixin in the amount of ethanol used was enough to extract all the colorant, while also maintaining a very low dissolution of the matrix of the candy.

High Performance Liquid Chromatography HPLC

The use of HPLC was intended to monitor the individual colorants inside the extract and to evaluate possible degradation products. Furthermore, in Paper I, HPLC was intended to monitor other minor components such as phenolic compounds in the samples collected from the different municipalities in La Paz. The concentration of bixin was obtained by comparing the area of the reference peak with a pure bixin standard. Peaks were monitored at 458 nm for carotenoids (Bixin, norbixin, etc.) and 280 nm for phenolic compounds and other metabolites [62]. All samples were analyzed in an HPLC from Agilent Technologies (Santa Clara, CA). A C-18 column (Purospher STAR RP-18 endcapped (3 µm)) was employed. The solutions prepared for the spectrophotometric measurements were filtered with a 0.22 μm pore size PTPF filter and introduced to the HPLC.

Paper I used a modified version of the method reported by Chisté et al. [5], using acetonitrile as solvent instead of methanol. The samples were run using 0.01% acetic acid aqueous solution (Solvent A) and Acetonitrile (Solvent B) with a gradient flow (A:B) as follows: 70:30 to 40:60 from 0 ' to minute 15 ' (0.8 ml/min), 40:60 to 20:80 from 15 ' to minute 25' ' (0.8 ml/min), 20:80 was kept constant from 25 ' to minute 35 ' (1ml/min), 20:80 to 70:30 from 35 ' to minute 37 ' (1ml/min). A 3-minute equilibrium time was set between runs. Papers II and III used a modified version of the method reported by Rios & Mercadante [107]. The samples were run in isocratic flow of 1 ml/min with a mobile phase of (65:35 v/v) acetonitrile –0.01% acetic acid aqueous solution.

The difference in both methods is that the method by Chisté et al. [5] makes it possible to monitor the phenolic compounds that are detected in the first minutes of the chromatogram. It provides a resolution of the small peaks in the first 10 minutes. The second method by Rios & Mercadante [107] is a method that makes it possible to monitor the major carotenoids of the extract in a shorter time. Thus, a method can be selected based on what compounds are of interest to monitor.

During the light degradation trials of the powders (Paper III), no degradation products were observed by HPLC. This may be due to a small concentration of the degradation products that was not detected by the instrument. The colorant concentration introduced to the instrument was small due to the limited amount of sample used for the irradiation trials. In addition, the sample had to be analyzed both by UV-vis spectroscopy and by HPLC. Thus, the same prepared (dissolved) sample concentration was measured by both techniques. Another explanation is that the degradation products may be colorless, with limited conjugated systems, and smaller volatile molecules that evaporate when formed. It is possible that a combination of both explanations is true.

Digital camera imaging

Digital Image Colorimetry (DIC) consists of measuring the color of samples by taking digital images with a camera or other electronic instruments. DIC is a versatile and inexpensive way of measuring the color of the surface of a food from an image [85, 86].

Pictures of the candies containing the annatto colorant formulations were taken before and after each irradiation period. Pictures were taken using a Nikon D3300 camera with Zoom-Nikkor AF-S 18-55mm / 3.5-5.6 lens. (NY, USA). The pictures were used to measure the color of the candies by measuring the $L^*a^*b^*$ parameters of the pictures of the candies. Monitoring the $L^*a^*b^*$parameters through time, the degradation of the color was measured. This color degradation was compared to the variation of the pigment concentration. Thus, the colorimetric parameters could be compared with concentration values to determine significant differences in color change in the candies in relation to colorant concentrations.

The color of the candies was obtained by measuring the RGB (Red, Green and Blue) parameters using ImageJ software (USA) [120]. These parameters were then converted to the more uniform $L^*a^*b^*$ parameters using ColorMine.org [121]. Then, the Hunter-Scotfield equation (Equation 14) was used for determining the difference in color, as delta ΔE of candies before (0) and after each irradiation period (i) [81]:

$$\Delta E = \sqrt{(a_i - a_0)^2 + (b_i - b_0)^2 + (L_i - L_0)^2} \quad (14)$$

The other calculated parameters were chroma $C_{a,b}$, the quantitative attribute of colorfulness that indicates the intensity of a hue (color) with respect to a gray color, was calculated by Equation 15;

$$\Delta C = \sqrt{(a_i - a_0)^2 + (b_i - b_0)^2} \quad (15)$$

The L*a*b* parameters were measured along the whole surface of the candy, except for the area where light was reflected (observed as a white halo in the candy). The colorimetric parameters were then measured inside a circle that was drawn inside the halo of the candy, and in a circular frame outside the halo of the candy (See Paper IV). Then these two parameters were averaged to determine the final value for each irradiation period. This way, a complete measurement of the variation of the colorimetric parameters was considered and compared to the total concentration of the colorant inside the candy.

For the sensorial analysis tests, the biggest area of the candies (inside the halo) was selected for measuring the $L^*a^*b^*$ parameters, since this area best described the variation in color perception in the candies. The biggest area inside the halo of the candies was selected to measure the color differences in the candies. It is important to state that differences considering the total color variation (inside and outside the halo of the candies) do not significantly change the results. They will only add to the scattering of the data.

The data collected in Paper IV with respect to the colorimetric measurements, is presented as chroma measurements. This is because we wanted to prevent the scattering of the data due to light changes. Nevertheless, there are no important differences between difference in chroma degradation curves and differences in color, ΔE, degradation curves. The standard deviation of L* parameters through time shows that it was relatively constant over time.

6 Main results

The summary of results presented in this thesis comprises the most important outcomes of the research work. These are schematized in Figure 7.

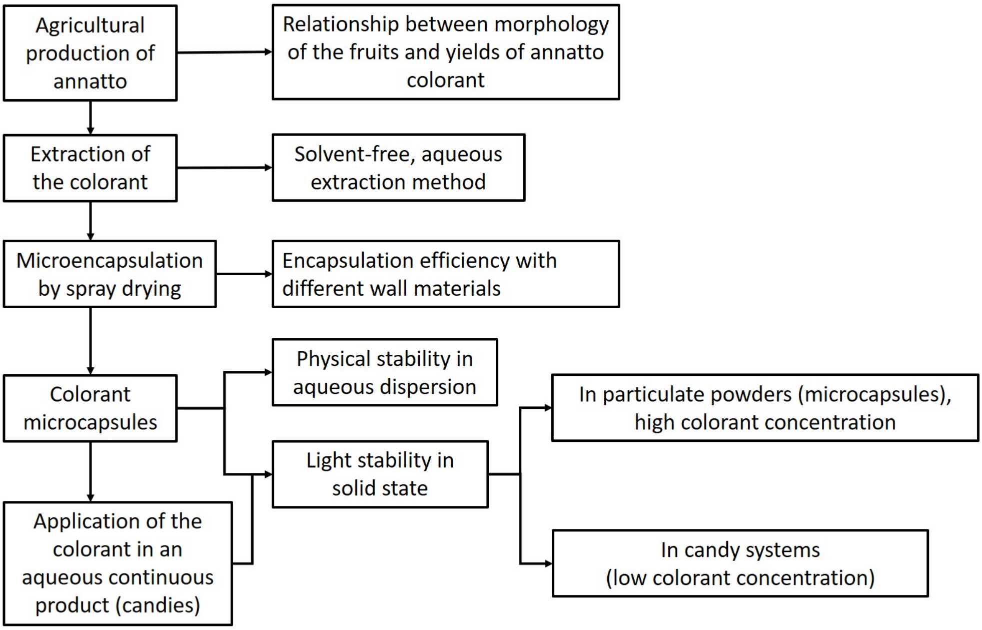


Figure 7: *Diagram of a production process of the annatto colorant. It schematizes the most important outcomes of each of the operational units developed in this thesis.*

Colorant extraction

Bixin can be applied in products as pure bixin crystals or as annatto extracts. Three different types of systems were used in the present thesis for different experiments. The first colorant was pure bixin crystals. The second colorant was the carbohydrate rich bixin "CRB" extract that was purified from the aril of the seeds (an annatto extract). The CRB was composed of bixin and the native carbohydrates of the aril of the seed. The third colorant was an annatto extract obtained directly from the seeds, which was the complete aril of the seeds. This annatto extract was composed of bixin, norbixin, native carbohydrates, phenolic components, and other minor components. Bixin and CRB were used in Paper II and Paper III. The annatto extract (aril of the seeds) was extracted and analyzed in Paper I and was used to give color to the candies in Paper IV.

The mild alkaline extraction method used for extracting the aril of the seeds (annatto extract) was applied in Paper I for analyzing the colorant composition of the aril of the seeds. In addition, the same extraction method was applied to obtain the annatto extract for Paper IV. On the other hand, the use of a mild base as Na_2CO_3 was used to disperse pure bixin crystals and the CRB extract in water after they were extracted and purified by solvent extraction methods (Papers II & III). Thus, the mild base can be used for the aqueous extraction of the annatto extracts and/or dispersing different annatto extracts and bixin in water.

According to the HPLC chromatogram, the bixin peak keeps the same retention time in bixin samples before and after its microencapsulation. To microencapsulate bixin, the mild alkaline colorant suspension was spray dried. Thus, no saponification of bixin is expected by this extraction method. The novelty of the extraction method developed in the present thesis is that it uses a mild base in small concentration and is applied in solid state. This allows for fast extraction of a high number of seeds by agitation with a rod. The pH of an aqueous solution of 0.3 mg/ml of microcapsules ranges from pH 7 to 9.

Relationship between morphology and colorant content

The 27 samples collected from Los Yungas of La Paz showed a high variation in morphological traits of the fruits of *Bixa orellana*. From the 16 morphological parameters measured in each of the samples, the most representative for describing the varieties of the fruits were the length, the width, the acuteness angle, the roundness of the fruit, the flatness, and the bristle density. From the 3 color parameters, the most representative were the color of the base of the fruit presented as a Red/Green ratio in RGB color space, the color of the apex, and the color of the hairs in Red/Green ratio as well. Then, the 4 phytochemical parameters measured in the aril of the seeds were the total colorant content, the bixin content, the norbixin content, a phenolic compound content, and minor components gathered under a same HPLC chromatographic peak. From these parameters, the samples were classified into 3 groups: A, B and C. Each group had subgroups, accounting for a total of 8 subgroups as shown in Figure 8. These 8 subgroups confirm the high phenotypic diversity (large number of morphotypes) of fruits of *Bixa orellana* collected in Los Yungas of La Paz.

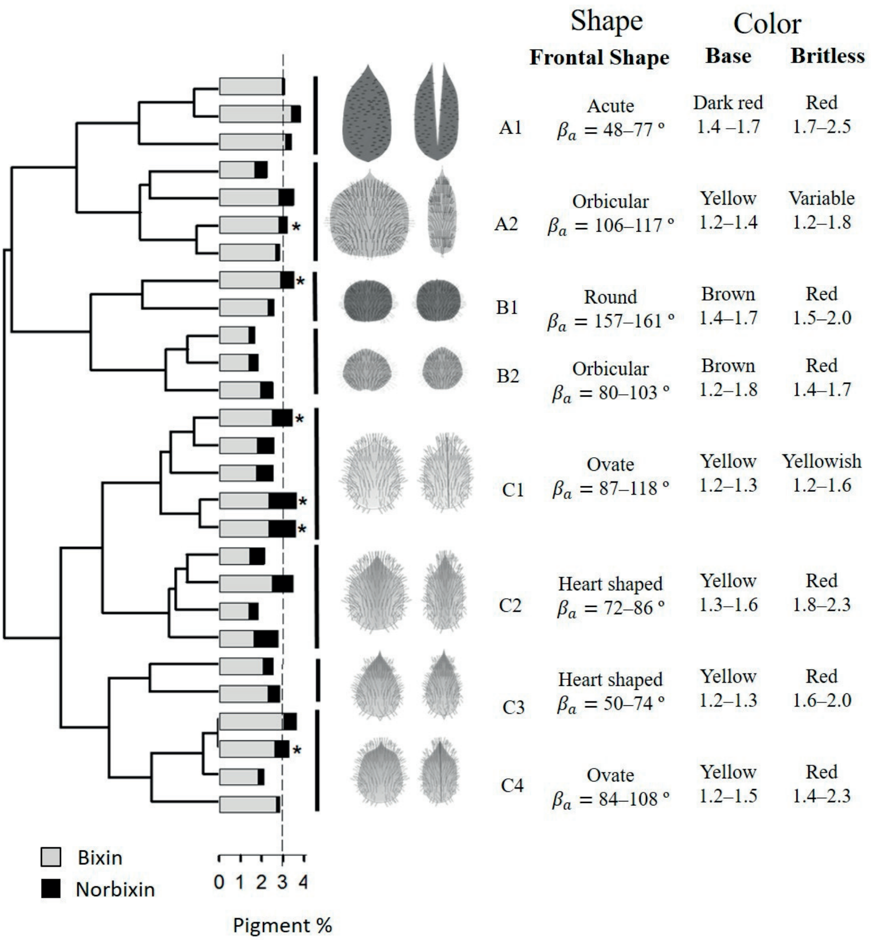


Figure 8: *Morphological and phytochemical characteristics of samples collected in Los Yungas of La Paz, Bolivia. Samples were classified into 3 groups. Group A has 2 subgroups, group B has 2 subgroups and group C has 4 subgroups. The most important characteristics of each group of the fruits are summarized to the right of the figure. The * symbol represents samples of managed cultivars. From Paper I.*

Group A presents two morphotypes A1 and A2. This group has as principal morphological characteristic big fruits with lengths between 4.5 and 5.5 cm. Morphotype A1 fruits are acute and ovate in their frontal and lateral shapes. The fruit is dark red in the base and has reddish bristles. The morphotype A1 fruits are dehiscent. Morphotype A2 has orbicular and flat frontal and lateral shape, respectively. They are yellow in the base of the fruit and the bristles can vary in color from yellow to red. This morphotype has soft bristles. The average total colorant content of Group A is 3.3±0.5%.

