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CARACTERIZACIÓN DEL PERFIL DE CAROTENOIDES DEL ZAPOTE (*Pouteria sapota*), EVALUACIÓN DE SU BIOACCESIBILIDAD Y DETERMINACIÓN DE LA ULTRAESTRUCTURA DE LOS CROMOPLASTOS

Tesis sometida a consideración de la Comisión del Programa de Estudios de Posgrado en Ciencia de Alimentos para optar al grado y título de Maestría Académica en Ciencia de Alimentos

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Dedicatory

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Resumen

La deficiencia de vitamina A es catalogada como un riesgo de moderado a severo para la salud humana, especialmente en países en vías de desarrollo. Esta deficiencia puede ocasionar enfermedades, como xeroftalmia, ceguera nocturna y anemia, así como debilitar el sistema inmune. Algunos carotenoides son conocidos por su actividad provitamina A; por lo tanto, existe interés en explorar nuevas fuentes vegetales ricas en carotenoides que sean más accesibles, como el zapote (*Pouteria sapota*). Éste constituye una fuente vegetal subutilizada con un perfil interesante de carotenoides, incluyendo algunos con grupos ceto en su estructura, como la sapotexantina. Aunque se han descrito diferentes genotipos de esta especie con variaciones en color de pulpa, no existen estudios que comparen la composición de sus pigmentos. Por lo tanto, el poder definir los patrones de carotenoides permitiría seleccionar genotipos promisorios como fuente de provitamina A. En el presente trabajo se describe el perfil y contenido de carotenoides de diferentes genotipos de zapote utilizando HPLC-DAD-MS^2 con trampa de iones. Además se evaluó la bioaccesibilidad *in vitro* de carotenoides en muestras de zapote fresco y cocinado, así como una comparación con otras fuentes ricas en cetocarotenoides. Por último, dado que la ultraestructura de los cromoplastos ha demostrado tener un gran impacto en la bioaccesibilidad de carotenoides en estudios previos, se caracterizó la estructura de los cromoplastos por medio de microscopía de luz y electrónica de transmisión.

Los pigmentos más importantes que se encontraron en los frutos de zapote fueron la sapotexantina y la criptocapsina, ambos con potencial actividad provitamina A. Altas concentraciones de estos pigmentos y de otros cetocarotenoides, como capsorubina y capsoneoxantina, fueron encontrados en los genotipos con pulpa de color rojo-anaranjado. Mientras que en el genotipo de pulpa amarilla-anaranjada predominaron los ésteres de neoxantina y otros epóxidos.

Los estudios de bioaccesibilidad *in vitro* en frutos de zapote y otras fuentes conocidas de cetocarotenoides, como el chile y el salmón, mostraron, en general, bajos niveles de bioaccesibilidad relativa, tanto en muestras frescas y después de cocción. El carotenoide más bioaccesible fue la capsantina presente en el chile, seguido por sapotexantina y los ésteres de criptocapsina a partir del zapote y por último astaxantina a partir del salmón. El tratamiento térmico de las muestras y la adición de grasa duplicó los niveles de bioaccesibilidad en casi todos los carotenoides analizados. La matriz de los alimentos mostró tener un gran impacto en los niveles de bioaccesibilidad de los carotenoides, particularmente en el salmón. Los carotenoides en los frutos de zapote se encuentran almacenados en cromoplastos del tipo globular-tubular, los cuales contenían grandes glóbulos lipídicos y acumulación de elementos tubulares. Este tipo de cromoplastos ha sido asociado con una mayor bioaccesibilidad de carotenoides.

Los cetocarotenoides, sapotexantina y criptocapsina, encontrados en frutos de zapote, son importantes por su potencial actividad provitamina A; sin embargo, es necesario la realización de otros estudios para determinar esta actividad. Las variaciones observadas tanto en el perfil como en el contenido de carotenoides de los genotipos analizados podrían estar relacionadas con diferencias en la expresión de genes o regulación postranslacional de las principales enzimas involucradas en la síntesis de carotenoides. Los ensayos de bioaccesibilidad *in vitro* mostraron una baja bioaccesibilidad de los cetocarotenoides presentes en zapote; estos niveles fueron aumentados al someter el fruto al tratamiento térmico y la adición de lípidos. La complementación de los estudios *in vitro* con ensayos *in vivo* son necesarios para determinar si la tendencia de absorción es similar, dado que otros factores inherentes del organismo del ser humano pueden afectar la absorción de estos cetocarotenoides.

Abstract

Vitamin A deficiency is a health problem affecting mainly developing or poor countries. Deficiency of this vitamin can cause severe health problems like xerophthalmia, night blindness and anemia. Some carotenoids are known sources of provitamin A; consequently, several efforts have been made to identify potential new sources of carotenoids that have provitamin A activity and are more accessible and less expensive than alternative known animal sources. Mamey sapote (*Pouteria sapota*) is one of them, being an underutilized carotenoid-rich fruit containing rare κ-ring carotenoids with potential provitamin A activity, including sapotexanthin. Although different genotypes showing distinct pulp colors are available, detailed knowledge on genotype-related differences in their carotenoid composition is still scarce. Being able to characterize the carotenoid profile will allow to identify those sapote genotypes better suitable as provitamin A sources. In this work, mamey sapote fruits from three genotypes were investigated to elucidate their detailed genuine carotenoid profile by HPLC-DAD-MS^2. Additionally, *in vitro* bioaccessibility was determined for carotenoids from raw and cooked samples and compared with other ketocarotenoid rich sources. Finally, since chromoplast ultrastructure has previously shown to exert an enormous impact on carotenoid bioaccessibility, carotenoid bearing cell structures in mamey sapote fruits were elucidated by light and transmission electron microscopy.

Two important pigments of this work found in mamey sapote were sapotexanthin and cryptocapsin, both potential provitamin A precursors. High amounts of these ketocarotenoids and others including capsorubin and capsoneoxanthin were observed in red-orange fleshed genotypes, while high concentrations of neoxanthin esters were the predominant carotenoids in yellow-orange fleshed fruits.

In vitro bioaccessibility studies in mamey sapote fruits and other known sources of ketocarotenoids, such as red bell pepper and salmon, indicated low carotenoid bioaccessibility from the raw and cooked fruit matrixes. The most bioaccessible carotenoid was capsanthin from red bell pepper, followed by sapotexanthin, cryptocapsin esters from mamey sapote, and finally astaxanthin from salmon. Thermal treatment and fat addition enhanced by around two-fold bioaccessibility of almost all carotenoids studied. Matrix appear to have a big impact on carotenoid bioaccessibility; this was particularly evident in salmon samples. Carotenoids in mamey sapote fruits appear to be deposited in globular-tubular chromoplasts containing large lipid globules and tubular elements. This type of carotenoid-deposition forms have been associated with higher carotenoid bioaccessibility.

The ketocarotenoids, sapotexanthin and cryptocapsin, found in mamey sapote fruits are important due to their potential provitamin A activity; however, further studies to determine this activity should be performed. Variations in both content and profile found in the carotenoids of the genotypes were hypothesized to be potentially related to differences in gene expression or post-translational regulation of the main enzymes for carotenoid biosynthesis. In vitro studies suggest a low bioaccessibility of ketocarotenoids from mamey sapote fruits, which was enhanced by thermal treatment and oil addition. Complementing *in vitro* models with *in vivo* bioavailability assays are also necessary to determine if absorption tendency is similar, since other factors related to the individual human organism could affect absorption of these ketocarotenoids.

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Abbreviations

APCI	Atmospheric-pressure chemical ionization
BHT	2,6-di-tert-butyl-p-cresol
CCS	Capsanthin-capsorubin synthase
DAD	Diode array detector
DMAPP	Dimethylallyl diphosphate
DOXP	1-deoxy-D-xylulose 5-phosphate
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose 5-phosphate synthase
EtOAc	Ethyl acetate
FAO	Food and Agriculture Organization
FW	Fresh weight
GGPP	Geranylgeranyl pyrophosphate
GGPS	Geranylgeranyl pyrophosphate synthase
HDR	1-hydroxy-2- methyl-2-(E)-butenyl 4-diphosphate reductase
HMPP	1-hydroxy-2- methyl-2-(E)-butenyl 4-diphosphate
HPLC	High performance liquid chromatography
IDI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl-diphosphate
LCY	Lycopene cyclase
MeOH	Methanol
MEP	2-C-methyl-erythritol-4-phosphate
mg	Milligrams
Min	Minutes
MS	Mass spectrometer
MWCF	Molecular weight correction factor
NXS	Neoxanthin synthase
PB	Petroleum benzin (light petroleum, b.p. 40-60 °C)
PSY	Phytoene synthase
PTFE	Polytetrafluoroethylene
RAE	Retinol activity equivalent
sec	Seconds
TBME	*tert* Butyl Methyl Ether
TRL	Triacylglycerol-rich lipoprotein
TSS	Total soluble solids
µm	Micrometers
UV-Vis	Ultraviolet–visible spectroscopy
VDE	Violaxanthin de-epoxidase
WHO	World Health Organization
ZEP	Zeaxanthin epoxidase
β-CH	β-carotene hydroxylase cyclase
β-LCY	Lycopene- β-cyclase
ε- CH	ε-carotene hydroxylase cyclase
ε- LCY	Lycopene-ε-cyclase

1. Introducción

Los carotenoides son pigmentos liposolubles derivados del isopentenil difosfato que se encuentran distribuidos ampliamente en la naturaleza y pueden ser encontrados en una gran diversidad de frutos (Ötles y Çagind, 2008). El ser humano no sintetiza estos compuestos, por lo que su obtención depende de la ingesta de alimentos ricos en carotenoides (Rodríguez-Amaya y Kimura, 2004). Estos pigmentos otorgan una serie de beneficios para la salud humana; por ejemplo, α y β-caroteno se utilizan como fuentes de provitamina A. Debido a su capacidad de captación de radicales libres, los carotenoides tienen una alta actividad antioxidante, también estimulan el sistema inmune y posiblemente ayudan a prevenir enfermedades cardíacas e inclusive el cáncer (Bendich, 1989; Deming et al., 2002). Los frutos constituyen una fuente importante de carotenoides; en las plantas estos compuestos son los encargados de darle color a los frutos, en conjunto con otros pigmentos como antocianinas y betalaínas.

Mientras que los alimentos de origen vegetal pueden proporcionar provitamina A en forma de carotenoides específicos, la vitamina A preformada se puede obtener también a partir de de fuentes de origen animal como el salmón, la yema de huevo, los crustáceos y la trucha (Rodríguez-Amaya, 2010). Sin embargo, el acceso a estas fuentes alternativas es más limitado para gran número de personas por su alto costo, en comparación con frutos o vegetales. Además, a partir de fuentes de origen animal se obtiene vitamina A preformada, forma en la que esta vitamina puede llegar a ser tóxica si se consume en altas cantidades. Igualmente se han encontrado efectos adversos en la salud con la suplementación con dosis altas (30 mg diarios) de β-caroteno en la dieta (The ATBC Cancer Prevention Study Group, 1994; Omenn et al., 1996). Mientras que un consumo excesivo de frutas y vegetales ricos en carotenoides no ha mostrado tener un efecto adverso sobre la salud. Además, el consumo de frutos y vegetales ricos en carotenoides constituye un mejor sistema de control, ya que, el metabolismo humano determina si es necesaria o no la conversión de carotenoides en vitamina A (FAO/WHO, 2002). Es por esto que existe un gran interés en explorar fuentes con alto contenido de carotenoides de origen vegetal.

Los países tropicales cuentan con una ventaja al ofrecer una gran variedad de frutos con altas concentraciones de carotenoides tales como la papaya y el mango; sin embargo,

muchos de estos países también cuentan con una alta deficiencia de vitamina A. La deficiencia de esta vitamina es un problema de salud que prevalece en al menos 122 países, en los cuales es considerado un riesgo de salud de moderado a severo (WHO, 2009). De ahí el interés en estudiar potenciales fuentes que sean más accesibles para países pobres. Se han realizado diversos estudios con el fin de caracterizar la composición de estos pigmentos en varios frutos, como en la naranja, el tomate y la zanahoria (Bramley, 2002; Frey-Wyssling y Schwegler, 1965; Hansen y Chiu, 2005; Hornero-Méndez y Mínguez-Mosquera, 2007; Pupin et al., 1999; Shi y Le Maguer, 2000; Thomson, 1966). No obstante, existe gran cantidad de frutos que todavía no han sido estudiados o se ha realizado una descripción muy básica de su composición, como el caso del zapote (Pouteria sapota (Jacq.) H.E.Moore & Stearn).

El zapote es un fruto tropical que se obtiene del árbol Pouteria sapota, nativo de México y Centroamérica, que crece en regiones tropicales y subtropicales, generalmente en climas cálidos y húmedos (Quesada, 1996; Yahia et al., 2011). Esta fruta presenta una cáscara áspera, semi-leñosa y firme con una tonalidad café oscura. El mesocarpio carnoso constituye la parte comestible, que presenta una coloración salmón claro/rosada antes de madurar, el cual se torna de un color anaranjado/rojo profundo y brillante cuando completa su maduración (Campbell et al., 1997). El zapote suele ser consumido como fruto fresco, aunque también ha sido empleado para la elaboración de helados, dulces y otros postres (Alia-Tejacal et al., 2007; Quesada, 1996).