Group B presents two morphotypes, B1 and B2. They have fruits with small sizes with lengths between 2.8 and 3.6 cm. Morphotype B1 has spherical fruits with brown colors at the base and red bristles. Its average colorant content is about 3.1 ± 0.6. Morphotype B2 has orbicular and round frontal and lateral shapes. They have the same color as B1 morphotype, but smaller colorant content (2.1 ± 0.4) The sphericity of this group is similar to the shape characteristics of *Bixa urucuana*, an ancestor plant from *Bixa orellana*.

Group C had medium-size fruits, with lengths ranging from 3.6 to 4.4 cm. This group has the highest number of morphotypes, C1, C2, C3, C4. Morphotypes C1 and C4 present ovate fruits and morphotypes C2 and C3 present a heart-shaped frontal plane and an ovate lateral shape. The base of the fruits of this group is yellow. However, the color of the bristles varies. Morphotype C1 has yellow bristles while the rest of the morphotypes have red bristles. Morphotypes C1, C3 and C4 have an average colorant content of 3%.

The morphological parameters that had a correlation with the colorant content were the width (R^2= 0.16, $F_{1,25}$ =4.61= P= 0.0416) and the length length (R^2= 0.10, P= 0.1002) of the fruits. The results show that the fruits that are wider may contain higher amounts of colorant. Fruits within the Group C showed that the yellower the color of the base of the fruit (C1 & C2), the higher content of norbixin. Within the Group C, which accounts for 15 samples, results showed that more cordate fruits tend to have higher colorant contents.

The average mass balance shows that the average content in the 27 samples was 3.0% total colorant content (2.4 Bixin and 0.6 Norbixin). The dry aril of the seed containing the colorants and native carbohydrates accounts for 10% of total weight. The dry colorless seeds represent 82%, and 5% corresponds to the moisture content with respect to the total weight of the seeds. A very important result shows that well-managed cultivars had higher bixin content (>3%) regardless of the group variety.

Microencapsulation by spray drying

Microencapsulation by spray drying is an effective technique for microencapsulating a suspension of bixin particles, CRB particles and annatto particles. Bixin particles can be encapsulated with macromolecules as maltodextrin (MD) and whey with encapsulation efficiencies of 77% and 62%, respectively (Figure 9). CRB particles containing bixin and the native carbohydrates can be also efficiently encapsulated by spray drying. The results show that encapsulating agents as MD, whey, and Sucrose offer encapsulation efficiencies above 90%, except for whey (86%). This means that over 90% of bixin will be protected inside the microcapsules and is not solvent extractable from the surface of the microcapsules.

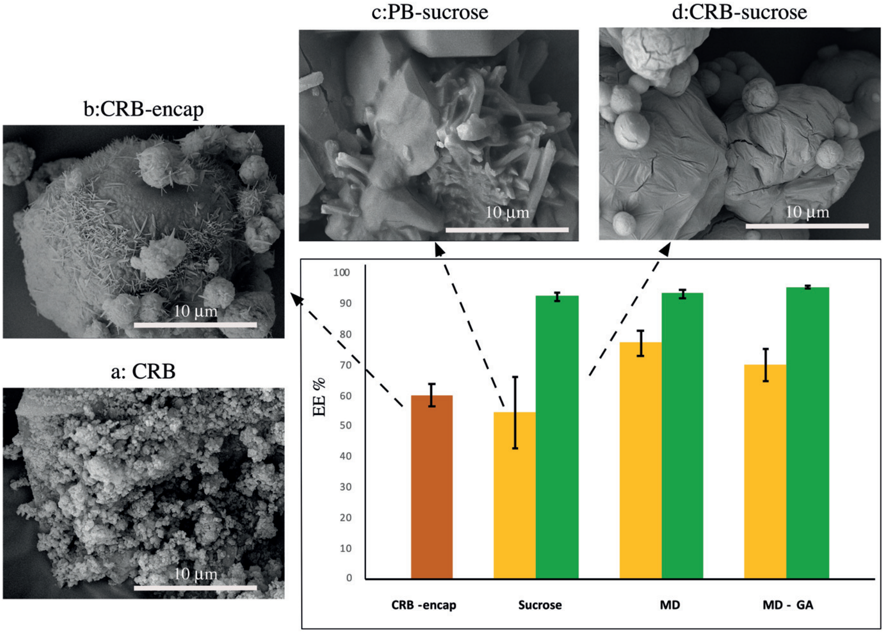


Figure 9: *Encapsulation efficiency of colorant particles that have comparable particle sizes. The figure shows encapsulation efficiencies of colorant particles encapsulated with native carbohydrates (CRB-encap, $D_{3,4}$ = 7.7 µm), sucrose ($D_{3,4}$ ~ 7 µm), maltodextrin (MD, $D_{3,4}$ ~ 4.4 µm) and a mixture of maltodextrin and gum arabic (MD-GA, $D_{3,4}$ ~ 4.4 µm). Bixin particles (yellow) and CRB particles (green). Higher encapsulation efficiencies are obtained for colorant particles containing the native carbohydrates (CRB systems). From Paper II.*

Enhancers like GA, pectin and CMC offer very little improvement to the encapsulation of pure bixin and CRB colorant particles. However, the big difference in encapsulation efficiencies between CRB systems and pure bixin shows that the presence of the native carbohydrates offers higher encapsulation efficiencies to bixin. This is true even for particles of the same average volume fraction diameter as in the case of B-MD and CRB-MD, and B-MD-GA and CRB-MD-GA (particle sizes ~4 µm).

Scanning electron microscopy images were obtained for CRB and for the spray-dried systems. An interesting case is the encapsulation of colorant with sucrose. Figure 9 shows the extracted and purified CRB before (9a) and after (9b) spray drying. The CRB before spray drying is composed of small particles (~1 µm) agglomerated into amorphous bigger aggregates ($D_{4,3}$~70 µm). After spray drying, the native carbohydrates act as an encapsulating agent. However, the relation of the amount of native carbohydrates to the amount of bixin after extraction was not high enough to reach a high encapsulation efficiency. As can be observed, bixin crystals are not encapsulated at the surface of the capsule.

Figure 9c and Figure 9d show the comparison between pure bixin crystals encapsulated with sucrose (9c) and CRB encapsulated with sucrose (9d). Sucrose crystallizes along with pure bixin, producing a low encapsulation efficiency of Bixin. This means that bixin is highly available for solvent extraction due to extensive fractures and a loss of capsular structure. In the case of CRB-sucrose microcapsules, an encapsulation efficiency of 92% was achieved. Figure 8d shows smooth microcapsules with no bixin crystals on the surface. It was concluded that sugar acts as an interacting filler [122] with the native carbohydrates to give an efficient spherical structure that protects bixin.

Dispersion stability

The dispersion of bixin crystals and CRB extract in acid aqueous media (pH = 3) was evaluated by means of a light scattering method using the equipment Turbiscan through time. The method consisted of scanning the transmittance of the samples over the length of the cells. The difference in transmittance between scans showed sedimentation of the particles and flocculation. Sedimentation was observed in the bottom of the cells, while flocculation, which led to further sedimentation, took place in the middle region of the cell.

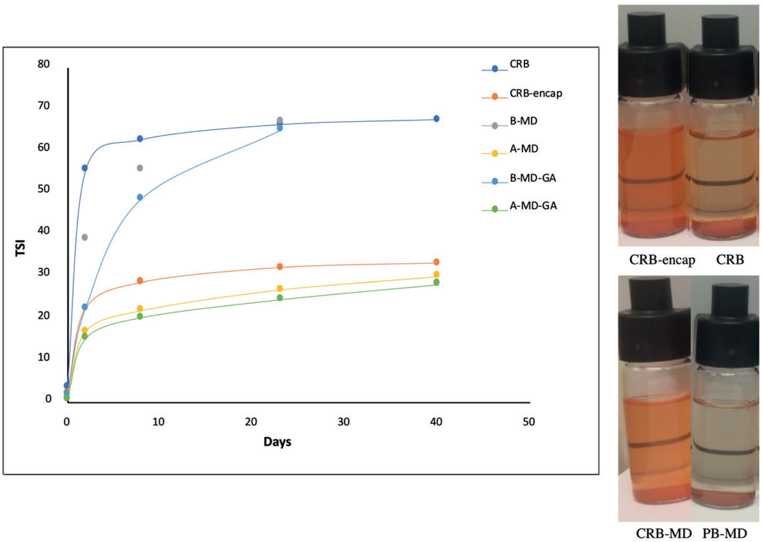


Figure 10: *Physical stability of bixin and annatto (CRB) colorant particles aqueous dispersions obtained by dissolving the colorants' microcapsules. Turbiscan Stability Index is graphed as a function of time (Days). Higher indexes are observed for bixin particles compared to annatto (CRB) particles for the same particle sizes. The systems presented are: Annatto (A), A-encap, B-MD, B-MD-GA, A-MD and A-MD-GA in 0.3 mg/ml. From Paper II.*

Furthermore, the sedimentation and/or destabilization profiles were analyzed to obtain a Turbiscan Stability Index (TSI). Figure 10 shows the Turbiscan Stability Indexes (TSI) of dissolved microcapsules of bixin crystals and CRB extract that were microencapsulated with two encapsulants: maltodextrin, and a mixture of maltodextrin and gum arabic. Both colorants had similar volume average particle diameters, 4.23 µm for B-MD and B-MD-GA, 4.54 µm for CRB-MD and 4.78 µm for CRB-MD- GA. The results show, that for the same particle sizes, CRB colorant particles had lower TSI values (higher physical stability) than bixin particles through the 40 days. Since the TSI values indicate an instant speed at which the particles destabilize, every measurement done shows that the sedimentation rate for bixin particles was higher than for CRB particles.

This strongly suggests that the native carbohydrates of the aril of the seeds play a major role in stabilizing the colorant particles in aqueous media. The zeta potential for these dispersions had a value of – 15 mV. This value is not high enough to prevent complete sedimentation; however, it illustrates a significant stability of CRB particles after 40 days. After this time, the presence of small CRB particles ($D_{3,4} = 0.3\ \mu m$) were still detected, which kept the orange color of the dispersions. In contrast, no bixin particles were detected after 40 days for dissolved bixin microcapsules.

The microcapsules are not only an efficient way of obtaining water dispersible formulations, but they are also intended to protect the colorant against light degradation. Thus, for describing the degradation process of the colorants, or in general of photolabile substances, a kinetic model was stablished. This model describes the decrease in colorant concentration as a function of the irradiation dose. The considerations of the model are that the powder bed can be described as colorant inclusions inside the microcapsules. This powder is analyzed as the integration over a group of layers that conforms the whole system for which their irradiating properties are considered. These properties include: the absorption, scattering and transmittance of the powder bed. Moreover, the scattering comprises the diffusive reflection and the scattering at every angle of the particles in the powder bed.

Conceptual model for light stability of photolabile substances in solids

The spray-dried powder bed is comprised of particles that absorb light due to the bixin pigment in the region of the blue-green light. On the other hand, the candies may be considered as a layer where the annatto colorant particles are dispersed. Given that the particles of the powders are, on average, >>10 times higher than the average wavelength that impinges the powder bed, optical geometrics are used to describe both systems. The degradation of a photolabile substance in a bed of a solid matrix exposed to light has been recently analyzed by Carvalho et al. [106] and our results are comparable to theirs. The kinetic model reported in Paper III describes the degradation of a photolabile substance in a powder bed. The sample is described as a powder bed with total depth Z counted downwards from the surface of the powder bed (z axis), as show in Figure 11. The incident lamp light has an intensity $I_q(z,t)$ that decreases over the path length of the powder bed depending on the powder's absorption and scattering characteristics. Moreover, the colorant load $B(\mathrm{t})$ was modeled to decrease through the depth (z) of the powder bed as a function of the total irradiation dose Q. The total bixin load $B(t)$ is the total amount of colorant in the sample used for the irradiation experiments.

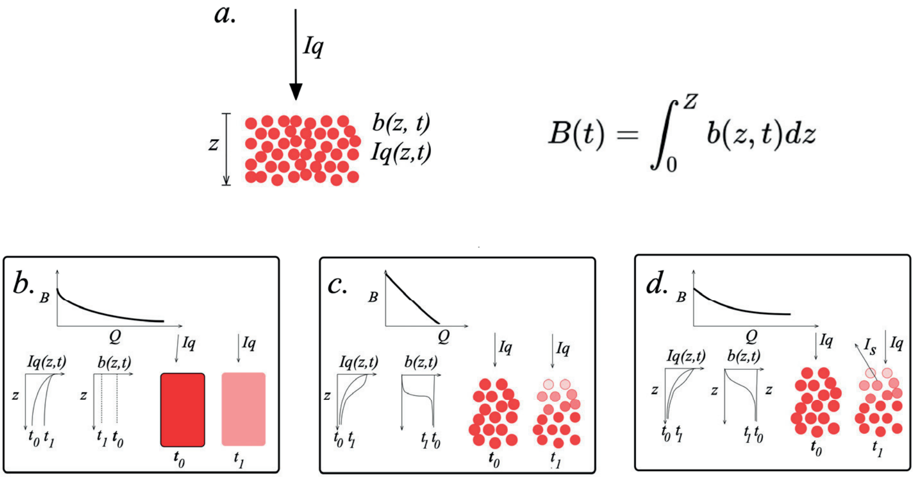


__Figure 11:__ Photobleaching modeling on powder bed microcapsules. Optical geometrics are used to describe the absorption and scattering characteristics of the powder beds under a total irradiation dose. __a)__ Iq is the light intensity of the lamp, and z axis describes the depth of the powder bed. The total Bixin surface load B(t) is the integration of the local concentration of bixin b(z,t) over the depth of the powder bed. B(t) decreases with the total irradiation dose Q. __b)__ The graphs show exponential degradation of bixin B(t) in a liquid exposed to a radiation dose Q; local concentration b(z,t) as a function of depth (z) and time (t); and the local light intensity Iq as a function of depth at time t(0) and t(1) (Model 1). __c)__ Linear decay of the bixin load as a function of irradiating dose, with only absorbance and no light scattering of the powder bed (Model 2). __d)__ Exponential degradation of bixin load as a function of the irradiation dose, with absorbance and light scattering of the powder bed (Model 3). From Paper III.

The total light that impinges a multiphase solid system, as the powders or the candies, is divided into reflected, absorbed, scattered, and transmitted light. In the present thesis, the reflected light is included in the scattering parameter as backscattered light. The light will be absorbed by the chromophores present in the solid multiphase system (bixin and annatto colorants) as a function of the colorant's absorptivity though wavelength (eq. 16). The transmitted light will be given by equation 17. Then, the attenuated light equals the non-transmitted light as shown in equation 18. The attenuated light equals the absorbed (Eq. 19) and scattered light (eq. 20) inside the system.

$$A(\lambda, t) = \int_{z=0}^{Z} \big(\epsilon(\lambda) \cdot b(t, z)\big) dz \qquad (16)$$

$$\frac{I_T(\lambda,t)}{I_q(\lambda)} = 10^{-(A(\lambda,t)+Z\cdot S)} \qquad (17)$$

$$\frac{I_a(\lambda,t)}{I_q(\lambda)} = 1 - 10^{-(A(\lambda,t)+Z\cdot S)} \qquad (18)$$

$$\frac{I_A(\lambda,t)}{I_q(\lambda)} = \rho_A(\lambda, t) \qquad (19)$$

$$\frac{I_S}{I_q(\lambda)} = \rho_S(\lambda, t) \qquad (20)$$

Here, A refers to the absorbance of the colorant in the solid system, $\epsilon(\lambda)$ refers to the absorptivity of the colorant, $b(t, z)$ is the local bixin load through the thickness Z of the powder bed. S is the scattering of the powder bed per length unit, I_T, I_a, I_A, I_S, I_q are the transmitted, attenuated, absorbed, scattered and incident light. The numerical solution of the modeling in Paper III gives the absorbed fraction of light $\rho_A(\lambda, t)$ as a function of wavelength and time and thus, the scattered fraction of light $\rho_S(\lambda, t)$. Notice that equation 20 can be approximately solved when there is a constant concentration of the colorant through the thickness of the layer by the Kubelka Munk theory (equation 10).

In Paper III, the modeling considered an average absorptivity of bixin over the spectra. In contrast, in Paper IV, bixin's degradation was modeled through the whole spectra to be able to model the change in color of the candy based on bixin's UV-vis spectra. In the case of the experimental calculations for powders and candies, the calculations of the degradation process were performed using the absorptivity of bixin at every wavelength of its spectra (340nm to 650 nm).

For the modeling of the colorant photobleaching in Paper III and IV, the load of bixin was modeled as a function of total irradiation dose that reaches the sample, considering different scattering parameters. On the other hand, the calculations of the degradation process, based on the experimental data for powders and candies, were performed as a function of the absorbed photons. The absorbed photons were required to determine the quantum yield of the colorant in each system. Equation 21 shows the attenuated photons $Q(t)$ through the multiphase system (powder or candy). When light scattering is negligible, the attenuated photons become proximate to the absorbed photons. This is the case at the beginning of the system's irradiation for high colorant loads (where the quantum yield was measured).

Moreover, the colorant loads used in the experiments in powders and candies were high enough to prevent blue-green light transmittance. In the same way, the scattering of the systems turned out to prevent light transmittance.

$$Q(t) = \sum_{t=0}^{t=t} \sum_{\lambda=340}^{\lambda=650} I_q(\lambda) \cdot \left(1 - 10^{-B(t)\cdot\epsilon(\lambda)+Z\cdot S}\right) \cdot \Delta t \qquad (21)$$

The absorbed accumulated light dose per area unit is $Q(t)$ when the term $Z \cdot S$ is negligible. B is the total surface load of bixin (total bixin content per area unit $mmol/m^2$).

For powder beds (Paper III), given a high colorant concentration $B(t) = \sum b_j(t)\Delta z$ and $B(0) > 20\ mmol/m^2$, and the thickness of the powder bed Z ~$0.3 - 1\ mm$, depending on the powder density, the transmittance of light is negligible. By accounting for the powder density, the colorant load can be employed per distance unit. Then, the shape of the degradation curves of the studied systems depended on the absorption and scattering properties of the systems. In the case of the candies, the modeling in Paper IV shows that low colorant loads $B < 1\ mmol/m^2$ may produce light transmittance and a decrease in the absorption curve of bixin. After irradiation, the non-absorbed light may increase in the range of wavelengths where the colorant absorbs light (blue-green light). However, the non-absorbed light may be attenuated in the matrix of the candy by light scattering, impeding the light transmittance. For these cases, the absorption curve has an exponential like curve (initial linear behavior) that depends on its colorant load. In the irradiation experiments, the lowest colorant concentration in the candies was about 0.6 $mmol/m^2$. This concentration allowed for almost total absorption of the light in the blue-green region. Thus, negligible light transmittance was obtained in this range according to the modeling results. After color degradation, both light scattering and light transmittance increase significantly.

When there is no transmitted light, all the incident light will be attenuated in the powder. For higher doses where the scattering becomes important, the $Z \cdot S$ parameters was still neglected for the experimental calculations assuming that $Q(t)$ is the absorbed photons. This is a pessimistic approach since it is assumed that lower irradiation dose is needed for degradation. It is assumed that the remaining colorant after each irradiation period will absorb all the photons, although the light scattering is present offering higher protection to the colorant against photobleaching.

The mathematical modeling and their formalisms presented in Paper III and IV resulted in 3 scenarios based on the characteristics of the irradiated systems: Model 1, where the system is liquid or and there are no concentration gradients (the concentration is very low and homogeneous because it equilibrates between each time step), there is no light scattering, and there is light transmittance; Model 2, where the system is solid, and there are concentration gradients through the powder depth, but no light scattering is present; and Model 3, where the system presents both concentration gradients through the powder depth, scattering, and no light transmittance (see Figure 11).

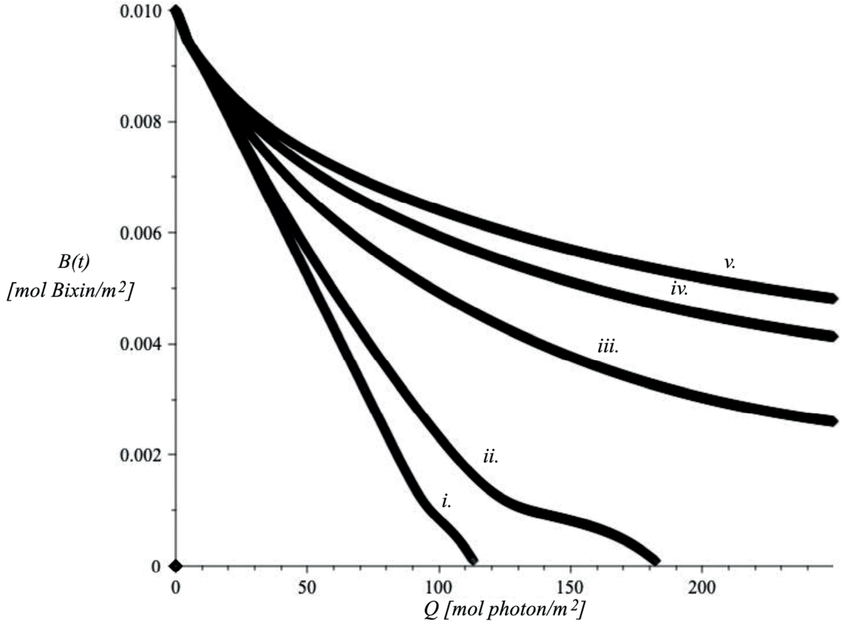


Figure 12: *Total content of bixin,* $B(t) = [mol\ Bixin/m^2]$*, in a sample as a function of accumulated total exposure,* $Q = [mol\ photons/m^2]$*. The slope of the curve corresponds to bixin quantum yield, which is the same in all curves,* $Y = 10^{-4}$*. The curves are constructed based on the numerical solution of the modeled equations in paper III. In graph the scattering of the full sample, S, is varied.* $\boldsymbol{i}.\ \hat{S} = 0$ *(no light scattering is assumed, Model 2),* $\boldsymbol{ii}.\ \hat{S} = 0.3$ $\boldsymbol{iii}.\ \hat{S} = 1.5$*,* $\boldsymbol{iv}.\ \hat{S} = 3$*,* $\boldsymbol{v}.\ \hat{S} = 9$ *(***ii.***,* $\boldsymbol{iii}., \boldsymbol{iv}., \boldsymbol{v}.$ *corresponds to Model 3,* $\hat{S} = S \cdot Z$*. The total bixin load* $B(0) = 10\,mmol/m^2$ *or* $4\ g/m^2$ *for a powder bed of 70 g/m²and about* 164 μm thickness. *From Paper III.*

Model 1 leads to the well-known exponential decay of the colorant with time, Model 2 leads to a linear decay as a function of the irradiation dose. In this case, the light is absorbed by the powder bed given a high concentration of absorbent, and/or the powder bed is thick enough, and no light scattering is observed. The slope is a direct measurement of the quantum yield of the photoreaction of bixin in the powder bed. The third model (Model 3) leads to an initial linear decay that is a direct measurement of the quantum yield, followed by a decrease in the slope, which is produced by the scattering effects of the powder bed. This happens when the absorption of the powder decreases with the decay in the colorant concentration. Thus, the light scattering of the powder becomes significant, decreasing the sensitivity of the remaining colorant towards light degradation (Figure 12).

Degradation curves of colorants in powders and candies

The systems can be classified as powder beds with a high load of colorant particles and candy systems with a low colorant load. Within the powders there are three groups: unprotected colorants, bixin microcapsules and annatto microcapsules (Figure 13).