El zapote sufre cambios drásticos en la firmeza, coloración y contenido de carotenoides durante el periodo de maduración (Villanueva-Arce et al., 2000). La cantidad de carotenoides totales se incrementa considerablemente, llegando inclusive a triplicarse cuando se compara el fruto recién cosechado con un fruto en periodo pos-climatérico. Este efecto resulta evidente con el cambio de coloración que experimenta el mesocarpio (Alia-Tejacal et al., 2007).

Desde el punto de vista nutricional, el zapote contiene vitamina C, carotenoides, tiamina, riboflavina y niacina, además de tener un alto porcentaje de fibra, calcio, hierro y potasio. Estudios preliminares atribuían su coloración a la presencia de α- y β-caroteno (Alia-Tejacal

et al., 2007); sin embargo, nuevos hallazgos apuntan a la presencia de cetocarotenoides, sapotexantina y criptocapsina, como los principales pigmentos presentes en el fruto (Murillo et al., 2011; Murillo et al., 2013). El árbol de zapote presenta una alta variabilidad dado que la propagación de este árbol se realiza mayoritariamente por semilla, lo cual permite una alta heterogeneidad genética con la consecuente variabilidad en las características de los frutos (Alia-Tejacal et al., 2007). Se han descrito diferentes genotipos de zapote (Monge y Guevara, 2000). Se pueden apreciar diferencias como cambios en el tamaño, forma, cantidad de semillas, diferente coloración de la cáscara y pulpa (Quesada, 1996). Se han descrito distintas coloraciones en la pulpa como, anaranjado, salmón y rojizo, lo que podría representar una diferencia en el contenido de carotenoides; así como en los valores de azúcares reductores, proteínas, azúcares totales, carbohidratos, pH y grados Brix (Gazel Filho et al., 1999). Estudios con otros frutos, como chile y pitanga, que comparan diferentes genotipos, han encontrado variaciones en el contenido y perfil de carotenoides (de Azevedo-Meleiro y Rodríguez-Amaya, 2009; De Rosso y Mercadante, 2005; Farnham y Kopsell, 2009; Levy et al., 1995). El zapote presenta perfiles interesantes de carotenoides; sin embargo, existe poco conocimiento acerca de la bioaccesibilidad de estos pigmentos y el potencial beneficioso para el ser humano. Al comparar diferentes genotipos de este fruto se podría determinar el más promisorio como fuente de carotenoides.

Una parte esencial para establecer los beneficios que se pueden obtener a partir de un fruto, es determinar la biodisponibilidad y bioaccesibilidad de sus compuestos bioactivos. La biodisponibilidad de los carotenoides corresponde a la fracción de una dosis oral de un compuesto o metabolito activo que llega al sistema circulatorio (Schumann et al., 1997), mientras que la bioaccesibilidad corresponde a la capacidad del proceso de digestión para liberar los carotenoides de la matriz (Goñi et al., 2006). Al estar ambos conceptos tan relacionados, una alta bioaccesibilidad puede implicar una alta biodisponibilidad. Ambas se ven afectadas por diversos factores, entre ellos su estructura molecular, propiedades físico-químicas, la matriz en la que se encuentren, ultraestructura de los cromoplastos, contenido de lípidos y el procesamiento que reciban (Deming et al., 2002). El contenido de lípidos, ya sea propio de la matriz o por adición, puede incrementar la bioaccesibilidad y biodisponibilidad de carotenoides, puesto que promueven la liberación de las sales biliares

y el aumento de tamaño de las micelas, optimizando la absorción de estos compuestos (Canene-Adams y Erdman, 2009; Mu y Høy, 2004; van den Berg, 1999). En cuanto al procesamiento, cuando los carotenoides se encuentran dentro de la matriz de los alimentos, estos están protegidos por la estructura celular y son menos vulnerables a sufrir alteraciones o degradación. Esta protección ocasiona que su biodisponibilidad disminuya (Rodríguez-Amaya, 1999). Cuando los frutos son pelados, cortados o preparados en forma de pulpa, las proteínas se empiezan a desnaturalizar y se rompen las membranas celulares, haciendo que los carotenoides sean más bioaccesibles; sin embargo, los carotenoides también se exponen a la acción del oxígeno o a la oxidación por parte de lipooxigenasas, lo que potencialmente podría disminuir su disponibilidad (Britton y Khachik, 2009; Mercadante, 2007; Pénicaud et al., 2011; Rodríguez-Amaya, 1999).

La distribución de carotenoides en los frutos y su estado de deposición en las células vegetales deben ser considerados por su impacto sustancial en la bioaccesibilidad y biodisponibilidad de los mismos. Los carotenoides, por lo general, son biosintetizados y depositados en plastidios (Cazzonelli y Pogson, 2010), en tejidos no fotosintéticos, éstops se almacenan en cromoplastos. El efecto de diferentes tipos de cromoplastos en la bioaccesibilidad de β-caroteno y licopeno ha sido descrito en un estudio comparativo entre zanahoria, mango, papaya y tomate (Schweiggert et al., 2012). En este trabajo, la liberación de los carotenoides acumulados en cromoplastos cristaloides fue menor que la obtenida a partir de cromoplastos globulares o globular-tubulares. Debido a lo anterior, la caracterización ultraestructural, así como los estudios de bioaccesibilidad resultan útiles para evaluar el potencial de una fruta en particular como fuente de carotenoides, lo que a su vez permite su utilización como base para la predicción y diseño de estudios en humanos.

En Costa Rica no se conocen plantaciones extensas en monocultivo de zapote. Frecuentemente se cultiva esta especie en asociación con otros frutales (cacao, aguacate o papaya). También es común observarlos en potreros y patios de casas, principalmente en las regiones del Pacífico Norte y el Atlántico (Quesada, 1996). El poder confirmar un alto aporte de carotenoides y una alta bioaccesibilidad, podría incrementar el interés en el aprovechamiento de este fruto e incentivar su cultivo.

En el siguiente estudio, los frutos de zapote fueron evaluados para elucidar el perfil detallado de carotenoides con análisis de HPLC-DAD-MS^2 (Cromatografía de liquidos de alta resolución con detector de arreglo de diodos y espectrometría de masas), para determinar la bioaccesibilidad de los carotenoides correspondientes, y para caracterizar la estructura de sus cromoplastos por medio de microscopía de luz y electrónica de transmisión. Con el fin de lograr los objetivos, se utilizaron metodologías muy diferentes; por lo tanto el trabajo de tesis fue dividido en cinco capítulos. El primero incluye aspectos generales de los carotenoides, seguido por un capítulo que detalla características morfológicas y físico-químicas de los frutos de zapote. El tercer capítulo se concentra en la estandarización de metodologías para la extracción de carotenoides y análisis por medio de HPLC-DAD-MS^2. El cuarto capítulo incluye un ensayo para la determinación in vitro de la bioaccesibilidad de los carotenoides presentes en el zapote comparado con otras fuentes como el chile y el salmón, así como la elucidación de la ultraestructura de los cromoplastos presentes en el zapote.

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2. Introduction

Carotenoids are pigments derived from isopentenyl diphosphate widely distributed in nature and responsible for the red, orange and yellow colors in numerous fruits, flowers and other plant parts (Ötles and Çagind, 2008). Humans are unable to synthetize vitamin A *de novo* and, therefore, must derive vitamin A from the diet (Rodríguez-Amaya and Kimura, 2004). These pigments can provide numerous health benefits; for example, α- and β-carotene are known for their provitamin A activity. Due to their efficient radical scavenging properties, carotenoids exert a high antioxidant activity, boosting the immune system, possibly helping to prevent heart diseases and even cancer (Bendich, 1989; Deming *et al.*, 2002). Fruits and vegetables represent an important dietary source of carotenoids (Ötles and Çagind, 2008).

While plant foods provide provitamin A in form of specific carotenoids, preformed vitamin A can also be obtained from animal sources like salmon, egg yolk, crustacean and trout (Rodríguez-Amaya, 2010). However, these sources are more expensive than vegetable or fruit alternatives. Overdose of preformed vitamin A can be toxic and supplementation with high daily doses of β-carotene (30 mg) has shown negative effects on health (Omenn *et al.*, 1996; The ATBC Cancer Prevention Study Group, 1994). However, excessive consumption of fruits rich in carotenoids have not proven to be detrimental for human health. Additionally, ingestion of fruits and vegetables rich in carotenoids constitutes a better control system, since human metabolism regulates the conversion of carotenoids to vitamin A (FAO/WHO, 2002). Therefore, there is great interest to explore new plant sources with high carotenoid content.

Tropical countries possess a wide variety of fruits with high carotenoid content including papaya and mango. However, many of these countries also have a high vitamin A deficiency rate. This deficiency has been catalogued as a moderate to severe health risk in at least 122 countries (WHO, 2009). Hence the interest to study promissory carotenoid sources that are more accessible for poor countries. Fruits and vegetables, like oranges, tomatoes and carrots have been widely studied (Bramley, 2002; Frey-Wyssling and Schwegler, 1965; Hansen and Chiu, 2005; Hornero-Méndez and Mínguez-Mosquera, 2007; Pupin *et al.*, 1999; Shi and Le Maguer, 2000; Thomson, 1966). However, some other fruits, such as mamey sapote,

have not been fully studied or detailed information about their carotenoid composition is scarce.

Mamey sapote (*Pouteria sapota* (Jacq.) H.E.Moore & Stearn) is a tropical fruit native to Mexico and Central America, which generally grows under warm and humid climates (Quesada, 1996; Yahia *et al.*, 2011). This fruit has a rough brown peel, while the flesh is soft with color variations between light pink, red, orange or even yellowish tones when ripe (Campbell *et al.*, 1997). Mamey sapote fruits are usually consumed fresh, but they have also been used to prepare ice cream, sweets and other desserts (Alia-Tejacal *et al.*, 2007; Quesada, 1996).

From the nutritional point of view, this fruit contains vitamin C, carotenoids, thiamine, riboflavin and niacin, in addition to high fiber, calcium and iron contents (Alia-Tejacal *et al.*, 2007). Preliminary analysis of carotenoid contents attributed the flesh color to the presence of α- and β-carotene (Alia-Tejacal *et al.*, 2007); however, recent studies point out to the presence of ketocarotenoids, like sapotexanthin and cryptocapsin, as main pigments (Murillo *et al.*, 2011; Murillo *et al.*, 2013). Mamey sapote plants present high variability, because they are usually propagated through seeds allowing wide heterogeneity between fruits (Alia-Tejacal *et al.*, 2007). Variations in size, shape, number of seeds and flesh color for different genotypes have been described (Monge and Guevara, 2000; Quesada, 1996). Another study also reported differences in pH, proteins, reducing sugars, total sugars and soluble solid contents (Gazel Filho *et al.*, 1999). Genotype variations in other fruits, like pepper and acerola, have been associated with differences in the carotenoid profiles and contents (de Azevedo-Meleiro and Rodríguez-Amaya, 2009; De Rosso and Mercadante, 2005; Farnham and Kopsell, 2009; Levy *et al.*, 1995). Therefore, it is expected to see these variations when evaluating mamey sapote individuals with different flesh colors. *P. sapota* fruits present an interesting carotenoid profile including numerous esters; however, little is known about potential benefits of their consumption. By comparing different genotypes, it is expected to determine its potential as dietary carotenoid source.

In order to establish potential benefits that can be obtained from a food, bioavailability and bioaccessibility of the bioactive compounds should be assessed. Bioavailability corresponds

to the "fraction of an oral dose of a parent compound or active metabolite that reaches the systemic circulation" (Schumann *et al.*, 1997), while bioaccessibility involves the capacity of the digestion process to free the carotenoids from the food matrix (Goñi *et al.*, 2006). Since these concepts are so related, high bioaccessibility could imply high bioavailability. Both can be affected by several factors like carotenoid molecular structure, characteristics of the matrix, chromoplast ultrastructure, lipid content and processing effects (Deming *et al.*, 2002). The addition of lipids to the ingested food increments both bioaccessibility and bioavailability of carotenoids, since lipids promote the secretion of bile salts, lipases and help to carry carotenoids and incorporate them into mixed micelles (Mu and Høy, 2004; van den Berg, 1999). The formation of micelles in the small intestine is a prerequisite for efficient carotenoid absorption, depending on the presence of bile salts (Canene-Adams and Erdman, 2009). Regarding processing, carotenoids inside their matrix are protected by the cellular structure and are less vulnerable to degradation; however, this also reduces their bioaccessibility (Rodríguez-Amaya, 1999). When fruits are peeled, chopped or blended, proteins are denaturalized and cellular membranes are disrupted; therefore, carotenoids are liberated and more bioaccessible. However, they are also exposed to oxidation, which could potentially reduce their bioavailability (Britton and Khachik, 2009; Mercadante, 2007; Pénicaud *et al.*, 2011; Rodríguez-Amaya, 1999).

Carotenoid distribution and their physical deposition state within the plant cell has to be considered due to its substantial impact on bioaccessibility and bioavailability. Carotenoids are generally biosynthesized and deposited in plastids (Cazzonelli and Pogson, 2010). In non-green plant tissues, the carotenoid-bearing structures are called chromoplasts. The effect of different types of chromoplasts on β-carotene and lycopene bioaccessibility has been already described when comparing carrot, mango, papaya and tomato (Schweiggert *et al.*, 2012). Liberation of carotenoids from crystalloid chromoplasts is lower than that from globular or globular-tubular chromoplasts. Therefore, ultrastructural characterization as well as bioaccessibility studies are useful to evaluate the potential of particular fruits as carotenoid sources and, in addition, to deduce measures for the prediction and design of human intervention studies.