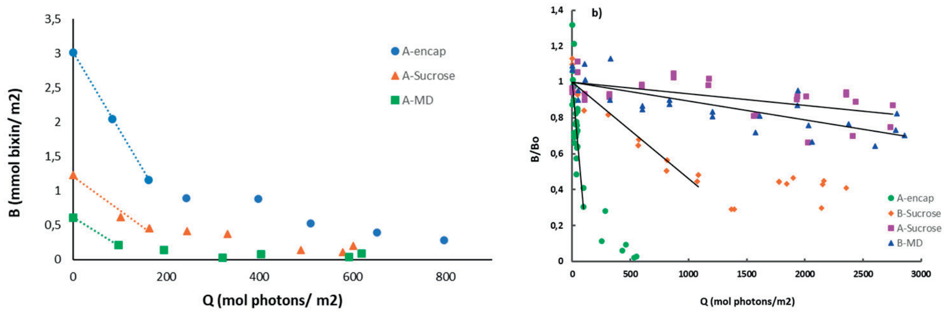


Figure 13: *a) Light degradation of colorant microcapsules applied in candies. The graphs present bixin fraction B/B(0) as a function of absorbed irradiation dose* Q $(mmol\,photons/m^2)$. *Extracted from Paper IV. b) Light degradation curves of the powder beds of unprotected colorants and colorant microcapsules. Extracted from Paper III and IV.*

Among the powdered systems, the unprotected colorants Bixin, Annatto and spray-dried Annatto (A-encap) presented fast degradation. At the beginning of the degradation process, Bixin and A-encap systems followed a linear curve (Figure 13b). Then, at about 20% of the remaining colorant ($400\,mol\,photons/m^2$ of irradiation dose), a decrease in the rate of colorant degradation was observed. This behavior is explained by Model 3 presented in the previous section. There, the light scattering of the powder bed becomes significant in protecting the color in some extent. Another explanation may be an increase in light transmittance. Thus, a slower degradation rate is observed. Annatto followed a mostly linear degradation curve (Model 2) until total degradation, which indicates no light scattering effects in the powder were present (Figure 13b).

The same degradation behavior as Model 3 is observed for B-Sucrose. Bixin spray dried with sucrose gave a poor encapsulation efficiency. As can be observed in Figure 8. The degradation of bixin is linear until about 50% of the remaining colorant ($1000\,mol\,photons/m^2$). Then, there is a decrease in the degradation rate. Hence, the remaining colorant concentration becomes constant up to 2500 $mol\,photons/m^2$. In this system, strong scattering effects are observed given by sucrose (Figure 13b).

Microencapsulated systems such as bixin microcapsules and annatto microcapsules followed a linear degradation process (Model 2) except for P-Whey microcapsules, which followed a Model 3 type of degradation curve. For P-Whey microcapsules a bend in the curve is observed at about $2000\ mol\ photons/m^2$ of irradiation dose. Systems that followed Model 2, such as A-Sucrose, A-MD and B-MD, had a linear degradation curve up to $3000\ mol\ photons/m^2$ (Figure 13b). The linearity of these systems is explained by the small degradation rate compared to unprotected colorants, and the high colorant load present in the powder. It is possible that when the colorant degradation progresses, the light scattering effects of the encapsulants will produce a bend in the curve as in Model 3 for P-Whey. Note that whey had a lower encapsulation efficiency compared to the other microencapsulated systems.

The same linear degradation behavior is observed at the beginning of the degradation experiments for annatto microcapsules dispersed in candies. Figure 13a also shows the deviation from linear behavior, for the three systems (candies containing A-encap, A-Sucrose and A-MD) caused by the light scattering effects in the candies after $100\ mol\ photons/m^2$ (Model 3). The three types of candies contained different colorant concentrations. Figure 13a shows that, with degradation rates being similar, a lower colorant concentration will result in the colorant degrading in a shorter time (absorbed irradiation dose). This is the case for A-Sucrose and A-encap for example. The results from the modeling in Paper IV, confirmed the initial linear degradation of the colored candies, followed by a bending in the curve when light scattering effects become important. The modeling also shows that for the same degradation rate, candies with lower colorant concentration will degrade with a lower total irradiation dose.

Quantum yields

The quantum yields of the different systems are compared in Figure 14. The error bars observed were calculated from the standard deviation of the slope. This figure presents the quantum yields of unprotected colorants as Bixin (B), Annatto (A) (native carbohydrate rich bixin extract) and spray dried annatto (A-encap), denoted by the blue markers; it presents bixin microcapsules as B-sucrose, B-whey, B-MD, B-MD-GA and B-MD-CMC denoted by the orange markers; the annatto microcapsules as A-Sucrose, A-Whey, A-MD, A-MD-GA, A-MD-Pectin, and A-MD-CMC, denoted by the green markers; and finally presents the quantum yields of annatto microcapsules A-encap, A-Sucrose and A-MD applied to candy systems.

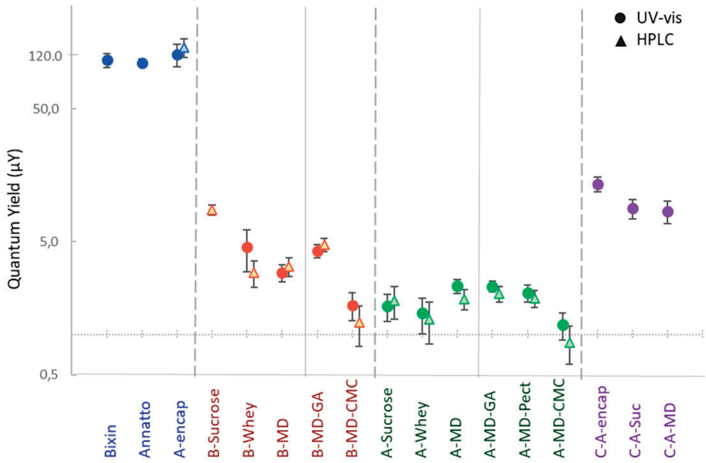


Figure 14: *Quantum yields of unprotected colorants Bixin (B), Annatto (A) and spray-dried Annatto (A-encap) in solid state (blue markers), as well as quantum yields of bixin microcapsules (orange markers) and annatto microcapsules (green markers). Purple dots are quantum yields of A-encap, A-Sucrose and A-MD dispersed in candy (C) matrix. The error bars observed were calculated from the standard deviation of the slope of the degradation process of each system. Degradation processes monitored by UV-vis spectroscopy are denoted by circles and processes monitored by HPLC are denoted by triangles. The y axis is presented in logarithmic scale. From Papers III and IV.*

The unprotected colorants Bixin, Annatto and A-encap have similar quantum yields (Figure 14). This means that they have similar degradation rates around $120\ \mu mol\ bixin/mol\ photons$. This range of results is comparable to what has been previously observed for carotenoids in solution [91]. The values obtained were 40 µmol carotenoid/mol quanta for β-carotene and $30\ \mu mol\ carotenoid/mol\ quanta$ for lutein in aqueous solutions [91]. A study by Petersen et al. on light sensitivity of annatto in cheddar cheese reported an annatto's quantum yield in solution at 436 nm of $190\ \mu mol\ bixin/mol\ photons$ [105]. These values are comparable to the ones obtained in the present thesis.

Experiments with protected colorants as bixin microcapsules and annatto microcapsules show that microencapsulation is an efficient technique for preventing light degradation in colorants such as bixin and annatto. Microencapsulation offers 30 to 100 times higher colorant stability against light degradation. Quantum yields for bixin microcapsules were found to have values between 2 and 9 $\mu mol\ bixin/mol\ photons$. Annatto microcapsules have quantum yields between 1 and 2 $\mu mol\ bixin/mol\ photons$ (Figure 14).

Microcapsules containing native carbohydrates are less sensitive to light degradation than microcapsules containing pure bixin. Materials as sucrose, maltodextrin, and mixtures with enhancers like GA, pectin and CMC have proven to be efficient encapsulating agents. Furthermore, these encapsulating agents have proven to offer protection to the colorants against light degradation. This is probably due to the ability of these materials to block the interaction of the colorants with oxygen. In this sense, oxidation reactions mediated or not by light are impeded. CMC has proven to be a better microencapsulation enhancer than GA and pectin, offering better protection to the colorants bixin and annatto against light degradation.

Measured quantum yields of A-encap, A-Sucrose and A-MD dispersed in candy matrix were found to be close to 13, 9 and 8 $\mu mol\ bixin/mol\ photons$, respectively (Figure 14). The smaller quantum yield of these colorants added to the candies compared to unprotected colorants can be explained by the protection offered by the candy matrix. The candy matrix is composed of sugars that cover the colorant particles. The protection mechanism may have to do with the prevention of oxidation reactions as mentioned before.

Relationship between the quantum yield and the encapsulation efficiency

The average particle diameter of the colorant particles (Bixin and Annatto systems) was measured by light scattering techniques. Moreover, the encapsulation efficiency of these colorant particles microencapsulated with different wall materials was determined by spectrophotometric techniques. The colorant present in the surface of the microcapsules was dissolved with solvents and measured by UV-vis spectrophotometry. This amount was then compared to the total amount of colorant measured in the capsule. This encapsulation efficiency indicated the fraction of colorant that remains inside the microcapsules and is assumed to be protected. Thus, the higher the encapsulation efficiency, the higher the expected protection of the colorant. This hypothesis was proven by comparing the quantum yield of the different colorant microcapsules against its encapsulation efficiency.

The results show that there is a linear relation between the quantum yield and the encapsulation efficiency of the microcapsules by Fisher's test $\left(F_{1,9} = 18.6, P < 0.05\ and\ R^2 = 0.67\right)$. The quantum yield will decrease by increasing the encapsulation efficiency of the colorant particles (Figure 15). This proves that the sensibility of the colorant particles against light degradation is smaller as the encapsulation efficiency increases. Furthermore, the encapsulation efficiency has a higher impact on the protection of the colorant than the variation in the particle size of the colorants. Note in Figure 15 that higher particle sizes do not imply higher stability of the colorants and vice versa.

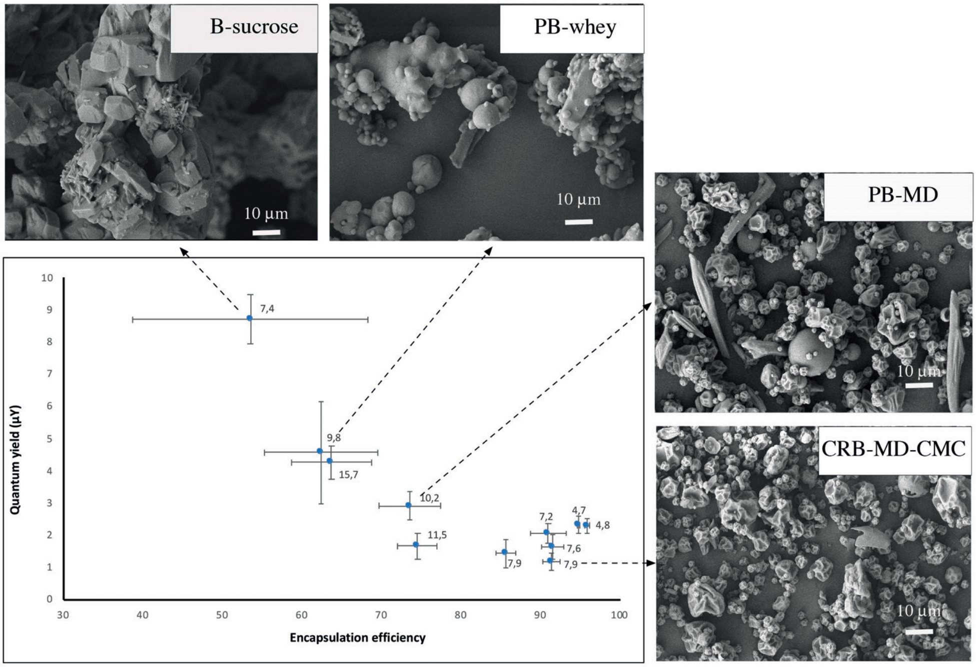


Figure 15: *Quantum yield of bixin and annatto microcapsules as a function of their microencapsulation efficiency EE, obtained from Curi-Borda et al. (20). A significant correlation with* $F2,9 = 18.6$*,* $P < 0.05$ *and* $R^2 = 0.67$ *is observed. The label subscripts are the sample's colorant particle size diameter* $D_{4,3}$*. Error bars of the quantum yield represent the standard deviation of the slope of each degradation process. Error bars for the encapsulation efficiency represent the standard deviation of several measurements of EE. Extracted from Paper II and III.*

Differences in color in the candies colored with annatto formulations

Differences in color have been classified as small differences for $\Delta E < 1.5$, distinct color intensities in the range of $1.5 < \Delta E < 3$, and very distinct differences for $\Delta E > 3$. Moreover, color differences noticeable through visual perception were found for differences in color of $\Delta E > 2$ in many studied products (37). The results obtained from the sensorial analysis performed in Paper IV showed that noticeable color differences in the pictures of the candies were perceived for $\Delta E > 4$. Pairs of candies with $\Delta E > 4$ were the ones that contained a difference in color concentration between candies of double or quintuple times, as in the case of A-Sucrose with A-MD and A-encap and A-MD, respectively. However, at higher colorant loads, as for A-encap (3 $mmol/m^2$) and A-Sucrose (1.3 $mmol/m^2$), the differences obtained below $\Delta E > 4$

may not be perceived. This may be explained by the absorption bands obtained by the modelling results in Paper IV. For colorant loads above 1 $mmol/m^2$, the intensity of the absorbed light curve remains constant, which indicates the nonlinearity of the color as a function of colorant concentration. Thus, similar intensities in color may be obtained, and thus no perceivable color differences. The nonlinearity of color has been previously reported for different industrial formulations that difficulted a perceivable differentiation of color intensities [81, 123-125].

The variation in color in the irradiated candies after $60\,mol\,photons/m^2$ (equivalent to an irradiation of 24 hours in southern Europe) produces a color difference with a ΔE = 8 (Figure 16). This means that the candies colored with annatto formulations may be irradiated for several hours of intense irradiation before the difference in color becomes visually perceivable during the product's consumption. However, it is recommended that candies colored with annatto formulations be packed in opaque packaging materials to prevent color degradation in the candies during the shelf life of the product.

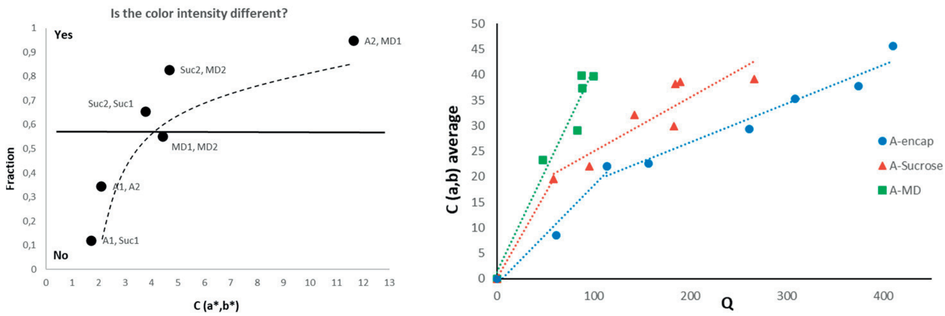


Figure 16: *a) Fraction of panelits who perceived a color difference between pairs of candy systems containing A-encap, A-Sucrose and A-MD as coloring agents. The concentration of the colorants in the candies were 3, 1.3, and 0.6* $\mathrm{mmol/m^2}$ *for A-encap, (A), A-Sucrose (Suc) and A-MD (MD), respectively. The numbers represent the replicate. b) Differences in color C(a*,b*) as a function of absorbed irradiation dose* $\mathrm{mol\,photons/m^2}$ *for candies containing A-encap, A-Sucrose and A-MD as coloring agents. Extracted from Paper IV.*

The time of color variation will be directly proportional to the colorant concentration. The higher the colorant concentration, the longer it will take for the color to fade away. This is a direct result of the half-life of a zero-order kinetic reaction $t_{1/2} = B(0)/2Y$ which depends on the initial concentration of the substance and on its degradation rate (quantum yield) [126]. It is important to note that light scattering phenomena decrease the speed of color degradation in the candies as in the case of A-encap. For this system, a clear inflexion in the degradation curve is observed when the light scattering of the candy becomes important around 100 $mol\,photons/m^2$.

7 Discussion

The importance of determining a fruit's morphological or color traits that would explain higher colorant contents translates in information for the construction of breeding programs. This allows for improvement of the species of *Bixa orellana* to produce higher yields of colorant, which turns into higher revenues for the producers. Several authors agree that higher colorant content can be expected for wider and longer fruit sizes [35, 37, 127]. Our results support this conclusion. In the case of color, literature offers contrasting results [11, 38, 40]. The newest results obtain through genetic experiments show that heterosis in the plants (different colors in the base and the bristles) may result in higher colorant contents [43]. Our results show that the color of the fruits may be related to norbixin/bixin content, however, this result is limited by the assumption that all samples had the same maturity. It is possible that bixin was not fully converted from norbixin when samples were collected. Moreover, the bixin content may increase with total colorant content in a rate of 0.8 with respect to total colorant content. Furthermore, regardless of the morphotype of the fruits, well-managed cultivars offer higher colorant content. It is important to note that the samples were analyzed 1 year after extraction, at which time double colorant contents may be expected in the seeds according to the degradation rate presented by Mantovani et al. [35].

The use of bixin, and annatto formulations containing bixin, has been limited in a major part to oily and fatty products such as margarines, pastries, and cheeses [7]. Higher concentration limits of bixin than of norbixin are allowed for coloring different products [18, 19]. Furthermore, we have shown that bixin is the major component in the seeds' aril (80% Bixin – 20% Norbixin). There is then motivation to achieve bixin water dispersible colorant formulations to expand the use of bixin in aqueous-based products. Extensive literature is available showing microencapsulation by spray drying as a technique for obtaining water dispersible formulations [67, 68, 128]. However, the limiting step is to obtain oil in water emulsions or dispersions that can be introduced into the equipment without using solvents that might increase the production and safety costs [129]. Consequently, our results show an alternative for producing aqueous dispersions of bixin and annatto extracts using a mild alkaline base and water. Thus, this dispersion can be directly introduced into the spray dryer or analyzed by different techniques.

Encapsulant materials like MD and enhancers like GA and CMC proved to be efficient encapsulants of pure bixin and annatto extracts. Comparable encapsulation efficiencies are obtained in previous studies by Barbosa et al. for a mixture of encapsulating agents, such as 95% GA and 5% sucrose [57]. The mixture of native carbohydrates and sucrose poses a very interesting high encapsulation efficiency and light stability. It was shown that sucrose may act as an interacting filler of other macromolecules [122]. Moreover, the native carbohydrates are important for increasing the encapsulation efficiency of bixin. Thus, its use is recommended for coloring formulations. It not only increases bixin protection, but eases the extraction process, reducing costs. Spray drying was easy to use as an encapsulating technique, giving high encapsulation efficiencies in most cases.

The direct mild alkaline extraction makes it possible to extract the colorants and the native carbohydrates. This process makes it possible to have very small colorant particle sizes. After introducing this dispersion into the spray dryer, higher encapsulation efficiency is expected. That is because for a particular encapsulant material, smaller particle sizes will produce higher encapsulation efficiencies in the range of 4– 15 µm. The microcapsules can be readily re-dispersible in water. Therefore, smaller particle sizes will produce higher physical stability in aqueous dispersions [73]. Furthermore, there is some evidence that the native carbohydrates may aide the dispersibility of the pigment in water. When there are no native carbohydrates, the bixin crystals will sediment under acid conditions. In contrast, after 40 days of the stability study, only the set of CRB dispersions had color remaining and submicron particles (0.3 and 0.7 µm). These results allow for the use of annatto colorants in aqueous products, such as sodas, juices and other beverages.

The model for light stability of the colorants provides an easy model for analyzing the photodegradation of photolabile substances. This may be of great use in the pharmaceutical industry for assessment of the photostability of photolabile drugs. There are very few studies that consider the powder as a system, considering the absorption and scattering effects of the powder [106, 130]. The absorption of the molecules in high concentration will prevent the light transmittance. This was also observed by Carvalho et al. [106], as they describe a possible shadowing effect when the photodegradant presents higher absorption than the matrix for the deeper layers of the powder bed. Then, our results show that significant scattering effects decrease the rate of colorant degradation, creating a deviation from linear behavior. Exponential-like degradation curves may be observed in previous studies in solid powder beds [63, 131]. Since the quantum yield imposes a linear degradation behavior, our model explains how the light scattering of the powder bed (when colorant concentration decreases), produces a decrease in the slope of the curve. Furthermore, our model is of great use for calculating the quantum yields of molecules of interest to enable determination of the shelf lives of the molecules.

Bixin and CRB degrade with similar quantum yields. This means that the native carbohydrates do not offer real protection against light colorant interactions. In contrast, microencapsulation by spray drying with encapsulant materials offers protection to the colorants, which prevents light degradation. In this regard, the native carbohydrates offer higher encapsulation efficiencies that offer higher light stability to the colorant. Protection against light degradation by spray drying is supported by a previous study of light degradation of potassium norbixinate microcapsules by freeze drying [75]. The study showed higher stability of the colorant in the microcapsules under light exposure. Our results show that the quantum yield decreases with the increase in encapsulation efficiency. Carboxymethylcellulose, CMC, offered the highest protection to both bixin and CRB due to a higher encapsulation efficiency compared to other encapsulants, respectively (Figure 14). These results support the use of microencapsulated powders in cosmetic formulations such as eye shadows, foundation, lipsticks, etc.

Furthermore, spray-dried annatto microcapsules can be readily applied in aqueous dispersible products such as candies. The stability tests of the colorant formulations in the candies give some insight about the colorant concentrations and the color of the candies; the duration of the color under light irradiation; and about the type of packaging to be used. An opaque package would be enough to protect the colorants during their shelf life. Moreover, during an average day of light irradiation of 5 kWh/m^2 (0.99 $mmol\ photons/m^2 s$), for instance, which corresponds to southern Europe (Spain) [132], and a colorant concentration of 3 $mmol/m^2$, the candies may be exposed to approximately 8 hours of intense light (midday light) without a perceivable difference in color change. After this time, color differences may increase to values higher than $\Delta E > 4$. Then, these color differences may be perceived by consumers. Consequently, these results make it possible to design the amount of colorant to be added during the formulation of product.

8 Conclusions

The aim of the present thesis was to generate comprehensive knowledge that would facilitate the applicability of the annatto colorant in aqueous liquid and solid product formulations by means of technological approaches. To fulfill this aim, 5 main objectives were specified. The first intended to characterize *Bixa orellana* fruit morphotypes at different altitudes to find traits that would correlate to higher bixin contents. The second and third objective searched to achieve aqueous dispersibility of the annatto colorant using microencapsulation by spray drying, and further evaluate the physical stability of the colorant's aqueous dispersions. The fourth objective was to study the photostability of the colorants' microcapsules (powder formulations) to evaluate the improvement to its light stability with respect to unprotected colorants. And the fifth objective was to evaluate the light stability and color stability of the pigments applied in an aqueous continuous product formulation such as candies. These objectives searched to satisfy the main requirements of the colorant applied in a product formulation during intended use.

The journey of this work started with the collection of twenty-seven samples of fruits of *Bixa orellana* from municipalities of La Paz, Bolivia. The fruits were classified into 3 groups according to 16 morphological parameters, 4 color parameters and 4 phytochemical parameters. The eight morphotypes found within each group confirm the high phenotypic diversity (large number of morphotypes) of fruits of *Bixa orellana* collected in La Paz. The morphological traits that had a correlation with bixin content were the size (width and length). Higher bixin contents can be expected from longer and wider fruits. In addition, wider fruit shapes could indicate higher bixin content. Furthermore, well-managed cultivars lead to higher bixin content regardless of the variety (above 3% after a year of sample collection). Our results show that the altitude does not have an impact on bixin and norbixin concentration.

Bixin and the annatto pigment are insoluble in water. Moreover, the several layers of pigment that cover the annatto seeds (aril) make the extraction process very laborious using solvent extraction methods. The pigment extraction and the colorant content analysis require an efficient extraction method. This was achieved through the development of an exaction method based on solid-liquid mild alkaline aqueous extraction. This method was developed based on methods reported in literature, which extract the colorant by abrasion with mild alkaline solutions. The novelty of the method presented in this thesis is that the mild base (Na_2CO_3) was added in solid state to the seeds and then water was added gradually to extract the pigment. Several aqueous extractions are performed, and sodium carbonate is added by parts when needed. This method allows a fairly rapid extraction procedure of a high number of seeds. This mild alkaline solid-liquid extraction procedure allowed to obtain an aqueous suspension of pigment (annatto). Moreover, this method was found useful for dispersing the bixin crystals and the purified native carbohydrate rich bixin extracts in water. This way, bixin, carbohydrate rich bixin extracts, and other annatto extracts can be microencapsulated by spray drying from aqueous dispersions of the pigments.