In Costa Rica, mamey sapote trees are usually cultivated as shadow trees for important cultives such as cacao, avocado, papaya and coffee (Quesada, 1996). Farmers consider harvesting mamey sapote fruits as secondary or as an additional income source associated to the main crop. An interesting profile of highly bioaccessible carotenoids could increase the interest for its consumption and encourage its cultivation.

In the present work, mamey sapote fruits were investigated to elucidate their detailed genuine carotenoid profile by HPLC-DAD-MS^2 (High performance liquid chromatography with diode array detector and mass spectrometry), to determine the bioaccessibility of the corresponding carotenoids, and to characterize their chromoplast ultrastructure by light and transmission electron microscopy. In order to achieve these goals, different methods were applied and, therefore, this thesis was divided into five chapters. The first one comprises general aspects of carotenoids, followed by a chapter studying physicochemical characterization of mamey sapote fruits. The third chapter focuses on the development of a method for carotenoid extraction and HPLC-DAD analysis. In the fourth chapter, genuine carotenoid profile of three mamey sapote genotypes will be discussed in detail. The final chapter provides insights into the *in vitro* bioaccessibility of mamey sapote carotenoids as compared to those from red pepper and salmon, including the characterization of chromoplast ultrastructure of mamey sapote fruits.

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3. Objectives

a. General objective

Determine the carotenoid profile, chromoplast ultrastructure and *in vitro* bioaccessibility of the carotenoids present in different genotypes of sapote (*Pouteria sapota*) fruits, in order to evaluate its potential as provitamin A and carotenoid source.

b. Specific Objectives

- Morphologically and physicochemically characterize mamey sapote fruits.
- Elucidate the carotenoid profile and concentration of carotenoids in fruits from three mamey sapote genotypes by HPLC-DAD-MS^2.
- Describe the ultrastructure of the chromoplasts present in sapote fruits by light and transmission electron microscopy.
- Compare the bioaccessibility of the carotenoids present in sapote and other sources of related carotenoids using an in vitro digestion model.

4. Chapter I: State of the Art: Definition, biosynthesis, localization, bioaccessibility and bioavailability of carotenoids

4.1. Introduction

Carotenoids are lipid-soluble pigments widely distributed in nature and biosynthesized by plants and microorganisms (Ötles and Çagind, 2008). Animals are not able to biosynthesize these pigments; however, they can accumulate or transform them when they are ingested (Rodríguez-Amaya and Kimura, 2004). Most carotenoids are generally conformed by 40 carbon atoms in form of eight isoprene units bonded by tail–head bonds on the outside and tail-tail bonds on the inside (Delgado-Vargas and Paredes-López, 2003). In the middle of the structure a series of double bonds are found constituting the chromophore, which gives them the capacity to absorb light and determine its molecular structure and chemical activity (Fig. 4.1) (Namitha and Negi, 2010; Ötles and Çagind, 2008). All carotenoids derivate from lycopene; further reactions are carried out to obtain different carotenoid structures (Ötles and Çagind, 2008; Rodríguez-Amaya and Kimura, 2004). Carotenoid functions in plants include photo oxidation protection as well as providing color to flowers to attract pollinators to fruits for seed dispersal (Ötles and Çagind, 2008).

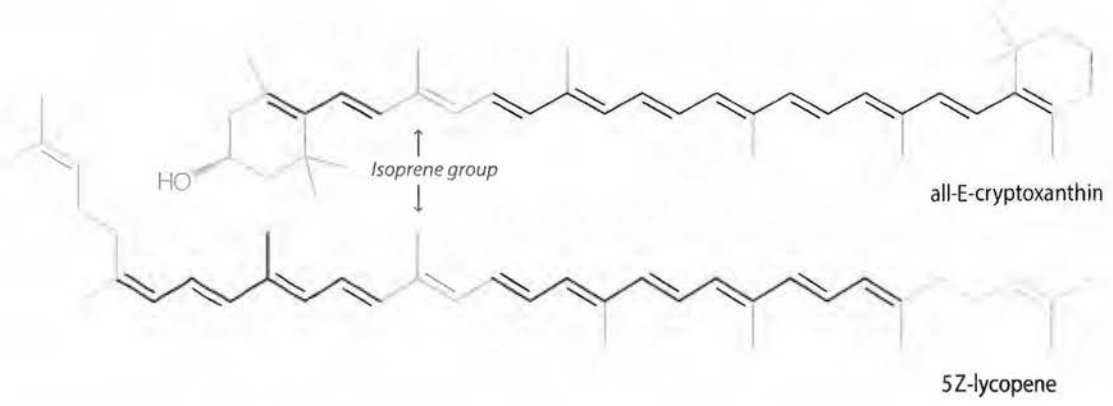

Figure 4.1. Structure of (*all*-E)-cryptoxanthin (xanthophyll) and (*5Z*)-lycopene (carotene). The black highlighted structure corresponds to the chromophore of each pigment.

The great benefit of carotenoids for the human health has been well documented. The provitamin A activity is the most studied health benefit of carotenoids (Deming *et al.*, 2002). Carotenoids, including β-carotene and those with a β-ionone end ring, act as vitamin A precursors (Woollard, 2012). These carotenoids are cleaved by a carotenoid oxygenase at

the central 15-15′ double bond to obtain retinal; in the case of β-carotene, two molecules of retinal can be obtained (Fig. 4.2).

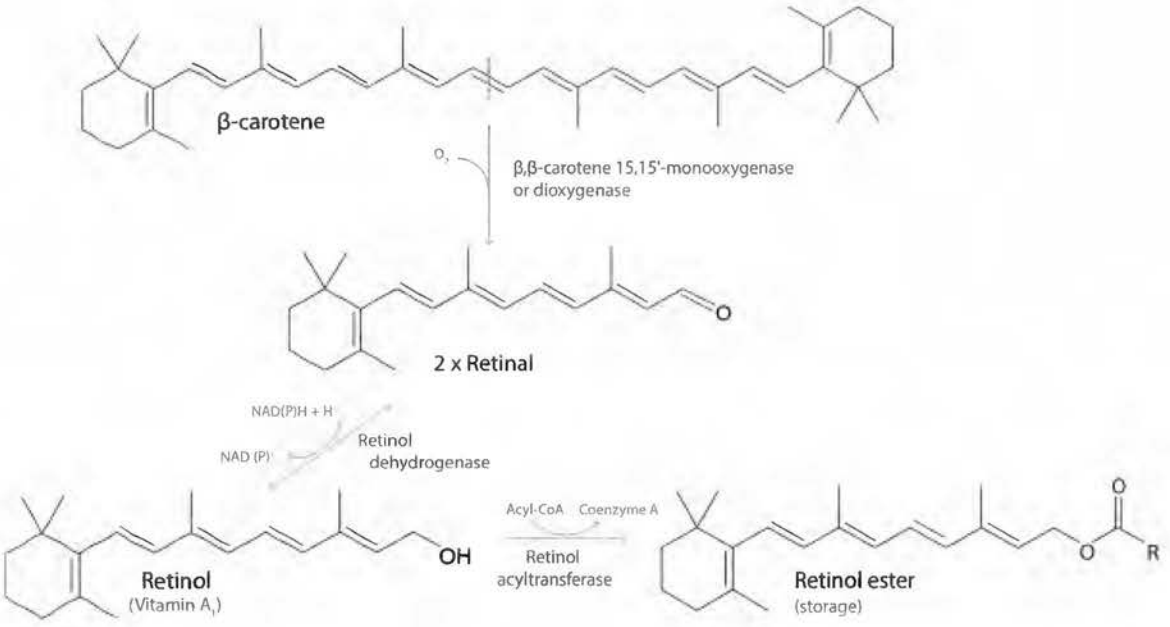

Figure 4.2. Conversion of β-carotene to retinal, retinol and retinol esters.

Vitamin A deficiency has been catalogued as a moderate to severe health issue, especially in developing countries. Deficiency of this vitamin may cause xerophthalmia, night blindness, anemia, slow growth rates, poor reproductive health and even a weakened immune system (FAO/WHO, 2002). Humans are not able to synthetize these compounds; therefore, food rich in carotenoids needs to be included in the diet (Rodríguez-Amaya and Kimura, 2004). Other biological actions of carotenoids include antioxidant activity, improvement of intercellular communication, cell differentiation inhibition during mutagenesis and immune response enhancement (Deming *et al.*, 2002).

4.2. Classification

Carotenoids are separated in two main types according to their structure; carotenes and xanthophylls (Fig. 4.1). Carotenes correspond to pure hydrocarbons, like α- and β-carotene, as well as lycopene. Xanthophylls contain at least one oxygen atom in their molecule; common substitute groups include hydroxyl, methoxyl, carbonyl, keto or epoxy groups (Lee

and Schwartz, 2005). Examples of xanthophylls are cryptoxanthin, neoxanthin, violaxanthin and astaxanthin (Rodríguez-Amaya and Kimura, 2004).

Regarding their health benefits, carotenoids can also be divided depending if they are provitamin A precursors or not. Provitamin A precursors include β-carotene, α-carotene, β-cryptoxanthin, β-canthaxanthin and β-echinenone, due to the presence of a β-ionone end ring in their structure (Woollard, 2012). Non-provitamin A carotenoids include lycopene, zeaxanthin and lutein (Olson, 1994).

4.3. Biosynthesis

Carotenoids cannot be synthetized by humans and there has been only one report of carotenoid biosynthesis in animals by pea aphids and the spider mites (Cobbs *et al.*, 2013). In plants, biosynthesis is carried out in plastids, mainly in chloroplasts and chromoplasts. The pathway starts with the condensation of pyruvate and glyceraldehyde-3-phosphate to form 2-C-methyl-erythritol-4-phosphate (MEP) with the help of two enzymes, 1-deoxy-D-xylulose 5-phosphate (DOXP) synthase (DXS) and DOXP reductoisomerase (DXR) (Bramley, 2002) (Fig. 4.3). Next, the MEP molecule is reduced by the 1-hydroxy-2- methyl-2-(E)-butenyl 4-diphosphate (HMBPP) reductase (HDR) to obtain the HMBPP. From the latter, the isomers, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are obtained; they are continuously interconverting by the action of the isopentenyl-diphosphate isomerase (IDI) (Cuttriss *et al.*, 2006). The binding of four molecules of IPP and one of DMAPP next to the action of the geranyl phosphate synthase (GGPS) produce the geranyl pyrophosphate (GGPP). Two molecules of GGPP are converted by the phytoene synthase (PSY) into phytoene, considered the first colorless carotenoid (Cazzonelli and Pogson, 2010). This last step is regulated by external stimuli like temperature, photoperiod, exposure to light and drought (Cazzonelli and Pogson, 2010; Cuttriss *et al.*, 2006). The phytoene molecule goes through a series of desaturation steps until it is converted into lycopene. Further cyclization steps result in the formation of α- and β-carotene, from which xanthophylls are synthetized (Cazzonelli *et al.*, 2010).

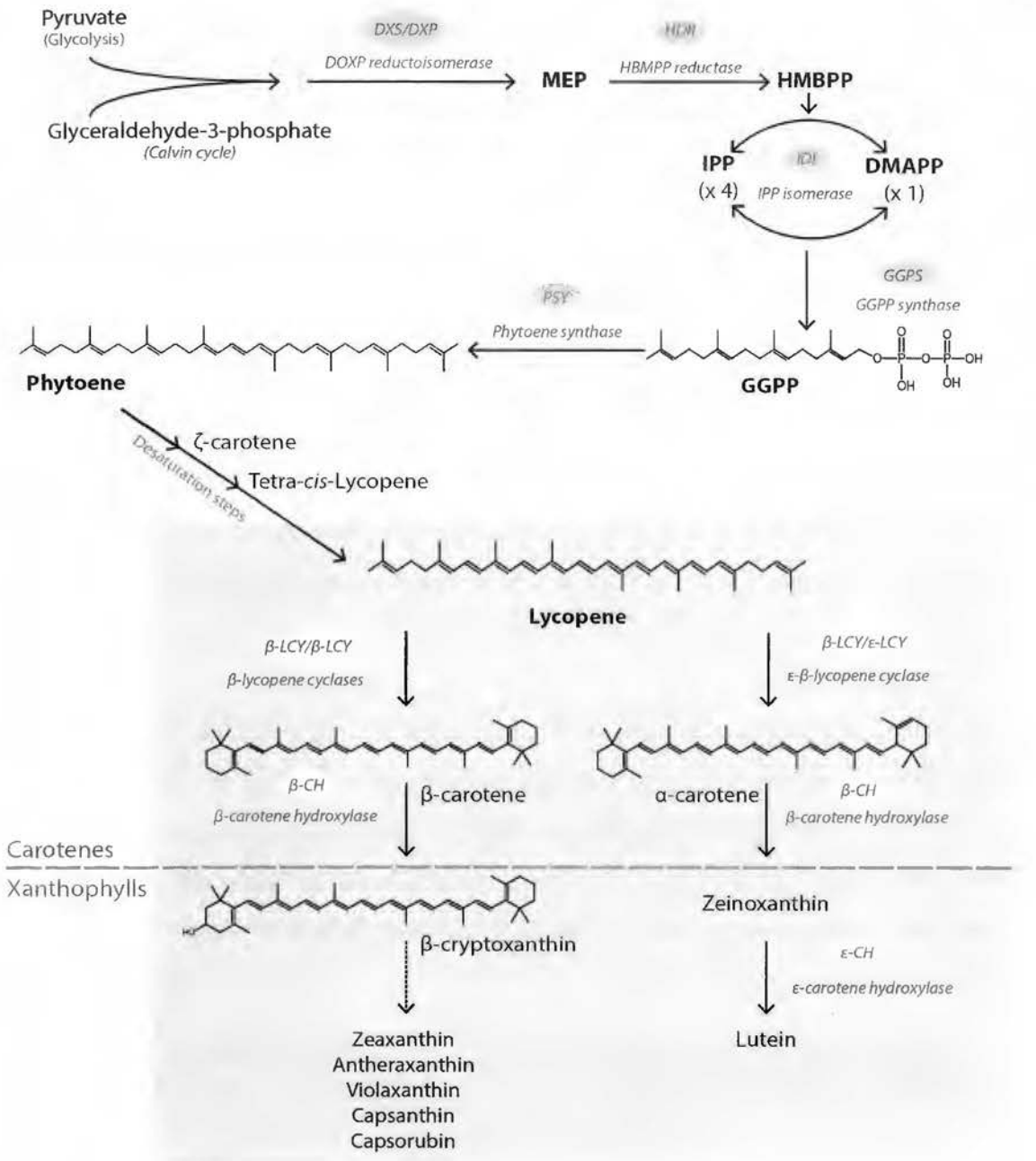

Figure 4.3. Carotenoid biosynthetic pathway in plastids. DOXP: 1-Deoxy-D-xylulose 5-phosphate, MEP: 2-C-Methylerythritol 4-phosphate, HMBPP: 1-hydroxy-2- methyl-2-(E)-butenyl 4-diphosphate, IPP: Isopentyl Diphosphate, DMAPP: Dimethylallyl diphosphate, GGPP: Geranylgeranyl pyrophosphate.