Microencapsulation by spray drying was found to be an efficient technique for obtaining aqueous dispersible formulations of the different pigments from annatto. The physical stability of the dissolved/dispersed colorant microcapsules will depend on the pH of the aqueous dispersion. Both annatto and pure bixin can be dispersed in mild basic pH (pH = 7– 9). However, at acid pH (pH = 3), pure bixin will sediment readily, while carbohydrate rich bixin microcapsules are dispersed in water for at least 40 days. This dispersion is attained through several factors. The first is the modification of the native carbohydrate structure after spray drying. The second is the small particle size observed in the carbohydrate rich bixin colorant particles (<1 μm). While flocculation and long-term sedimentation remain a challenge for future studies, these microcapsules have potential for use in aqueous products such as soft drinks, juices, and aqueous continuous formulations, among others.

Microencapsulation by spray drying not only makes it possible to obtain dispersible annatto formulations, but it is also an effective method for protecting the colorants (bixin and annatto extracts) from light degradation. The light stability of the pigment in solid state will be directly proportional to the encapsulation efficiency. The higher the encapsulation efficiency, the lower the sensitivity of bixin towards light. The quantum yield of bixin and annatto was found to be 30–100 times lower when microencapsulated compared to unprotected colorants. Native carbohydrates increase bixin stability against light by increasing its encapsulation efficiency.

The photostability studies contributed to the development of a model to describe the degradation of solid-state photolabile substances based on analytical techniques and using computational tools. The model comprises three scenarios. The first is the exponential degradation of colored substances in homogeneous liquids. The second scenario is the linear degradation of colored powders when light scattering in the powder is not significant. And the third scenario is the exponential degradation in solid state due to a significant light scattering of light in the solid. The annatto extract or microcapsules follow the second and third scenarios of the solid-state degradation model. Degradation will be linear until the scattering of light in the system becomes significant.

To finalize this journey, microcapsules – spray dried annatto, annatto microencapsulated with maltodextrin, and annatto microencapsulated with sucrose – were used to color candies. Because the encapsulating agents of the capsules were dissolved when preparing the candies, the sensitivity of the annatto extract in the three formulations used produced very similar quantum yields. These quantum yields were around 12 times smaller than for unprotected colorants. The smaller quantum yields were attained because of the caramel matrix that acted as a protector of the pigments, decreasing their sensitivity to light. These results allow to plan the packaging of the sweets colored with annatto to prevent color degradation.

Different concentrations of annatto can produce similar chroma in the candies, i.e., present similar color parameters a^*, b^*, and L^*. The differences between the intensity of chroma are visually noticeable for color differences greater than $\Delta E > > 4$. These results are valid considering that our sensory study was conducted in digital format. In general, the color of the candies had a good acceptability by the participants in the sensory analysis. Then, it is a good indicator for expecting good acceptability of the color of the candies in a wider population.

The results of this thesis offer a wide spectrum of possibilities of application of the colorant annatto in aqueous continuous product formulations in the food industry and in cosmetics. Furthermore, this thesis opens the door for experimental research in a rising field that has to do with photolabile solid formulations and their interaction with light.

9 Future Outlook

Based on the journey and results obtained in the present thesis, a number of questions were raised and there is an acknowledgment for further studies in several aspects viewed during this work. Some important aspects are discussed below.

It is necessary to optimize the amount of mild alkaline base to be added, in solid state, to extract the pigments from the seed's aril. This optimized pigment/mild base ratio is important to decrease extraction times. Moreover, for analytical purposes, the quantity of seeds to be used may be decreased as well. Studies regarding this ratio will make it possible to disperse purified bixin, and other annatto extracts in water in a more efficient way, i.e., preventing undesired saponification, reducing the particle average diameter of the colorant particles, and reducing the reactant amount and analysis times.

This extraction method facilitates further research in the development of bixin or annatto water dispersible formulations. Studies are needed to prevent flocculation and sedimentation. These studies may include techniques that make it possible to reduce the particle size of the pigments. These may include the mild alkaline extraction method, the increase of the viscosity of the liquid where the colorant is intended to be applied, or studies on emulsions are also possible.

Previous studies have reported the presence of proteins and phenolic components in the aril of the seeds of *Bixa orellana*. It would be interesting to analyze these contents in Bolivian samples.

With respect to the light stability studies in solid state, topics of interest would focus on the effect of different particle sizes in the light stability of pure colorant powders. Variations in particle sizes may be considered above 4 µm to be in the range of optical geometrics, and below 4 µm where Rayleigh theory approaches may be considered.

Another topic of interest would be studies regarding the matrices used for coating or encapsulating the colorants. Matrices with reflecting, fluorescent or scattering properties would be interesting to consider as encapsulating agents in order to evaluate their impact on the color stability. Moreover, there is a need to study the minimal thickness of excipients needed to cover a photolabile substance inside a pharmaceutical tablet, to protect it against light degradation. These studies are of significance for offering safer and quality products to patients.

The model for light stability of the colorants provides an easy model for monitoring the degradation of photolabile substances when the concentration of the photolabile substance can be measured, or the concentration of a degradation product. The model considers the light scattering of the matrix of the powder bed, which can be measured by separate means. This way, the quantum yield of the photoreaction can be measured. Conversely, the scattering of the powder excipients may be calculated by means of photodegradation experiments.

Further studies are needed with respect to the relationship between the reflectance curves as a function of color concentration of bixin, and how the color changes with higher concentration. Different concentrations may produce different colors regardless of the system that contains the colorant, i.e., liquid dispersions or solid matrix as candies.

Acknowledgements

This PhD journey has been a unique and very special stage of my life. I got to travel to the other side of the world for the first time. I got to experience and live in a new country, make new friends, and meet new people who I shared new experiences with and who taught me valuable things. I also got to strengthen friendships and shared new experiences with them. I have grown and learned about science, about life and about myself in so many aspects, so, I would like to thank some people that have been present in a special way during this PhD journey.

Björn Bergenståhl, my Swedish supervisor, thank you for supporting me during my PhD studies. Thank you for sharing your knowledge, thank you for your time, your dedication, all your effort put in the Papers, the fruitful discussions, your perspectives and insights about science and life. I have learned a lot from you.

Juan Antonio Alvarado, my Bolivian supervisor, thank you for actively supporting me and for your dedication during this time. Thank you for making the research process and administrative processes in Bolivia, possible.

Ignacio Chirico, and the headquarters of DIPGIS-UMSA, thank you for enabling the administrative processes, and facilitating the ASDI-UMSA cooperation program in order to make the present research possible.

Niko Gentile and Henrik Davidsson from the Department of Architecture and Built Environment, Division of Energy and Building Design, LTH, *many* thanks for providing the access to the solar laboratory used in the irradiation experiments and for all the collaboration during this time.

Luis Marconi, from the National Herbarium of Bolivia, thank you for the time and dedication spend during the analysis of the collected annatto fruits.

Javier Linares-Pastén and to the laboratory of Biotechnology at the Department of Chemistry at Lund University, thank you for the collaboration, time and efforts put into the analysis of the native carbohydrates of the annatto seeds' outer layer.

Many thanks to the ***Swedish National Center for High-Resolution Electron Microscopy (nCHREM)*** for the Scanning Electron Microscope expertise, the effort given during the training time and the assistance during image acquisition.

Yvonne Granfeldt, Marie Wahlgren, Ingegerd Sjoholm, Malin Sjöö, Eva Tornberg, I feel great admiration for all of you. Thank you for your kindness, thank you for sharing your knowledge with me, for the nice talks, and the thoughts exchanged.

Marilyn Rayner, thank you for offering the right words at the right times, for your strong spirit, and support.

Federico Gomez, thank you for your friendship and the lunches shared.

Hans Bolinsson, and Dan Johansson, thank you very much for your efficient assistance regarding the handling and maintenance of the laboratory materials and equipment. Thank you for your dedication to allow and facilitate a proper, efficient, and adequate laboratory work. It was very nice to share some good times with you during my PhD studies.

Rubén Choque, thank you for your willingness and patience of driving us through Los Yungas of La Paz during the collecting trips.

To my co-authors: Vandana Tannira, Tuba Tat, Ninoska Chino, Rosmery Tarqui, Cai Yang, Mina Haijan, Javier Linares-Pastén, Evelyn Mendoza, Luis Marconi, Marco Quino, Niko Gentile, Álvaro Salinas. Thank you for the efforts, the good work, and the dedication put in our research work. Thank you for the listening, for the productive discussions, for your trust in me and in our work.

Many thanks to the ***PhD student colleagues***, researchers, educators, professors, and all the people who are part of the department of Food Technology, Engineering, and Nutrition for making my passage through the department a journey full of learning, enjoyable “fika” times and after-works, memorable department trips, and a journey full of fun activities.

To the educators, professors, and administrative personnel of the ***Carrera de Ciencias Químicas*** of UMSA, many thanks for the access to the installations and laboratories, for the assistance and collaboration. Thanks to Ms. ***Helena*** and Ms. ***Gloria*** for all the administration assistance.

Mauricio Peñarrieta, thank you for your assistance, support and collaboration with respect to technical considerations, equipment, and materials. It has been great to share good talks and life histories.

Patricia Mollinedo, thank you for the access to the microscope and materials.

To all the people who are part of the ***Laboratory of Bioorganic*** in the Career of Chemistry in La Paz, thank you for the assistance and collaboration with materials and equipment during my studies.

A very special thanks to Álvaro Salinas for being the greatest support during the process of my PhD studies. All the efforts and accomplishments of this thesis owe great gratitude to your back up and support.

Claudia Ziekle, thank you for being a loving and caring friend. I am very happy to have meet you and have enjoyed so many great talks by the sea during the long Swedish summer days. From trips to thoughts and fun, thank you for all the great times shared.

Edita Demir, thank you for your generous heart. It has been encouraging to share great discussions about life opportunities and perspectives. Thank you for your friendship and all the great experiences shared.

Claudia Lazarte, Noor Liyana Yusof (hermanita, Yanita), Kati Aalaei, Hizfacila Sarai, thank you for all the laughing during our dinners. I am very grateful for our friendship, your support, for opening your home to me, and for all our shared experiences.

Rodrigo Villagomez, thank you for being a lifetime friend and for your support during my research work. You are one of the kindest, most knowledgeable, and most capable persons I know. Thank you for sharing that with me. It has been great to share talks, activities and experiences in this other part of the world.

Teresa Canedo, thank you for opening your home to me. Thank you for your friendship, for your support and all the learning during this time. I feel blessed to have shared this stage of my life with you.

Vanesa Castro, thank you for your kind heart, for being there when I needed help, and for all the times shared.

Oscar Rollano, Daniel Osorio, Atma Bustos, Catalina Fuentes, Juan Salinas, Fabrizio, Maribel Lozano, Carla Fernandez, Sander Perez, and all the Bolivian friends that I got close to during this time, thank you for all the learning together. Thank you for your friendship and for supporting me. It was great for me to enjoy Sweden with you from the perspective of the eyes that see interesting things for the first time.

Enrique Joffré and Graciela Terán, thank you for the great times shared, the memorable experiences, the trips and the wonderful talks about life. I feel very grateful for your friendship and for having the doors of your home, opened for me.

Randi Phinney, thank you for your cheerfulness and your active dedication to interesting activities. It has been great to share so many talks and exchange ideas about life and dreams. Thank you for our beautiful friendship.

Ida-Marie Andersson, Kajsa Nilsson, Stina Burri, Shuai Bai, thank you for your friendship, for sharing your Swedish traditions and your home with me. I have enjoyed the Swedish woods, the singing, and the schnapps. I have loved the Swedish crayfish parties and sharing all the dancing together through the PhD's dissertations.

To my friends who I have got to meet in this "ancient world" called Europe, heartfully, many thanks for the drinks and talks shared, the dinners, the dancing, the trips, the festivals, the great times shared, the walks, the hikes, the wall climbing, the new perspectives. It has been great to getting to know you and growing a friendship :)

To my mom and dad, I could have not asked for better parents. I feel very blessed for having you in my life. I feel proud of you. Thank you for guiding me and supporting me.

To my brothers and sister, thank you for our comradery and your assistance when distance make processes more difficult. Thank you for your support and love.

Alejandro Hurtado, thank you for reminding me of the beautiful things of life.

A mis grandes amigos de la vida de Bolivia, que están dentro y fuera del país, ustedes saben quiénes son, muchas gracias por tu amistad, por tu hermandad, por tu cariño, por apoyarme durante este tiempo. Gracias por compartir tu vida conmigo, por las risas, las tristezas, la confianza, por quererme y aceptarme como soy. ¡Te quiero mucho!

References

1. Agócs, A. and J. Deli, *Pigments in your food.* Journal of Food Composition and Analysis, 2011. **24**(6): p. 757-759.
2. McAvoy, S.A., *Global regulations of food colors.* The Manufacturing Confectioner, 2014. **94**(9): p. 77-86.
3. Meticulous Market Research. *Global Market Opportunity Analysis and Industry Forecasts*. 2020.
4. Green, C., *Natural colourants and dyestuffs: A review of production, markets and development potential*. 1995: Food and Agriculture Organization of the United Nations.
5. Chisté, R.C., et al., *Simultaneous extraction and analysis by high performance liquid chromatography coupled to diode array and mass spectrometric detectors of bixin and phenolic compounds from annatto seeds.* Journal of Chromatography A, 2011. **1218**(1): p. 57-63.
6. Amaral, L., et al., *Anatomical and biochemical changes in the composition of developing seed coats of annatto (Bixa orellana L.).* Trees, 2009. **23**(2): p. 287-293.
7. Satyanarayana, A. and P.G. Prabhakara, *Chemistry, Processing and Toxicology of Annatto (Bixa orellana L.* J. Food Sci Technol, 2003. **Vol 40**(No 2): p. 131-141.
8. Galindo-Cuspinera, V., M.B. Lubran, and S.A. Rankin, *Comparison of volatile compounds in water-and oil-soluble annatto (Bixa orellana L.) extracts.* Journal of Agricultural and Food Chemistry, 2002. **50**(7): p. 2010-2015.
9. Jondiko, I.J. and G. Pattenden, *Terpenoids and an apocarotenoid from seeds of Bixa orellana.* Phytochemistry, 1989. **28**(11): p. 3159-3162.
10. Vilar Dde, A., et al., *Traditional uses, chemical constituents, and biological activities of Bixa orellana (L).: A review.* Scientific World Journal, 2014: p. 857292.
11. Fabri, E.G. and J.R.S. Teramoto, *Urucum: fonte de corantes naturais.* Horticultura brasileira, 2015. **33**(1): p. 140-140.
12. Dias, N.O., et al., *Morpho-agronomic characterization and estimates of genetic parameters in annatto plant.* Horticultura Brasileira, 2017. **35**(2): p. 242-246.
13. Smith., J., - 67th Joint FAO/WHO Expert Committee on Food Additives (JECFA) Meeting *Annatto Extracts.* Chemical and Technical Assessment (CTA) - Food and Agriculture Organization of the United Nations, 2006.
14. Commission Regulations (EU) No 1129/2011, *Regulations: Amending Annex II Regulation (EC) No 1333/2008 of the European Parliament and of the Council by a Union list of food additives.* 2011.
15. H.D. Preston, M.D.R., *Extraction and chemistry of annatto.* Food chemistry, 1980. **5**: p. 47-56.

16. INE. *Instituto Nacional de Estadística, Exportaciones, Bases de Datos Exportaciones*. 2020 [cited 2020; Available from: https://www.ine.gob.bo/index.php/estadisticas-economicas/comercio-exterior/bases-de-datos-exportaciones/.
17. Julie N. Barrows, A.L.L., Catherine J. Bailey. *Color Additives History*. [cited 2020 April, 4th]; Available from: https://www.fda.gov/industry/color-additives/color-additives-history#authors.
18. Additives, E.P.o.F. and N.S.a.t. Food, *The safety of annatto extracts (E 160b) as a food additive.* EFSA Journal, 2016. **14**(8): p. e04544.
19. Tennant, D.R. and M. O'Callaghan, *Survey of usage and estimated intakes of annatto extracts.* Food Research International, 2005. **38**(8-9): p. 911-917.
20. Kiokias, S. and M.H. Gordon, *Antioxidant properties of annatto carotenoids.* Food Chemistry, 2003. **83**(4): p. 523-529.
21. Rivera-Madrid, R., et al., *Carotenoid derivates in achiote (Bixa orellana) seeds: synthesis and health promoting properties.* Frontiers in plant science, 2016. **7**: p. 1406.
22. Nathan, V.K., et al., *Antioxidant and antimicrobial potential of natural colouring pigment derived from Bixa orellana L. Seed Aril.* Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 2019. **89**(1): p. 137-143.
23. Delgado-Vargas, F. and O. Paredes-Lopez, *Natural colorants for food and nutraceutical uses*. 2002: CRC press.
24. Additives, J.F.W.E.C.o.F. and F.A.O.o.t.U. Nation, *Compendium of Food Additive Specifications: Combined Specifications from 1st Through the 67th Meeting.* FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 2006. **Part 3**.
25. Breukers, S., et al., *Hydrophilic carotenoids: surface properties and aggregation behavior of the potassium salt of the highly unsaturated diacid norbixin.* Helvetica Chimica Acta, 2009. **92**(9): p. 1741-1747.
26. Cardarelli, C., M. Benassi, and A. Mercadante, *Characterization of different annatto extracts based on antioxidant and colour properties.* LWT - Food Science and Technology, 2008. **41**(9): p. 1689-1693.
27. Scotter, M.J., *The chemistry and analysis of annatto food colouring: a review.* Food Additives & Contaminants: Part A, 2009. **26**(8): p. 1123-1145.
28. Lauro, G. and J. Francis, *Natural food colorants: science and technology*. 2000: CRC Press.
29. Satyanarayana, A., P.P. Rao, and D.G. Rao, *Influence of source and quality on the color characteristics of annatto dyes and formulations.* LWT - Food Science and Technology, 2010. **43**(9): p. 1456-1460.
30. Scotter, M., et al., *Method development and analysis of retail foods for annatto food colouring material.* Food Additives & Contaminants, 2002. **19**(3): p. 205-222.
31. Romero, A. and S.E. West, *Environmental issues in Latin America and the Caribbean*. 2006: Springer.
32. Villegas, P. and M. Gandarillas, *Los recursos naturales en Bolivia*. 2008: Centro de Documentación e Información Bolivia, CEDIB.

33. Choquecallata, H.R., *Comercialización del achiote (Bixa orellana L.) en el cantón Rosario-Entre Ríos, Provincia Caranavi*. 2008, Universidad Mayor de San Andrés, La Paz (Bolivia). Facultad de Agronomía.
34. Aparnathi, K., R. Lata, and R. Sharma, *Annatto (Bixa orellana L.)-its cultivation, preparation and usage.* International journal of tropical agriculture, 1990. **8**(1): p. 80-88.
35. Mantovani, N.C., et al., *Avaliação de genótipos de urucum (Bixa orellana L.) por meio da caracterização morfológica de frutos, produtividade de sementes e teor de bixina.* Ciência Florestal, 2013. **23**(2): p. 344-362.
36. Joseph, N. and E.A. Siril, *Floral Color Polymorphism and Reproductive Success in Annatto (Bixa orellana L.).* Tropical Plant Biology, 2013. **6**(4): p. 217-227.
37. Taboada, C. and H. Salm, *Caracterización morfológica y contenido de bixina en diferentes variedades de achiote (Bixa orellana) de los Yungas de La Paz.* Ecología en Bolivia, 1997. **29**: p. 45 - 50
38. Akshatha, V., P. Giridhar, and G.A. Ravishankar, *Morphological diversity in Bixa orellana L. and variations in annatto pigment yield.* The Journal of Horticultural Science and Biotechnology, 2011. **86**(4): p. 319-324.
39. Nogueira, P.R., Gomez, Martha., Gomes, Eliane., Da Rocha, Paulo., Mello, Antonio., Spatti, Leandro., *Concentração de bixina e lipídios em sementes de urucum da coleção do instituto agronômico (IAC).* 2010. **69**(3): p. 519-524.
40. Rodríguez-Ávila, N.L., et al., *Regulation of pigment-related genes during flower and fruit development of Bixa orellana.* Plant molecular biology reporter, 2011. **29**(1): p. 43-50.
41. Rivera-Madrid, R., et al., *Preliminary studies toward genetic improvement of annatto (Bixa orellana L.).* Scientia horticulturae, 2006. **109**(2): p. 165-172.
42. Moreira, P.A., et al., *The domestication of annatto (Bixa orellana) from Bixa urucurana in Amazonia.* Economic Botany, 2015. **69**(2): p. 127-135.
43. Trujillo-Hdz, J.A., et al., *Functional polymorphism in lycopene beta-cyclase gene as a molecular marker to predict bixin production in Bixa orellana L.(achiote).* Molecular Breeding, 2016. **36**(9): p. 135.
44. Dardanelli, J.L., et al., *Soybean maturity groups, environments, and their interaction define mega-environments for seed composition in Argentina.* Crop Science, 2006. **46**(5): p. 1939-1947.
45. Carrera, C.S., J.L. Dardanelli, and D.O. Soldini, *Genotypic and environmental variation in seed nutraceutical and industrial composition of non-transgenic soybean (Glycine max) genotypes.* Crop and Pasture Science, 2014. **65**(12): p. 1311-1322.
46. Rohloff, J., et al., *Nutritional composition of bilberries (Vaccinium myrtillus L.) from forest fields in Norway–Effects of geographic origin, climate, fertilization and soil properties.* 2015.
47. Li, H., R. Tsao, and Z. Deng, *Factors affecting the antioxidant potential and health benefits of plant foods.* Canadian journal of plant science, 2012. **92**(6): p. 1101-1111.
48. Gouvinhas, I., et al., *Enhanced phytochemical composition and biological activities of grape (Vitis vinifera L.) Stems growing in low altitude regions.* Scientia Horticulturae, 2020. **265**: p. 109248.

49. Körner, C., *The use of 'altitude'in ecological research.* Trends in ecology & evolution, 2007. **22**(11): p. 569-574.
50. Martz, F., et al., *Phenolic composition and antioxidant capacity of bilberry (Vaccinium myrtillus) leaves in Northern Europe following foliar development and along environmental gradients.* Journal of chemical ecology, 2010. **36**(9): p. 1017-1028.
51. Taquichiri, M., et al., *High levels of resveratrol in grapes cultivated at high altitude valleys in Bolivia.* International Journal of Fruit Science, 2014. **14**(3): p. 311-326.
52. Peñarrieta, J.M., et al., *Phenolic compounds in food.* Revista Boliviana de química, 2014. **31**(2): p. 68-81.
53. Osorio-Macías, D.E., et al., *Resveratrol, phenolic antioxidants, and saccharides in South American red wines.* International Journal of Wine Research, 2018. **10**: p. 1-11.
54. Guerrero-Chavez, G., M. Scampicchio, and C. Andreotti, *Influence of the site altitude on strawberry phenolic composition and quality.* Scientia Horticulturae, 2015. **192**: p. 21-28.
55. Giuliano, G., C. Rosati, and P.M. Bramley, *To dye or not to dye: biochemistry of annatto unveiled.* TRENDS in Biotechnology, 2003. **21**(12): p. 513-516.
56. Rahmalia, W., et al., *Aprotic solvents effect on the UV-visible absorption spectra of bixin.* Spectrochim Acta A Mol Biomol Spectrosc, 2014. **131**: p. 455-60.
57. Barbosa, M.I.M.J., C.D. Borsarelli, and A.Z. Mercadante, *Light stability of spray-dried bixin encapsulated with different edible polysaccharide preparations.* Food Research International, 2005. **38**(8-9): p. 989-994.
58. Najar, S., F. Bobbio, and P. Bobbio, *Effects of light, air, anti-oxidants and pro-oxidants on annatto extracts (Bixa orellana).* Food Chemistry, 1988. **29**(4): p. 283-289.
59. Møller, A.H., et al., *Mechanism behind the degradation of aqueous norbixin upon storage in light and dark environment.* Food Chemistry, 2020. **310**: p. 125967.
60. Scotter, M.J., et al., *Analysis of annatto (Bixa orellana) food coloring formulations. 1. Determination of coloring components and colored thermal degradation products by high-performance liquid chromatography with photodiode array detection.* Journal of Agricultural and Food Chemistry, 1998. **46**(3): p. 1031-1038.
61. Zechmeister, L. and R. Escue, *A stereochemical study of methylbixin.* Journal of the American Chemical Society, 1944. **66**(3): p. 322-330.
62. Scotter, M., et al., *Characterization of the principal colouring components of annatto using high performance liquid chromatography with photodiode-array detection.* Food Additives & Contaminants, 1994. **11**(3): p. 301-315.
63. Balaswamy, K., et al., *Stability of bixin in annatto oleoresin and dye powder during storage.* LWT-Food Science and Technology, 2006. **39**(8): p. 952-956.
64. Berton-Carabin, C.C. and K. Schroën, *Pickering emulsions for food applications: background, trends, and challenges.* Annual review of food science and technology, 2015. **6**: p. 263-297.
65. Mcclements, D.J., *Critical review of techniques and methodologies for characterization of emulsion stability.* Critical reviews in food science and nutrition, 2007. **47**(7): p. 611-649.

66. Lobato, K.B., et al., *Characterisation and stability evaluation of bixin nanocapsules.* Food Chem, 2013. **141**(4): p. 3906-12.