4.4. Carotenoid localization

Carotenoids in green plant parts are considered accessory pigments for photosynthesis and, therefore, they are accumulated in specialized subcellular organelles that belong to photosynthetic tissues like the chloroplast (Ötles and Çagind, 2008; Wieslaw, 2010). In general, carotenoids have an hydrophobic nature and, therefore, they are commonly found associated to the plastid's lipid membranes (Wieslaw, 2010). The location of the carotenoids within the membrane is determined by their structure. For instance, those with a more polar structure, e.g. with hydroxyl substitutes at one or both sides of the molecule, tend to accommodate their chromophore within the hydrophobic nucleus of the membrane (Fig. 4.4) (Deming *et al.*, 2002). The orientation will rely mainly on the substitutes present and their capacity to establish hydrogen bonds with the polar zones of the membrane (Wieslaw, 2010). In the case of lutein, it has been proven that it can be oriented both horizontally and vertically due to the presence of the –OH groups on both ends of the molecule (Pasenkiewicz-Gierula *et al.*, 2012; Sujak *et al.*, 1999). Similarly, zeaxanthin can also attach to the membrane in both orientations; depending if it is in *cis* or *trans* configuration (Widomska and Subczynski, 2008). Apolar carotenoids, like β-carotene, have certain movement freedom within the hydrophobic region of the membrane as they cannot establish hydrogen bonds with the polar lipids at the surface of the membrane or the polar phase (Wieslaw, 2010).

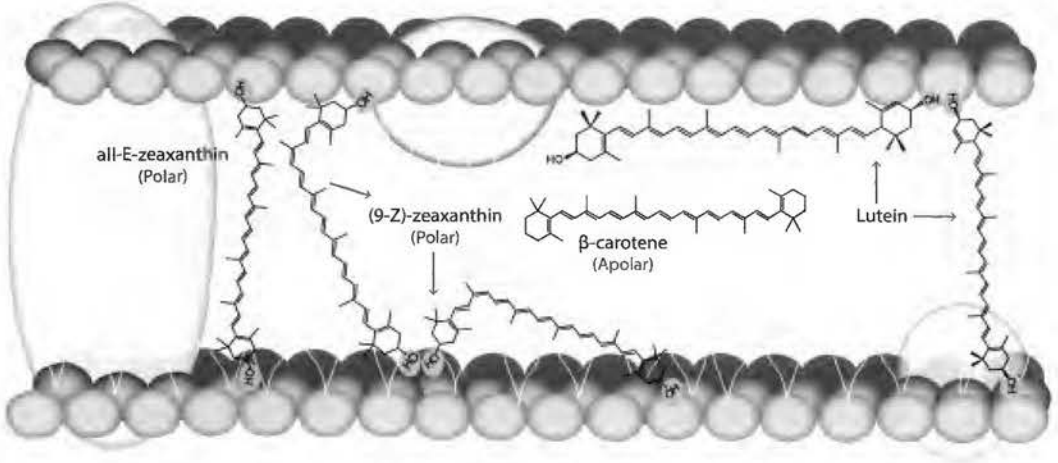

Figure 4.4. Orientation of polar (zeaxanthin, lutein) and apolar (β-carotene) carotenoids within the lipid membrane. Circles around -OH end groups indicate bonding with membrane polar zones.

In non-photosynthetic carotenoid-rich plant tissues, carotenoids are commonly found inside specialized plastids called chromoplasts. When fruits ripen, the photosynthetic system is degraded and the thylakoid membranes disintegrate allowing the transformation of chloroplasts into chromoplasts (Britton, 2008; Hudák *et al.*, 2005). Chromoplasts can also originate *de novo* from undifferentiated plastids called proplastids (Evert, 2006). Chromoplasts constitute the group of plastids with the highest morphological variation (Fig. 4.5), thus they usually are classified according to their structure (Evert, 2006). Globular chromoplasts are the most common ones, and are present in oranges (Hudák *et al.*, 2005) and mango fruits (Vásquez-Caicedo *et al.*, 2006). Other chromoplast structures may be membranous, tubular, globular-tubular and crystalloid (Egea *et al.*, 2010; Hudák *et al.*, 2005). Inside the chromoplasts, carotenoids are frequently found in plastoglobules (Fig. 4.6), these globular lipid deposits formed by a lipid monolayer that vary in size (Bréhélin *et al.*, 2007). The number of plastoglobules increase during fruit ripening; with accumulating carotenoid concentrations, their crystallization may be induced as for example in the case of β-carotene in carrots or lycopene in tomatoes (Ben-Shaul and Shimon, 1965; Harris and Spurr, 1969). Distribution of carotenoids in fruits is complex and heterogeneous, varying depending on the species, ripening stage, planting location and postharvest handling (Rodríguez-Amaya and Kimura, 2004).

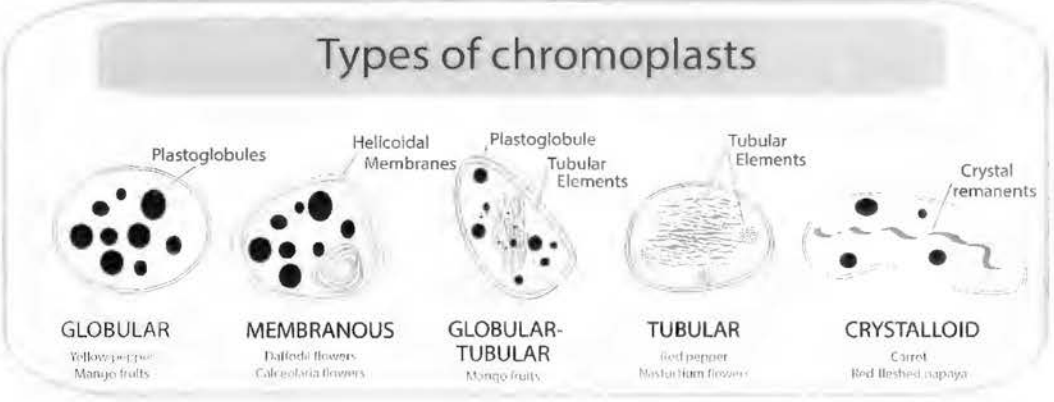

Figure 4.5. Different chromoplast structures found in plants including some examples. Examples described in literature for mango (Vásquez-Caicedo *et al.*, 2006), yellow pepper (Evert, 2006), daffodil flowers (Liedvogel *et al.*, 1976), calceolaria flowers (Wrischer and Ljubesic, 1984), nasturtium flowers and red bell pepper (Deruère *et al.*, 1994), papaya (Schweiggert *et al.*, 2011a) and carrot (Straus, 1950).

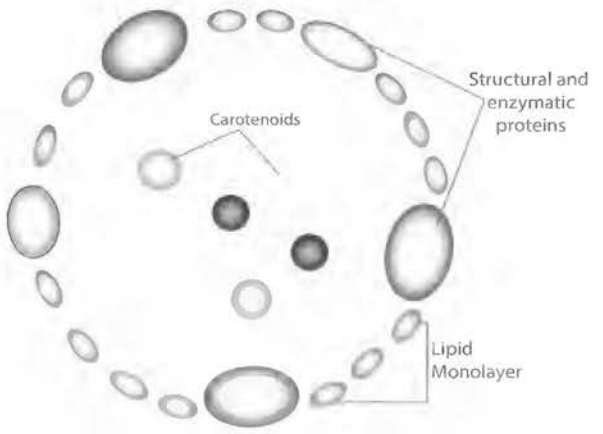

Figure 4.6. Plastoglobule structure model containing carotenoids.

4.5. Bioavailability and bioaccessibility

In order to be beneficial for human health, carotenoids need to be bioavailable, i.e. liberated from the food matrix and absorbed to the human blood stream (Huo *et al.*, 2007). Bioavailability has been defined and perceived differently between researchers; in general, it has been described as "proportion of a nutrient in food that is utilized for normal body functions" (Fairweather-Tait and Southon, 2003). Most studies in carotenoids define bioavailability as "the fraction of an oral dose of a parent compound or active metabolite that reaches the systemic circulation" (Schumann *et al.*, 1997). Therefore, most bioavailability assessment studies focus on the carotenoid concentrations in plasma and plasma fractions, such as the triacylglycerol-rich lipoprotein (TRL) fraction containing newly absorbed carotenoids (Alminger, 2012; Asai *et al.*, 2008; Rock *et al.*, 1998; Schweiggert *et al.*, 2014). In figure 4.7, carotenoid absorption from the food matrix until the accumulation in human tissues is illustrated. Initially, carotenoids are deposited in the food matrix as described above. Carotenoids are partially liberated during food processing and kitchen preparation. In addition, chewing breaks down physical matrix components when they are ingested (Kotake-Nara and Nagao, 2012). Once in the stomach, these pigments are incorporated into lipid globules derived from dietary co-consumed lipids. In the small intestine, secreted bile salts and lipases help to break these globules and reduce their size in order to ultimately form micelles (Olson, 1994). Micelles are molecular aggregates able to transport hydrophobic compounds, acting as highly efficient emulsifiers

and encapsulating carotenoids for their transport through the small intestine and later incorporation into the intestinal mucosa. Micelles are constituted by bile salts, cholesterol, fatty acids, mono acyl glycerides and phospholipids. Carotenoid incorporation in the intestinal cells is mediated by simple or facilitated diffusion (Kotake-Nara and Nagao, 2012). After incorporation in the intestinal mucosa, the enterocyte's Golgi apparatus packs the compounds in chylomicrons to be transported into the lymph and blood torrent (Alminger, 2012; Parker, 1996). Once in the blood torrent, they can be distributed to the liver, where synthesis and accumulation of vitamin A takes place (Canene-Adams and Erdman, 2009; Deming *et al.*, 2002).

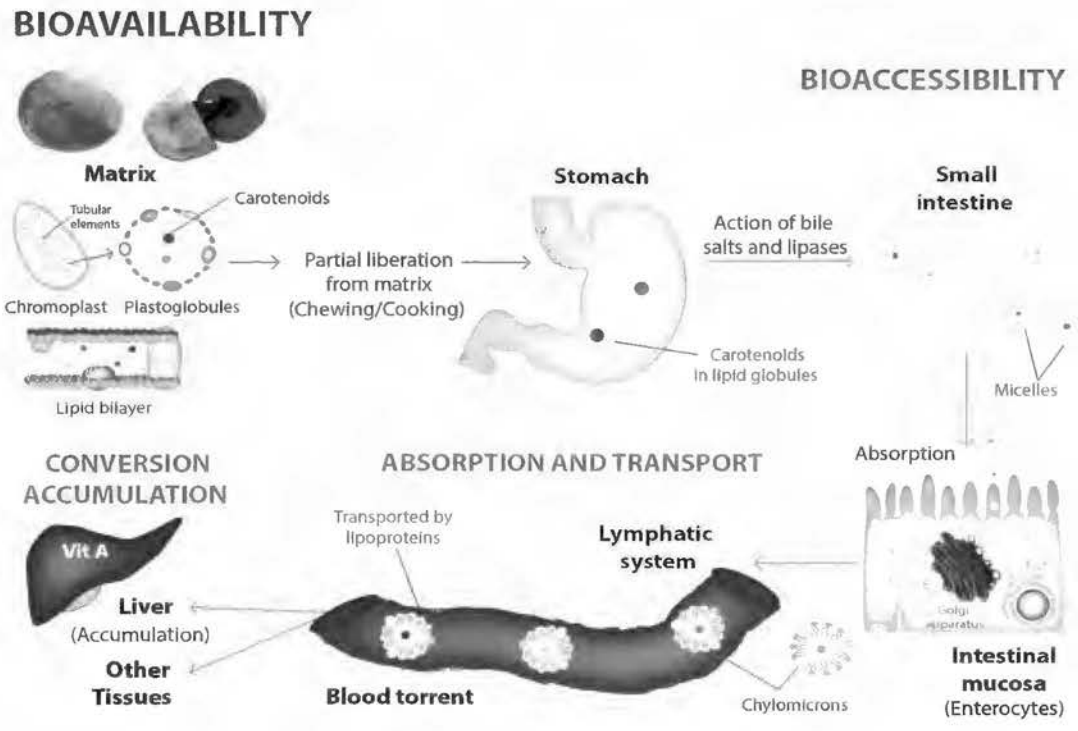

Figure 4.7. Carotenoid absorption process diagram, from food matrix to human body.

The absorption and conversion of carotenoids into vitamin A is affected by several factors, including their molecular structure, physico-chemical properties, food matrix, processing, addition of lipids, among others (Deming *et al.*, 2002). Addition of a lipid source can promote the formation of larger micelles and has been associated to an increase in membrane permeability leading to enhanced carotenoid absorption (Kotake-Nara and

Nagao, 2012; Ornelas-Paz *et al.*, 2008). The amount of carotenoids incorporated into micelles is also affected by the particular carotenoid polarity and the composition and saturation degree of the fatty acids constituting the micelles (Yeum and Russell, 2002). The use of heat can also enhance the carotenoid absorption; even though a portion of the carotenoids are degraded by this treatment, the amount released from the matrix is higher (Boon *et al.*, 2010). The combination of heat treatment and lipid addition has been proven to increase bioavailability of lycopene from tomatoes (Fielding *et al.*, 2005). Carotenoid polarity also has an effect on absorption: polar compounds can be incorporated more easily into micelles than apolar pigments, like β-carotene (Olson and Mello, 2012; Yeum and Russell, 2002). Another important factor is the food matrix and subcellular localization of the carotenoids; free carotenoids or those inside chromoplasts are easier to extract than those within lipid bilayers (During, 2007). In fruits, carotenoids are mainly stored inside chromoplasts: Changes within chromoplast types constitute one of the main barriers to access carotenoids.

4.6. Fruits as promissory carotenoid sources

Tropical fruits, in general, have been a target to carotenoid studies in order to find new accessible sources of these compounds. Recent studies have evaluated carotenoid contents in papaya (Schweiggert *et al.*, 2011b), mango (Vásquez-Caicedo *et al.*, 2006), peach palm (Hempel *et al.*, 2014; Rojas-Garbanzo *et al.*, 2011), naranjilla (Gancel *et al.*, 2008) and mamey sapote (Murillo *et al.*, 2011).

Mamey sapote (*Pouteria sapota*) fruit, the subject of study in this work, has an ovoid – elliptical form, brown and rough skin and a variety of flesh colors, from yellowish tones to red. The presence of this bright flesh color has attracted the attention for its potential use as a carotenoid source. Recent studies indicate the presence of ketocarotenoids, responsible for the red coloration (Deli *et al.*, 2011; Murillo *et al.*, 2011; Murillo *et al.*, 2012). Special emphasis has been given to the presence of a newly described carotenoid named sapotexanthin (Murillo *et al.*, 2011), which constitutes one of the main pigments in mamey sapote fruits and, due to its structure, has potential provitamin A activity (Fig. 4.8).

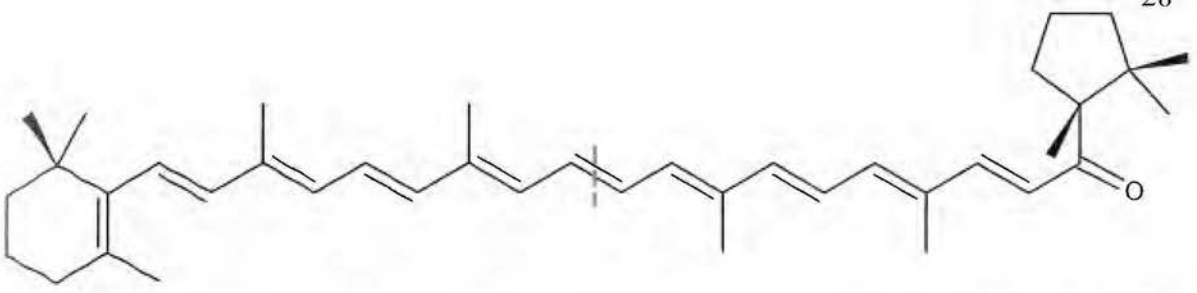

Figure 4.8. Sapotexanthin, ketocarotenoid of mamey sapote fruits. Orange dashed lines shows eventual cleavage for provitamin A activity.

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5. Chapter II: Physicochemical characterization of mamey sapote fruits, comparison with other Sapotaceae

5.1. Abstract

Mamey sapote (*Pouteria sapota*) is a tropical fruit native to Central America and Mexico, which sweet fruits present a variety of flesh colors from yellow to red or even salmon pink. The flesh color in mamey sapote fruits has been attributed to the presence of carotenoids. A characterization of morphological and physicochemical traits of mamey sapote fruits was performed in this work. The chemical composition of mamey sapote fruits is comparable to that of *Manilkara sapote* and *Pouteria viridis*. The fruits of the three species were characterized by high contents on total soluble solids, amounting 35.6 °Brix for mamey sapote. Mamey sapote fruits can be considered a good source of dietary fiber and protein with 7.65 and 2.30%, respectively. Low fat contents and a pH value near 7 are also characteristic of the fruits.

5.2. Introduction

Mamey sapote (*Pouteria sapota* (Jacq.) H.E.Moore & Stearn) is a tropical fruit native to Central America and Mexico. It belongs to the Sapotaceae family, which includes other fruits like "chicosapote" (*Manilkara sapota*), "caimito" (*Pouteria caimito*) "canistel" (*Pouteria campechiana*) and "zapote injerto" (*Pouteria viridis*) (Morera, 1992) (Fig. 5.1.). Members of the Sapotaceae family are known for their high latex content and also for their sweet edible fruits (Vaghani, 2003).

Flesh color from mamey sapote fruits ranges from yellow to red or even salmon pink (Morera, 1992). This fruit can be consumed fresh, in desserts like ice cream, jam and cakes or in drinks (Lim, 2013). Mamey sapote peel is brown with a rough texture, and between 1–2 mm thick (Lim, 2013). Fruits can vary in fruit shape, size and firmness of the pulp. There is little information about the genetic variability within members of this family. Some effort has been made to physically characterize these fruits and conduct a selection of the most promissory mamey sapote trees and evaluate the genetic diversity in the region (Campbell and Ledesma, 2006; Carrara *et al.*, 2004; Gazel Filho *et al.*, 1999).

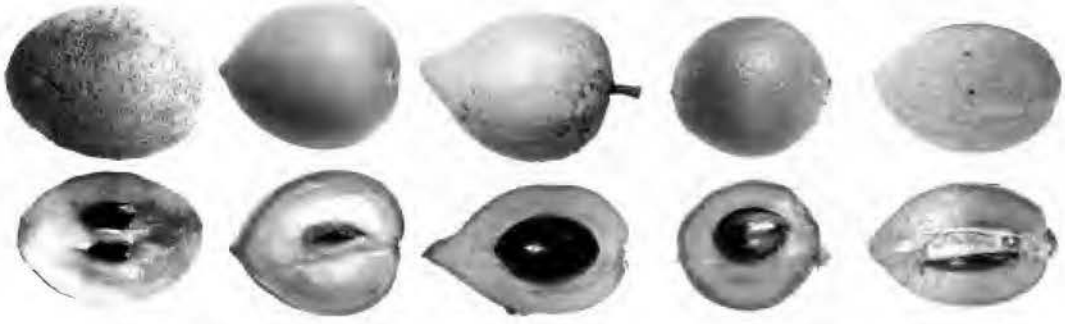

Figure 5.1. Fruits belonging to the Sapotaceae family, including "chicosapote", "caimito", "canistel", "zapote injerto" and "mamey sapote". Credit for some of the photos: ©toptropicals.com, ©Ian Maguire UF/IFAS/TREC.

In Costa Rica, sapote have two harvesting seasons; the main one, between December and March and other one between May and June. However, some trees can produce fruits all year long (Azurdia, 2006). Differences in fruit color, flesh sweetness, shape, fiber content and skin firmness have been observed; however, information about measurement of genetic diversity in mamey sapote is still scarce (Azurdia, 2006). In addition, climate can affect the fruit flesh color and sweetness; it has been described, that fruits growing in dry areas have a more intense flesh color and are sweeter (Azurdia, 2006; Quesada, 1996).

In Central America, the Tropical Agricultural Research and Higher Education Center (CATIE) has one of the most important collections of Sapotaceae from the region; it possess at least 200 partially characterized accessions of mamey sapote (Monge and Guevara, 2000). In this center, asexual propagation has been used to maintain the collection. This type of propagation can be also used to selectively propagate genotypes with better characteristics (Umaña, 1997).

The flesh color in mamey sapote fruits has been attributed to carotenoids. First reports pointed out to α- and β-carotene as main pigments (Alia-Tejacal *et al.*, 2007). However, recent findings also attribute the color to the presence of the ketocarotenoids, sapotexanthin and cryptocapsin (Murillo *et al.*, 2011; Murillo *et al.*, 2013).

This chapter focuses on the physicochemical and morphological characterization of mamey sapote fruits from one of the red-orange genotype (11129).

5.3. Materials and methods

5.3.1. Project location. Analyses were carried out at the School of Food Technology and the National Center of Science and Food Technology at the University of Costa Rica between September and October 2013.

5.3.2. Plant material. Four fruits from *P. sapota* 11129 genotype were sampled from the collection of the CATIE (Turrialba, Costa Rica). Only fruits with evident yellowish-orange flesh, as evaluated by making a little scratch on the skin, were collected. Fruits were left to ripe for 7 days at room temperature.

5.3.3. Morphological traits. Total length, diameter, total weight, pulp and seed weight, and number of seeds of the fruits were determined.

5.3.4. Physicochemical analysis.

Texture. Flesh and peel firmness were determined at ten different points around the mayor diameter of the fruit. Measurements were performed using a Texture Analyzer TA.XT Plus (Stable Microsystems, Surrey, UK) with a 2 mm diameter stainless steel probe. The speed was set to 1.5 mm/s with a penetration distance of 15 mm. Conditions were set during preliminary tests. Peel firmness was obtained with the measurent of the maximum force value (N) during the peel penetration, while the average value registered after peel penetration was considered as flesh firmness.

Color. Flesh color was determined with a color meter Color Flex, Hunter Lab (Hunterlab, Reston, USA) equipped with the software Spectra Manager, with a color scale D65/10°. A puree was prepared from different sections of each fruit by duplicate and the results expressed in CIE- L*, a* y b*. Measurements were performed immediately to avoid fruit oxidation.

Brix degrees. Total soluble solid (TSS) were determined by triplicate with an Abbé (Atago Co. Ltd., Tokio, Japan) refractometer directly from the sapote juice obtained by pressing the pulp with a thick fabric.

pH. Measurements of pH were performed by triplicate with pH meter (Metrohm 827, Herisau, Switzerland) directly to a fruit macerate.

Composition analysis. Parameters including water content, proteins, starch, carbohydrates, acidity, energetic value, fat and ashes were determined. Water content was determined by the method P-SA-MQ-002 based on AOAC 920.151 (AOAC, 2005), proteins by the method P-SA-MQ-003 based on AOAC 920.152 (AOAC, 2005), starch by the enzymatic method P-SA-MQ-18, acidity by the method P-SA-MQ-011 based on AOAC 942.15 (AOAC, 2005). Fat content by the method P-SA-MQ-005 based on Carpenter *et al.* (1993), dietetic fiber according to P-SA-MQ-007 based on AOAC 985.29 (AOAC, 2005), ashes with the method P-SA-MQ-004 based on AOAC 940.26 (AOAC, 2005). Finally, total carbohydrates were estimated by subtraction. Energetic value was estimated with the formula: (4 x % total carbohydrates x % protein) + 9 x % fat.

5.3.5. Statistical analysis. A one-way analysis of variance (ANOVA) test was conducted to determine significant differences between measurements of the same fruit. Shapiro-Wilk's test was conducted to test normality of the data, and homogeneity of variances was assessed by Levene's test. For non-parametric samples a Kruskall-Wallis test was conducted. When differences between samples were found, a Tukey test was applied. All analysis were performed with the program SAS JMP 8 (SAS Institute Inc. Cary, NC, USA).

5.4. Results and discussion

The selected accession of mamey sapote fruits presented a red-orange colored flesh (Fig. 5.2). Morphological traits are summarized in Table 5.1. Total fruit weight ranged between 341 g up to 506 g, presenting an ovoid shape and between 1 or 2 seeds. In general, size and shape of mamey sapote fruits can vary greatly; they can have round, ovoid or elliptic shapes; total length can also vary between 7.5 up to 22.8 cm. Size variation are reflected in their weight that has been found to be from 227 g and up to 2.3 kg (Morton, 1987).