67. Zhang, Y. and Q. Zhong, *Encapsulation of bixin in sodium caseinate to deliver the colorant in transparent dispersions.* Food Hydrocolloids, 2013. **33**(1): p. 1-9.

68. Gharsallaoui, A., et al., *Applications of spray-drying in microencapsulation of food ingredients: An overview.* Food Research International, 2007. **40**(9): p. 1107-1121.

69. Yadav, M.P., D.B. Johnston, and K.B. Hicks, *Corn fiber gum: New structure/function relationships for this potential beverage flavor stabilizer.* Food Hydrocolloids, 2009. **23**(6): p. 1488-1493.

70. Ebringerová, A. *Structural diversity and application potential of hemicelluloses*. in *Macromolecular symposia*. 2005. Wiley Online Library.

71. Mikkonen, K., et al., *Films from spruce galactoglucomannan blended with poly (vinyl alcohol), corn arabinoxylan, and konjac glucomannan.* BioResources, 2008. **3**(1): p. 178-191.

72. Hansen, N. and D. Plackett, *Sustainable films and coatings from hemicelluloses: a review.* Biomacromolecules, 2008. **9**(6): p. 1493-1505.

73. Curi-Borda, C.K., et al., *Multilayer Bixin Microcapsules: The Impact of Native Carbohydrates on the Microencapsulation Efficiency and Dispersion Stability.* Foods, 2019. **8**(3): p. 108.

74. Marcolino, V.A., et al., *Interaction of curcumin and bixin with beta-cyclodextrin: complexation methods, stability, and applications in food.* J Agric Food Chem, 2011. **59**(7): p. 3348-57.

75. Sousdaleff, M., et al., *Microencapsulation by freeze-drying of potassium norbixinate and curcumin with maltodextrin: stability, solubility, and food application.* J Agric Food Chem, 2013. **61**(4): p. 955-65.

76. Ozkan, G., et al., *A review of microencapsulation methods for food antioxidants: Principles, advantages, drawbacks and applications.* Food chemistry, 2019. **272**: p. 494-506.

77. Eun, J.-B., et al., *A review of encapsulation of carotenoids using spray drying and freeze drying.* Critical Reviews in Food Science and Nutrition, 2019: p. 1-26.

78. Jafari, S.M., et al., *Encapsulation efficiency of food flavours and oils during spray drying.* Drying Technology, 2008. **26**(7): p. 816-835.

79. P. Joshi, N.R.C., Lausanne and C. J. B. Brimelow, *Colour measurement of foods by colour reflectance, in Colour in Food* 2002, Shanghai: Nestle´ R&D Centre.

80. Hunt, R.W.G. and M.R. Pointer, *Measuring colour*. 2011: John Wiley & Sons.

81. MacDougall, D., *Colour measurement of food: principles and practice*, in *Colour Measurement*. 2010, Elsevier. p. 312-342.

82. Hutchings, J., *The perception and sensory assessment of colour*. in Colour in Food. 2002: CRC Press.

83. Malacara, D., *Color vision and colorimetry: theory and applications*. Color Research & Application: Endorsed by Inter-Society Color Council, The Colour Group (Great Britain), Canadian Society for Color, Color Science Association of Japan, Dutch Society for the Study of Color, The Swedish Colour Centre Foundation, Colour Society of Australia, Centre Français de la Couleur. Vol. 28. 2003. 77-78.
84. MacDougall, D., *The chemistry of colour and appearance.* Food chemistry, 1986. **21**(4): p. 283-299.
85. Leon, K., et al., *Color measurement in L∗a∗b∗ units from RGB digital images.* Food research international, 2006. **39**(10): p. 1084-1091.
86. Pathare, P.B., U.L. Opara, and F.A.-J. Al-Said, *Colour measurement and analysis in fresh and processed foods: a review.* Food and bioprocess technology, 2013. **6**(1): p. 36-60.
87. Zhao, Y. and R.S. Berns, *Predicting the spectral reflectance factor of translucent paints using Kubelka-Munk turbid media theory: Review and evaluation.* Color Research & Application: , 2009. **34**(6): p. 417-431.
88. Bandpay, M.G., et al., *Mathematical and empirical evaluation of accuracy of the Kubelka–Munk model for color match prediction of opaque and translucent surface coatings.* Journal of Coatings Technology and Research, 2018. **15**(5): p. 1117-1131.
89. Jelen, H., *Food flavors: chemical, sensory and technological properties*. 2011: CRC Press.
90. Lawless, H.T. and H. Heymann, *Sensory evaluation of food: principles and practices*. 2010: Springer Science & Business Media.
91. Jørgensen, K. and L.H. Skibsted, *Light sensitivity of carotenoids used as food colours.* Zeitschrift für Lebensmitteluntersuchung und-Forschung A, 1990. **190**(4): p. 306-313.
92. Allain, L., et al., *Implications of In-Use Photostability: Proposed Guidance for Photostability Testing and Labeling to Support the Administration of Photosensitive Pharmaceutical Products, Part 3. Oral Drug Products.* Journal of Pharmaceutical Sciences, 2016. **105**(5): p. 1586-1594.
93. Baertschi, S.W., et al., *Implications of in-use photostability: proposed guidance for photostability testing and labeling to support the administration of photosensitive pharmaceutical products, part 2: topical drug product.* Journal of Pharmaceutical Sciences, 2015. **104**(9): p. 2688-2701.
94. Albini, A. and E. Fasani, *Drugs: photochemistry and photostability*. 2007: Royal Society of Chemistry.
95. Center for Drug Evaluation and Research, C.f.B.E.a.R., *Q1B Photostability Testing of New Drug Substances and Products.* Food and Drug Administration FDA, 1996. **ICH**.
96. Baertschi, S.W., K.M. Alsante, and H.H. Tønnesen, *A critical assessment of the ICH guideline on photostability testing of new drug substances and products (Q1B): Recommendation for revision.* Journal of Pharmaceutical Sciences, 2010. **99**(7): p. 2934-2940.
97. Hippler, M., *Photochemical kinetics: Reaction orders and analogies with molecular beam scattering and cavity ring-down experiments.* Journal of Chemical Education, 2003. **80**(9): p. 1074.

98. Bohren, C.F. and D.R. Huffman, *Absorption and scattering of light by small particles*. 2008: John Wiley & Sons.
99. Hébert, M., R.D. Hersch, and P. Emmel, *Fundamentals of optics and radiometry for color reproduction.* Handbook of Digital Imaging, Wiley, 2015. **2**: p. 1021-1077.
100. Ma, L., et al., *Dependent scattering and absorption by densely packed discrete spherical particles: effects of complex refractive index.* Journal of Quantitative Spectroscopy and Radiative Transfer, 2017. **196**: p. 94-102.
101. Randrianalisoa, J. and D. Baillis, *Analytical model of radiative properties of packed beds and dispersed media.* International Journal of Heat and Mass Transfer, 2014. **70**: p. 264-275.
102. Diebold, M.P., *Application of Light Scattering to Coatings: A User's Guide*. 2014: Springer.
103. Mohan, S., et al., *Refractive Index Measurement of Pharmaceutical Solids: A Review of Measurement Methods and Pharmaceutical Applications.* Journal of Pharmaceutical Sciences, 2019. **108**(11): p. 3478-3495.
104. Kubelka, P., *New contributions to the optics of intensely light-scattering materials. Part I.* J. Opt. Soc. Am., 1948. **38**(5): p. 448-457.
105. Petersen, M., L. Wiking, and H. Stapelfeldt, *Light sensitivity of two colorants for Cheddar cheese. Quantum yields for photodegradation in an aqueous model system in relation to light stability of cheese in illuminated display.* Journal of dairy research, 1999. **66**(4): p. 599-607.
106. Carvalho, T.C., T.E. La Cruz, and J.E. Tábora, *A photochemical kinetic model for solid dosage forms.* European Journal of Pharmaceutics and Biopharmaceutics, 2017. **120**: p. 63-72.
107. Rios, A. and A. Mercadante, *Otimização das Condições para Obtenção de Padrão de Bixina e das etapas de Extração e Saponificação para Quantificação de Bixina em "Snacks" extrusados por CLAE.* Alim. Nutr, 2004. **15**(3): p. 203-213.
108. Manke Nachtigall, A., et al., *Estudo da saponificação em pigmentos de urucum.* Ciência e Tecnologia de Alimentos, 2009. **29**(4).
109. Silva, P.I., A.M. Nachtigall, and P.C. Stringheta, *Fatores que influenciam a reação de saponificação dos carotenóides presentes no urucum (Bixa orellana L.).* Ciência e Agrotecnologia, 2009. **33**: p. 1892.
110. Saifullah, M., et al., *Micro and nano encapsulation, retention and controlled release of flavor and aroma compounds: A critical review.* Trends in Food Science & Technology, 2019. **86**: p. 230-251.
111. Ruphuy, G., et al., *Encapsulation of poorly soluble drugs in yeast glucan particles by spray drying improves dispersion and dissolution properties.* International Journal of Pharmaceutics, 2020. **576**: p. 118990.
112. Jafari, S.M., Y. He, and B. Bhandari, *Role of powder particle size on the encapsulation efficiency of oils during spray drying.* Drying Technology, 2007. **25**(6): p. 1081-1089.

113. Mengual, O., et al., *TURBISCAN MA 2000: multiple light scattering measurement for concentrated emulsion and suspension instability analysis.* Talanta, 1999. **50**(2): p. 445-456.
114. Formulaction Smart Scientific analysis, T., *Stability of pigment injet inks* Application paper, 2009: p. 1-5.
115. Formulaction Smart Scientific analysis, *User Guide, Turbiscan* 2016.
116. McClements, D.J., *Theoretical prediction of emulsion color.* Advances in colloid and interface science, 2002. **97**(1-3): p. 63-89.
117. Blevin, W. and W. Brown, *Light-scattering properties of pigment suspensions.* JOSA, 1961. **51**(9): p. 975-982.
118. Kregiel, D., *Health safety of soft drinks: contents, containers, and microorganisms.* BioMed research international, 2015. **2015**.
119. Bittencourt, C., et al., *ToF-SIMS characterization of thermal modifications of bixin from Bixa orellana fruit.* Journal of agricultural and food chemistry, 2005. **53**(16): p. 6195-6200.
120. ImageJ. *An open platform for scientific image analysis*. 2021; Available from: https://imagej.net/Welcome.
121. ColorMine. *Accurate and Easy Color Calculations, ColorMine.org*. 2021; Available from: http://colormine.org/.
122. Embuscado, M. and K. Huber, *Edible films and coatings for food applications*. 2009: Springer.
123. Meléndez-Martínez, A.J., I.M. Vicario, and F.J. Heredia, *Application of tristimulus colorimetry to estimate the carotenoids content in ultrafrozen orange juices.* Journal of Agricultural and Food Chemistry, 2003. **51**(25): p. 7266-7270.
124. Gullett, E., F. Francis, and F. Clydesdale, *Colorimetry of Foods 5. Tang.* Canadian Institute of Food Science and Technology Journal, 1972. **5**(1): p. 32-36.
125. Little, A.C., *Color Measurement of Translucent Food Samples a.* Journal of Food Science, 1964. **29**(6): p. 782-789.
126. Vallance, C., *An Introduction to Chemical Kinetics*. 2017: Morgan & Claypool Publishers.
127. Arce Portuguez, J.A., *Caracterización de 81 plantas de achiote (Bixa orellana L.) de la colección del CATIE procedentes de Honduras y Guatemala y propagación vegetativa por estacas.* 1984.
128. Pan, K., Q. Zhong, and S.J. Baek, *Enhanced dispersibility and bioactivity of curcumin by encapsulation in casein nanocapsules.* Journal of agricultural and food chemistry, 2013. **61**(25): p. 6036-6043.
129. McConvey, I., M. Hoyle, and O. Nulty, *Reducing the hazards associated with spray drying from laboratory through to larger scale including considerations for outsourcing and secondary containment.* IChemE SYMPOSIUM SERIES NO. 155, Hazards XXI, 2009.
130. La Cruz, T.E., et al., *Implementation of a mathematical model for the photochemical kinetics of a solid form active pharmaceutical ingredient.* International journal of pharmaceutics, 2019. **566**: p. 500-512.

131. Marinkovic, V.D., et al., *Photochemical degradation of solid-state nisoldipine monitored by HPLC.* Journal of pharmaceutical and biomedical analysis, 2003. **32**(4-5): p. 929-935.

132. Solargis s.r.o. *Download solar resource maps and GIS data for 180+ countries*. Solar resource maps of Bolivia [cited 2020 January]; Available from: https://solargis.com/maps-and-gis-data/download/bolivia.