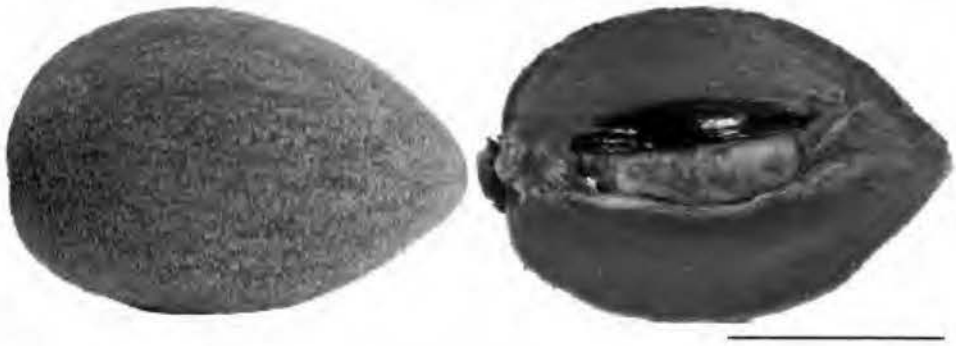

Figure 5.2. Mamey sapote fruits of evaluated genotype. Bar corresponds to 5 cm.

Table 5.1. Morphological traits and further characteristics of mamey sapote fruit analyzed (genotype 11129).

Parameter		Value
Total weigth (g)		378.46 ± 75.90
Seed weight (g)		43.53 ± 9.23
Number of seeds		1-2
Lenght (cm)		12.13 ± 1.53
Diameter (cm)		25.50 ± 1.58
Peel firmness (N)		9.91 ± 0.89
Flesh firmness (N)		1.23 ± 0.32
Color	L*	39.55 ± 2.17
	a*	42.72 ± 2.60
	b*	37.31 ± 0.3

Flesh firmness values are lower than those reported by Villanueva-Arce *et al.* (2000), who showed that unripe fruits firmness was around 35-45 N and after ripeness it went down to 3-5 N. Previous studies on mamey sapote fruit ripening have stablish a great influence of β-galacturonase with flesh softening (Arenas-Ocampo *et al.*, 2003). A significant difference was found in the peel and flesh firmness of the fruits analyzed in this work, that ranged from 8.7-10.2 N and 0.8-1.2 N, respectively.

Flesh color of the fruits analyzed was red-orange (Fig. 5.2). Color measurements based on the CIE L*a*b* system are shown in table 5.1, no significant difference ($p > 0.05$) was observed between measurements of the fruits analyzed. Lightness (L*) in mamey sapote fruits has been associated with phenolic compounds responsible for flesh darkening during ripening (Alia-Tejacal *et al.*, 2002; Díaz-Perez *et al.*, 2000), while high a* and b* values

are associated with the presence of carotenoids. Values obtained are consistent with the flesh coloration observed.

Physicochemical characteristics of the mamey sapote fruits analyzed 8genotype 11129) are described in Table 5.2 as well as data from studies in other Sapotaceae fruits for comparison. The majority of values of the parameters evaluated match the ones reported in the literature for mamey sapote fruits.

Table 5.2. Physicochemical parameters of mamey sapote (*P. sapota*) genotype 11129 and comparison with previously reported values for *P. sapota* and other Sapotaceae.

Parameter	Values for *P. sapota* fruits analyzed (genotype 11129)	*P. sapota* (Bayuelo-Jiménez et al., 2007; Gazel Filho *et al.*, 1999; Morton, 1987)	Other Sapotaceae			
			M. sapota (Andrade et al., 2009; Gazel Filho et al., 2002; Leung, 1961)	*P. caimito* (Leung, 1961; Morton, 1987; Muñoz Jáuregui et al., 2009)	*P. campechiana* (Dáger Jervis and Cornejo Hidalgo, 2003; Morton, 1987)	*P. viridis* (Crane et al., 2001; Leung, 1961; Morton, 1987)
Water content (%)	56.30 ± 1.04	55.30-73.10	71.10-75.40	74.10-87.35	60.60-87.50	60.60-69.50
Ashes (g/100 g)	1.22 ± 0.09	0.70-1.30	0.40-0.50	0.39-0.90	0.25-0.90	0.69-1.38
Protein (g/100 g)	2.30 ± 0.22	0.19-2.00	0.53-0.55	1.06-2.10	0.60-0.68	0.15-0.28
Fat (g/100 g)	0.20 ± 0.00	0.09-0.25	0.76	0.45-1.10	0.13-0.20	0.24-0.28
Dietetary fiber (g/100 g)	7.65 ± 0.25	1.20-3.20	1.60	0.90-3.00	1.00	1.20-1.60
Total starch (g/100g)	19.53 ± 1.12		-	-	-	-
Acidity (g/100 g) (expressed as citric acid)	0.075 ± 0.006	0.06-0.16 (as malic acid)	0.13	-	-	-
Total carbohydrates (g/100 g)	39.90 ± 1.09	30.00-34.80	21.00-27.80	11.10	11.40	28.60
Available carbohydrates (g/100 g)	32.23 ± 0.89	28.80-31.80	19.40-26.20	8.10	10.40	27.00-27.40
Energetic value (kcal/100 g)	140.25 ± 2.99	114.50	94.00	140	138.80-193.00	110.00
Energetic value as fat (kcal/100 g)	2.00 ± 0.00	-	-	-	7.00	-
TSS (g/100 g)	35.6 ± 0.74	30.10	16.80-22.00	13.00-18.00	11.80	25.40-32.00
pH	6.52 ± 0.083	5.80	4.70-5.10	-	-	-

Low moisture contents were observed for mamey sapote fruits analyzed in this study and reported in the literature, considering that water is one of the most abundant components of fruits and can reach up to 90 % (Vicente *et al.*, 2009). It is even lower than water content found in bananas (74 %) and plantains (62.5 %), whose values are already considered low for a fruit (Giami and Alu, 1994; Kayisu *et al.*, 1981). On the other hand, higher ash contents were found for *P. sapota* in comparison to those values reporter for other sapotaceae. Morton (1987)reported that the main minerals present in these fruits are calcium and phosphorus. Protein content in fruits and vegetables is around 1% (Vicente *et al.*, 2009), in mamey sapote this value reached up to 2.30 %. In general, fat content in fruits is also low (around 1%) and mamey sapote fruits are not the exception, with only 0.2 %. The same

behavior was observed in the other related species. Higher dietary fiber contents were established when compared to the other Sapotaceae species. Consumption of foods rich in dietary fiber has been recommended for its numerous benefits in human health, including improved digestion (Kendall *et al.*, 2010). Values obtained for mamey sapote fruits are comparable with those of guava (7.2 %) and avocado (6.8), both fruits considered high contributors to the dietary fiber daily dose (Mahattanatawee *et al.*, 2006; Vicente *et al.*, 2009).

Energetic value found in mamey sapote fruits is relatively high due to the presence of carbohydrates. This was also reflected in the elevated TSS measured around 35.6 (Table 5.2), similar to previously published data where TSS are above 30 % (Alia-Tejacal *et al.*, 2007; Díaz-Perez *et al.*, 2000; Gazel Filho *et al.*, 1999). These values are high compared to those of other fruits like papaya with just 11 ° Brix (Schweiggert *et al.*, 2012). Higher TSS contents were observed for mamey sapote, considering that values of 25.4-32 ° Brix were reported for *P. viridis* and 16.8-22 ° Brix for *M. sapota*. Sugars present in mamey sapote fruits are mainly sucrose, followed by glucose and fructose (Gazel Filho *et al.*, 1999; Villanueva-Arce *et al.*, 2000). A high content of starch, around 19 %, was observed in *P. sapote*. As far as our knowledge; there are no previous reports on the starch contents in the Sapotaceae fruits. Values obtained for this fruit are comparable with those reported for unripe banana (around 20%) (Kayisu *et al.*, 1981), which is among the fruits with highest starch content. After mamey sapote fruit ripening, a 6-fold increase in the sucrose content was reported (Alia-Tejacal *et al.*, 2005; Arenas-Ocampo *et al.*, 2003). As fruit ripens, starch is hydrolyzed to simple sugars; reduction of total starch is also expected for mamey sapote fruits. .

Protein content of mamey sapote fruits reached 2.30%, which are higher than values reported for other Sapotaceae fruits and comparable only with that of *P. caimito.* Protein contents in fruits are usually low, with values rounding 1 %. Only, scarce fruits, such as avocado, with high protein contents between 1.11-1.75 % (Mooz *et al.*, 2012).

Fruit acidity is the result of the presence of organic acids, mainly malic and citric acid (Etienne *et al.*, 2013). In mamey sapote fruits, acidity values expressed as citric acid, were

considered low (0.06%) when compared to fruits such as pineapple with 1% (Singleton and Gortner, 1965). Low acidity values (0.06-0.16) expressed as malic acid have been reported previously for mamey sapote fruits (Bayuelo-Jiménez *et al.*, 2007). Previous studies on mamey sapote fruit quality, determined that acidity values were constant throughout the maturation process (Arenas-Ocampo *et al.*, 2003; Díaz-Perez *et al.*, 2000). The low acidity values associated with a high TSS contents, resulting in an elevated ° Brix/acidity ratio, have been reported in other studies for mamey sapote fruits (Arenas-Ocampo *et al.*, 2003; Brito and Narain, 2002). A pH value near 7 can be explained due to the low titratable acid content.

5.5. Conclusions

The selected mamey sapote fruit accession 11129 presented relatively high levels of dietary fiber and protein than other fruits. Levels of total soluble solids are really high and comparable only with *P.viridis*, another member of the Sapotaceae family.Values obtained for the different parameters in the analyzed mamey sapote fruits were comparable with those previously reported in the literature. The only exception was the dietary fiber content, which was considerably higher in 11129 genotype than reported data. When compared to previous reports including other member of the Sapotaceae family, higher similarities were observed with *P.viridis* followed by *M. sapota*. Mamey sapote can be considered a fruit with a high energetic value, taking into account the starch and TSS contents, but also a source of protein and dietary fiber. Low moisture contents may be important to consider when analyzing the chemical composition of the fruit.

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6. Chapter III: Development of an extraction and quantification method for the determination of carotenoids from mamey sapote fruits by HPLC-DAD

6.1. Abstract

Carotenoids are lipid-soluble compounds abundant in plants. Extraction procedures need to be adapted according to the matrix and type of carotenoids present in the samples to be analyzed. Selection of suitable solvents may have a great impact on carotenoid analysis. Sampling steps and establishment of chromatographic conditions also need to be modified to better suit the respective individual compounds, due to variations in polarity. Therefore, a carotenoid extraction procedure and a HPLC method was developed and optimized for the analysis of carotenoids in mamey sapote fruits. Two extraction solvents were evaluated. The first one was based on acetone with subsequent separation with hexane and diethyl ether, while the second extraction solvent explored was a mixture of equivalent volumes of methanol, ethyl acetate and hexane. The first one was determined to be better for the type of carotenoids present in mamey sapote. Different HPLC programs were also evaluated for optimum separation of carotenoids facilitating further compound identification and quantification. Longer retention times and changes on the solvents gradients allowed the separation of the peaks.

6.2. Introduction

Carotenoids are lipid-soluble pigments found in nature, which are responsible for the red, orange and yellow coloring of many fruits. They are composed by a polyene chain and a highly insaturated middle section called chromophore, responsible of the different absorption spectra (Rodríguez-Amaya, 2001). According to their structure, they can be catalogued in two main groups: carotenes and xanthophylls. Carotenes are composed only by hydrocarbons, like α-, β-carotene and lycopene. On the other hand, xanthophylls are molecules with one or more oxygen atoms in their structure; examples of these ones include β-cryptoxanthin, violaxanthin, zeaxanthin and neoxanthin (Rodríguez-Amaya and Kimura, 2004). Carotenoid analysis can be challenging, considering that carotenoids are very stable in their natural matrixes, but when released by tissue disruption, such as peeling or chopping, they are susceptible to degradation or oxidation due to their labile structure (Boon

et al., 2010). Some considerations have to be taken while analyzing carotenoids. Avoiding light and oxygen exposure as well as pH control are crucial for successful carotenoid analyses. Moreover, sample processing and storage times should be reduced in order to avoid carotenoid degradation (Rodríguez-Amaya, 2001).

The choice of the extraction solvents may also have a great impact on carotenoid analyses. They need to be chosen depending on the sample matrix and the type of carotenoids present. Once released from the matrix, carotenoids need to be solubilized in the extraction solvent. Carotenes are more soluble in non-polar solvents like hexane, while xanthophylls dissolve better in slightly polar solvents, such as ethyl acetate and short chain alcohols. Most frequently, acetone is used since it solubilizes a wide range of carotenoids (Schiedt and Liaaen-Jensen, 1995).

Mamey sapote (*Pouteria sapota* Jacq. H.E. Moore & Stearn) is a tropical fruit native to Mexico and Central America (Quesada, 1996). It has a fleshy mesocarp, whose coloration varies between red and orange (Morera, 1992). Preliminary studies attributed the red color to the presence of α- and β-carotene (Alia-Tejacal *et al.*, 2005); however, recent studies determined that ketocarotenoids are main pigments and highly responsible of the red coloration in the fruit (Murillo *et al.*, 2011; Murillo *et al.*, 2013). These ketocarotenoids include sapotexanthin, described recently by Murillo *et al.* (2011), and cryptocapsin esters. Other more polar pigments have been described in this fruit, such as neoxanthin, violaxanthin and capsoneoxanthin (Turcsi *et al.*, 2010). Therefore, a mixture of solvents could be appropriate to guarantee extraction of all carotenoids in *P. sapota*.

After extraction, carotenoids are mostly separated and subsequently analyzed using High Performance Liquid Chromatography (HPLC). In order to achieve an efficient chromatographic separation, elution conditions need to be optimized, considering that food samples may contain a mixture of carotenoids, including carotenes and xanthophylls, and their isomers (Rodríguez-Amaya and Kimura, 2004). The type of column chosen can also allow better resolution of the sample profile. Particularly, a C_{30} reversed-phase column is recommended to enhance separation of geometrical carotenoid isomers (Sander *et al.*, 1994).

In order to guarantee representative carotenoid analyses, all pigments present in the sample need to be released from the matrix; great variations in composition between sample matrices make it necessary to adapt extraction protocols (Rodríguez-Amaya and Kimura, 2004). Not only extraction protocols need to be adapted, also chromatographic conditions need to be modified to better suit to the samples to be analyzed. Therefore, the optimization of extraction protocols and chromatographic conditions for the analyses of carotenoids from mamey sapote fruits was the main objective of this chapter.

6.3. Materials and methods

6.3.1. Project location. All analyses were carried out at the Institute of Food Science and Biotechnology of the University of Hohenheim, Stuttgart, Germany, from October 2012 until May 2013.

6.3.2. Plant material. Fruits from three *Pouteria sapota* genotypes were sampled from the collection of the Tropical Agricultural Research and Higher Education Center, CATIE (Turrialba, Costa Rica). The analyzed genotypes were 11163, 11129 and 8747; the first one with a yellow-orange pulp color and the others with a red-orange flesh color. Only fruits with evident yellowish-orange pulp, as evaluated by making a little scratch on the skin, were collected. The fruits were transported by plane to the University of Hohenheim, Stuttgart, Germany and stored wrapped in newspaper at 23 °C until fruit maturity. Pulp from ripe fruits was cut into small pieces, frozen with liquid nitrogen, vacuum packed in aluminum bags and stored at -80 °C until analysis.

6.3.3. Carotenoid extraction. In order to determine the most adequate method for carotenoid extraction from mamey sapote fruit tissues, experiments were conducted with two solvent mixtures. The first solution was a (1:1:1 v/v/v) mixture of methanol (MeOH), ethyl acetate (EtOAc) and light petroleum (LP) containing 0.1 g/L 2,6-di-tert-butyl-*p*-cresol (BHT), as described by Schweiggert *et al.* (2011) for papaya; while the second one contained acetone with 0,1% (w/v) BHT, according to the modified method reported by Murillo *et al.* (2011) for mamey sapote. The first practical steps with included samples thawing, shortly homogenization with a porcelain mortar were the same for both extraction solvent mixtures, the difference occurs during the phase separation steps.

Briefly, an aliquot of 1.00 ± 0.01 g of macerate was placed in a tube containing 0,1 g of $NaHCO_3$. Subsequent extraction was conducted with a probe sonicator (Sonopuls HD 3100, Germany), using a MS 72 probe with the each one of the above-mentioned solvent mixtures. After extraction for 30 s at 75% amplitude, the samples were centrifuged for 3 min at 3000 x g (Eppendorf Centrifuge 5804R, Eppendorf, Hamburg, Germany) to separate solids from the carotenoid-containing solvent phase. For acetone extraction, the colored fraction was collected in a tube after each extraction; the extraction was repeated until sample residues appeared colorless. Subsequently, one last extraction was conducted with n-hexane and the collected hexane phase was combined with the acetone extracts. Subsequent phase separation was enhanced with 4 mL EtO_2/n-hexane (0,1% w/v BHT), washing the organic phase twice with deionized water. Regarding the extraction with MeOH/EtOAc/PB, the procedure was carried out as described above, except for collecting only the upper hexane phase during probe sonication-assisted extraction. The combined upper phases were washed twice with water as well. After washing, the upper fraction of each extraction method was collected and evaporated under a gentle nitrogen stream and stored at -80 °C until saponification or direct HPLC analyses.

Saponified samples were obtained by re-dissolving the extract in 3 mL light petroleum or diethyl ether, for the MeOH/EtOAc/PB mixture or acetone, respectively. Moreover, 3 mL of potassium hydroxide (30% MeOH) was added and the mixture was stirred for three hours. KOH residues were eliminated by washing the extract three times with deionized water. The upper phases were collected and evaporated with a N_2 stream, and were subsequently stored at -80 °C until HPLC analysis. The method for extraction with acetone is illustrated in Figure 6.1.

Recovery assay. In order to validate the acetone extraction method, a recovery assay (standard addition experiment) was conducted. The standards used for this assay were (*all*-E)-violaxanthin, (*all*-E)-β-cryptoxanthin and (*all*-E)-β-carotene (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany), since these compounds represent carotenoids of a largely different polarity. Three sets of tubes were used, one with the standard, the second with the sample extract and the last one with the combination of both, sample extract plus the standard added. A small sample of each carotenoid standard was completely dissolved in

corresponding solvent: (*all*-E)-violaxanthin (acetone), (*all*-E)-β-cryptoxanthin and (*all*-E)-β-carotene (TBME). An equal volume of each carotenoid solution was transferred to the corresponding tube and immediately evaporated under a nitrogen stream. Tubes containing only the standards were stored at –80 °C until HPLC analysis. Samples were weighed in the corresponding tubes for carotenoid extraction. This assay was repeated under the same conditions in different days. The recovered fraction was estimated by subtracting the carotenoid area obtained from sample extract plus the standard (tube 3) and the sample extract (tube 2) and comparing it with the area in the standard (tube 1).

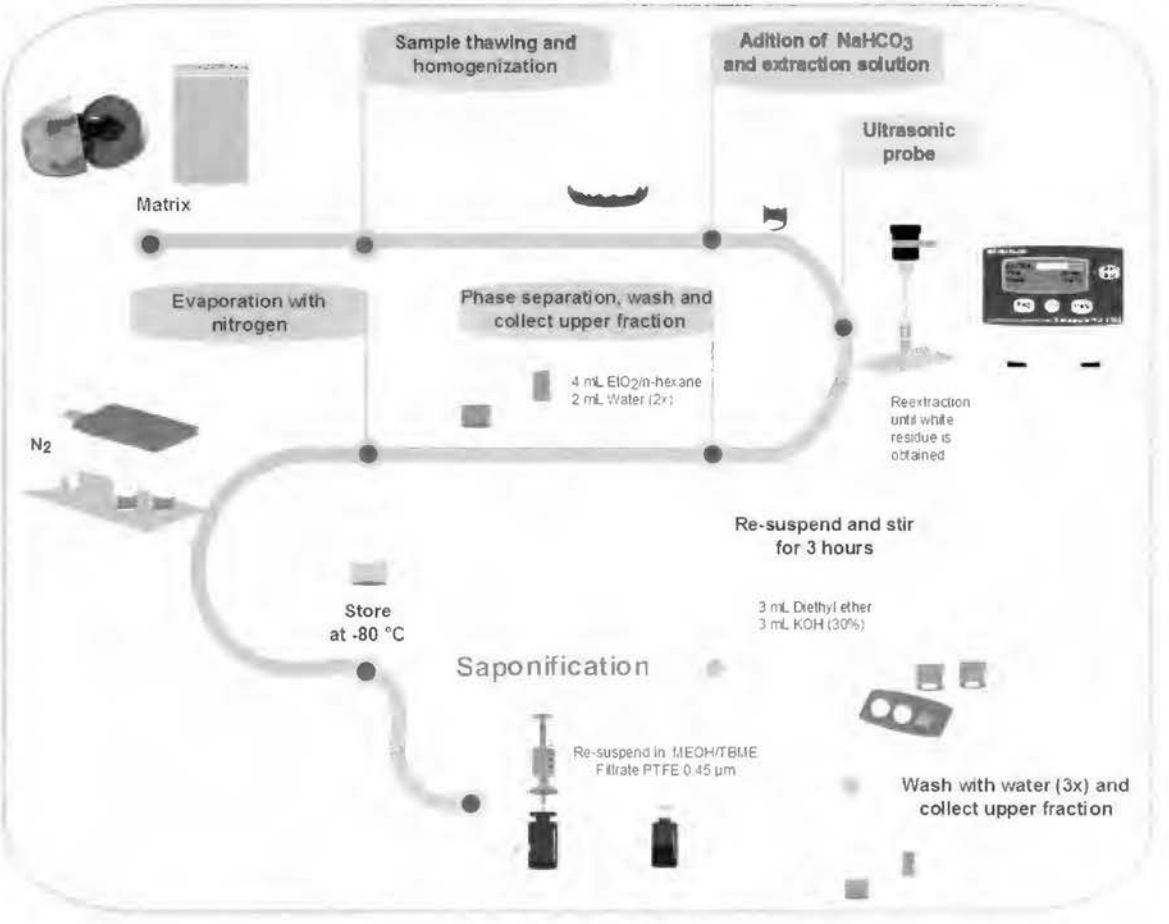

Figure 6.1. Procedure for carotenoid extraction with acetone as solvent; where EtO_2 is diethyl ether, N_2 nitrogen, KOH potassium hydroxide and PTFE is polytetrafluoroethylene filter.

In order to establish the adequate HPLC system parameters, several combinations of retention times and gradients were carried out (Table 6.1). Dried carotenoid extracts were re-suspended in 600 µL TBME/MeOH (1:1, v/v) and filtered with

a 0.45 μm pore PTFE filter for HPLC analysis. The carotenoid separation was achieved using a 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a G1379A degasser, a G1312A binary gradient pump, a G1313A autosampler, a G1316A column oven, and a G1315B diode array detector. The column was operated at 40 °C and consisted of an analytical scale YMC C_{30} reverse phase column (3 μm particle size, 150 x 3.0 mm i.d, YMC Europe, Dinslaken, Germany) and protected by a YMC C_{30} guard column (10 x 30 i.d., 3 μm particle size, YMC Europe). The eluents used were MeOH/H_2O (90:10, v/v/v, eluent A) and MeOH/TBME/ H_2O (20:78:2, v/v/v, eluent B), both containing 1.5 g/L ammonium acetate. Different gradient programs, as shown in Table 6.1, were compared. Carotenoids were monitored at 450 nm, and additional UV-Vis spectra were recorded in the range of 200–600 nm.

Table 6.1. Parameters for different programs in mamey sapote fruit samples HPLC run optimization. Eluent B composition: MeOH/TBME/$NH_4C_2H_3O_2$ (20:78:2).

Program	Parameters									
1	Time (min)	0	5	52	55	58	60		Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	100	100	0	0		0.5	60
2	Time (min)	0	5	27	70	75	80	82	Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	29	100	100	0	0	0.8	82
3	Time (min)	0	5	82	85	88	90		Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	100	100	0	0		0.8	90
4	Time (min)	0	5	82	85	88	90		Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	100	100	0	0		0.6	90
5	Time (min)	0	5	60	82	85	88	90	Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	80	100	100	0	0	0.8	90
6	Time (min)	0	5	70	82	85	88	90	Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	80	100	10	0	0	0.8	90
7	Time (min)	0	5	75	82	85	88	90	Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	80	100	10	0	0	0.8	90
8	Time (min)	0	5	78	82	90			Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	75	100	0			0.8	90
9	Time (min)	0	5	88	92	95	98	100	Flow rate (mL/min)	Total time (min)
	% Eluent B	0	0	75	100	100	0	0	0.8	100

6.4. Results and discussion

Carotenoid extraction is usually conducted with a mixture of solvents that penetrate the food matrix. In addition, they induce precipitation of some compounds, e.g. pectin, and therefore enhance the extraction of carotenoids (Rodríguez, 2001). It is important to consider the type of carotenoids present in a sample before selecting solvents. In mamey sapote fruits, the presence of both xanthophylls and carotenes has been previously reported (Alia-Tejacal *et al.*, 2005; Deli *et al.*, 2011; Murillo *et al.*, 2011). Therefore, solubility of both types of carotenoids needs to be considered.

In this work, when comparing saponified samples extracted with the two evaluated protocols, considerably higher values were obtained with acetone for the first two peaks, tentatively identified as neoxanthin or violaxanthin (Chapter IV), than with MeOH/EtOAc/BP. Other differences were not so clear (Fig. 6.2).

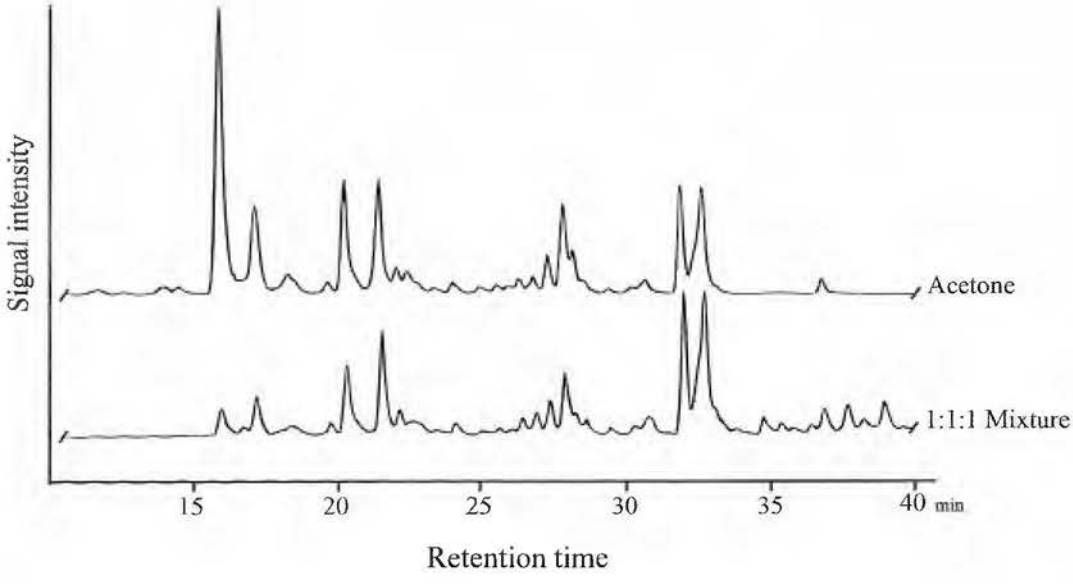

Figure 6.2. Comparison of peaks obtained from saponified samples of mamey sapote fruits using acetone or a mixture of methanol, ethyl acetate and light petroleum (1:1:1 v/v)

Carotenes are soluble in low polarity solvents like petroleum ether, hexane, and toluene while xanthophylls have proven to dissolve better in more polar solvents like methanol or ethanol (Rodríguez-Amaya, 2001; Schiedt and Liaaen-Jensen, 1995). The use of acetone has the advantage of having a drying effect on the tissue, which, in consequence, could

enhance the extraction of both carotenes and xanthophylls (Rodríguez-Amaya, 2010; Schiedt and Liaaen-Jensen, 1995). Additional solvent mixtures have been used elsewhere as well; for example, a mixture of acetone/hexane (4:6, v/v) and ethanol/hexane (4:3, v/v) were demonstrated by Taungbodhitham *et al.* (1998) to be good extraction mixtures for lycopene and β-carotene, when assessing different extraction methods for the carotenoid analysis in various foods and vegetables. One extraction solution that has proven to work well with a great variety of samples is the MeOH/EtOAc/LP (1:1:1 v/v) solution, previously used in papaya, carrots and tomatoes (Schweiggert *et al.*, 2011; Schweiggert *et al.*, 2014). In both extraction protocols the antioxidant BHT was used to prevent carotenoid oxidation. For mamey sapote fruits, it appears that acetone is the most appropriate solvent due to the presence of xanthophylls. In the 1:1:1 mixture, carotenoids with affinity to petroleum benzin would be extracted, while some polar compounds, like neoxanthin in mamey sapote fruits, will remain in the water phase due to higher affinity to MeOH and EtOAc.

The small peaks observed after 34 min of the 1:1:1 mixture (Figure 6.2) correspond to carotenoid esters that were not completely saponified. Since the time for saponification in both methods was the same, the differences may be due to a lower solubility of KOH in light petroleum than in diethyl ether. Therefore, diethyl ether and KOH may be a better mixture for saponification of esters in mamey sapote samples. According to the results of this experiment, acetone was chosen as the extraction solvent for the next experiments. Subsequent carotenoid identification, discussed in chapter IV, showed that all carotenoids present belong to the xanthophyll group, supporting the better extraction results with acetone.

After acetone was selected for carotenoid extraction from mamey sapote fruits, recovery assays were made with (*all*-E)-violaxanthin, (*all*-E)-β-cryptoxanthin and (*all*-E)-β-carotene standards, which comprises different carotenoid polarity ranges. Using the acetone-based extraction protocol, total recovery for all carotenoids evaluated was above 97.84 % (Table 6.2.). Small differences found in the recovery percentages could be due to evaporation of the sample before HPLC run, giving percentages higher than 100%. In general, the acetone extraction method proved to be adequate for the efficient carotenoid extraction in mamey sapote fruits.

Table 6.2. Acetone extraction recovery from mamey sapote samples with (*all*-E)-violaxanthin, (*all*-E)-β-cryptoxanthin and (*all*-E)-β-carotene standards.

Carotenoid standard	Recovery Day 1	Recovery Day 2
Violaxanthin	98.87%	104.23%
β-cryptoxanthin	98.85%	97.84%
β-carotene	119.34%	104.69%

After selecting the most appropriate extraction method and determining its recovery rate, the next step was to optimize the HPLC run. The eluents selected for this analysis were eluent A MeOH/H_2O (90:10) and B MeOH/TBME/H_2O (20:78:2), both containing 1.5 g/L ammonium acetate. This combination includes a larger percentage of methanol, a weak organic solvent, due to the presence of mainly xanthophylls in the sample. Several trials and changes on the elution time (Table 6.1) were conducted to accomplish better separation and the corresponding chromatograms are shown in Figure 6.3. Changes in run time, eluent concentration and flow rate helped to accomplish better carotenoid separation. It is clear that after retention time modification and consequently eluent gradient peaks appeared more separately, for example program number 1 versus the other 8 programs. Total time for the programs 3 to 8 was the same, 90 min, however chances in the gradient steps and flow rate allowed better separation of the peaks. The main changes are observed between 35 to 65 min, all peaks were recovered before 75 min. In this case, the selected program was number 8, which allowed a good carotenoid separation with 90 min running time. The next program with 100 min running time did not show a big difference in separation, therefore the 10 min shorter program was chosen.

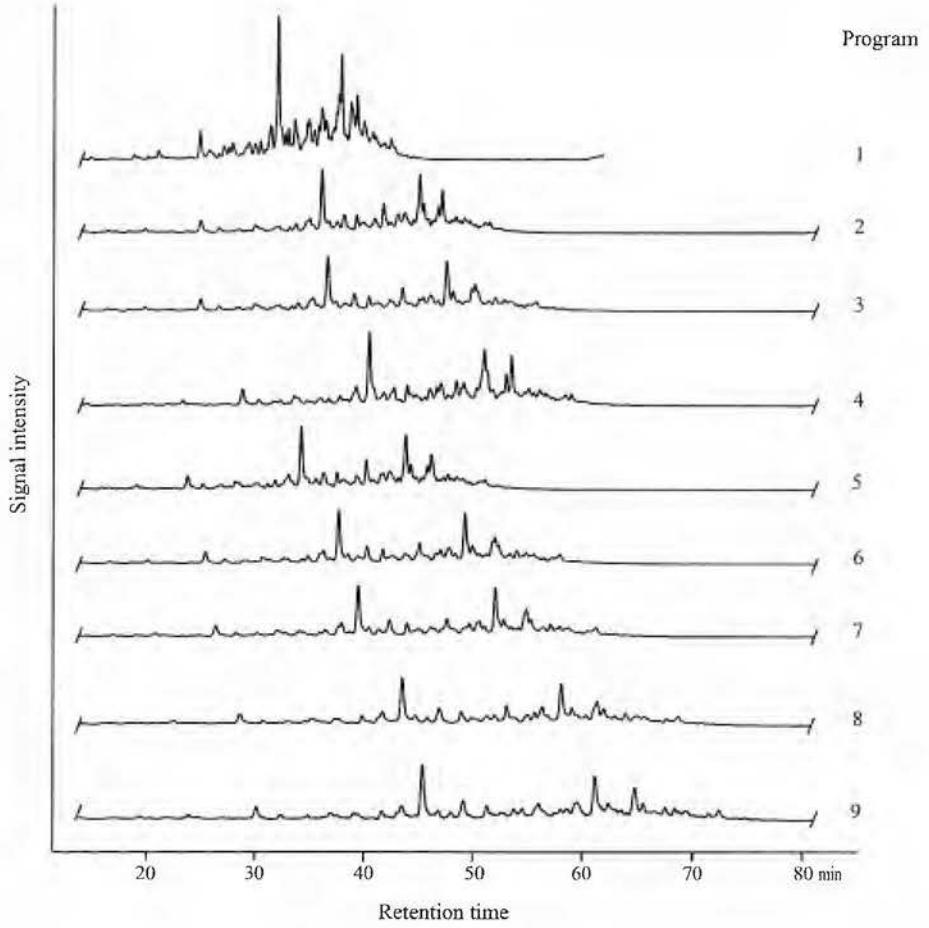

Figure 6.3. HPLC separation trials in mamey sapote fruit using non-saponified extracts. Individual chromatograms correspond to the programs described in Table 6.1.

6.5. Conclusions

Carotenoid analysis comprises a series of factors to consider that will vary depending on the food matrix to be analyzed. Mamey sapote fruits have the advantage of containing low fat levels, therefore, saponification might be only necessary for identification purposes. Moreover, their soft pulp facilitates homogenization and tissue disruption, allowing easier carotenoid liberation. As explained before, aside from food matrix, the extraction efficiency is determined by the type of carotenoids present; since sapote fruits present mainly xanthophylls, acetone was a better suitable extraction solvent than the MeOH/EtOAc/PB mixture for the analyses of fresh fruit samples in this work. HPLC can be an important tool to separate, identify and quantify carotenoids when it is adjusted to the analyzed matrix. Conditions optimization done in this work improved pigment peak separation in mamey

sapote samples. The methodology established here was used for carotenoid analyses, discussed in the next chapters.

6.6. References: Chapter III

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7. Chapter IV: Genuine carotenoid profile and concentration in sapote fruits

7.1. Abstract

Vitamin A deficiency has been catalogued as a moderate to severe problem in many developing countries. Fruits and vegetables constitute a great dietary source of provitamin A precursors, being metabolized by humans to vitamin A. These precursors naturally occurring in plants are yellow, orange, and red, and are called carotenoids. Several efforts have been made to identify potential new sources of these pigments. Mamey sapote (*Pouteria sapota*) is an underutilized carotenoid-rich fruit containing rare κ-ring carotenoids like the newly identified sapotexanthin. Although different genotypes showing distinct pulp colors are available, in-depth knowledge on genotype-related differences in their carotenoid composition is still scarce. Therefore, the goal of this research work was to qualitatively and quantitatively describe the carotenoid profile of different mamey sapote genotypes using HPLC-DAD coupled to multi-stage mass spectrometry. Two important pigments found in mamey sapote fruits were sapotexanthin and cryptocapsin, two potential provitamin A precursors. In this work, high amounts of these ketocarotenoids, including capsorubin and capsoneoxanthin, were observed in red-orange fleshed genotypes, while high concentrations of neoxanthin esters were the predominant carotenoids, besides minor amounts of sapotexanthin and cryptocapsin, in yellow-orange fleshed fruits. Sapotexanthin contents did not vary significantly between genotypes (289-371 μg/100 g of fresh weight, FW). Nevertheless, enormous differences were found in total carotenoid contents of the different genotypes, ranging from 1672 to 6533 μg / 100 g FW.

7.2. Introduction

Carotenoids are lipid-soluble plant pigments providing numerous benefits to human health, mainly because they are a source of provitamin A. Additionally, some are considered important due to their antioxidant activity and strengthening of the immune system (Bendich, 1989; Deming *et al.*, 2002). The study of potential new vegetable sources of carotenoids is of interest, considering that vitamin A deficiency has been catalogued as a moderate to severe health issue in at least 122 countries, most of them developing or transition countries (WHO, 2009). This deficiency can cause diseases such as

xerophthalmia, night blindness and anemia and also weakens the immune system (Lin and Liu, 2012). There are different sources of dietary vitamin A. Preformed vitamin A is obtained from animal sources, while vegetables and fruits are source of provitamin A (Barber *et al.*, 2012). The consumption of fruits and vegetables rich in carotenoids constitute a better control system, since the human metabolism regulates the conversion of carotenoids into vitamin A (Barber *et al.*, 2012; FAO/WHO, 2002; Zeb, 2012). Additionally, these sources are less expensive, making them more accessible in poor countries. The climate of tropical countries allows growing of numerous different fruits, some of them with high carotenoid contents, for example, papaya and mango. However, there are plenty of fruits that have not yet been studied or just a basic description of its composition is available. This is the case for mamey sapote (*Pouteria sapota* Jacq. H.E. Moore & Stearn).

Mamey sapote is native to Mexico and Central America and can be grown in tropical and subtropical regions with warm and humid climates (Quesada, 1996). The fleshy and edible mesocarp has deep orange/red coloration at full maturity (Campbell *et al.*, 1997). This fruit can be consumed fresh and can also be used to prepare ice creams, sweets and other deserts (Alia-Tejacal *et al.*, 2007; Quesada, 1996). Sapote represents another underutilized fruit rich in carotenoids, with an interesting carotenoid pattern (Alia-Tejacal *et al.*, 2007; Murillo *et al.*, 2010). Previous studies stated that the carotenoids present in this fruit correspond to β-carotene, lutein, α-carotene and ζ-carotene (Alia-Tejacal *et al.*, 2005; Yahia *et al.*, 2011). New findings point to the presence of ketocarotenoids such as a recently described carotenoid named sapotexanthin (Murillo *et al.*, 2011). Ketocarotenoids are xanthophylls rarely synthetized by plants. Examples of them are capsanthin, capsorubin and cryptocapsin present in red peppers (*Capsicum annum*) (Deli *et al.*, 2001).

Several genotypes of sapote fruits have been described, with differences in shape, size, sweetness, pulp color, etc. (Monge and Guevara, 2000; Quesada, 1996). Although different genotypes showing distinct pulp colors are available, no detailed studies on their pigment composition have been published up to now (Farré *et al.*, 2010; Monge and Guevara, 2000). Therefore, a comparison between different genotypes could help defining their specific carotenoid patterns, which could be determinant for the direct use of particular cultivars as a source of provitamin A and in future breeding programs as well. Hence, the goal of this

research was to qualitatively and quantitatively describe the carotenoid profiles of different sapote genotypes using HPLC-DAD coupled to mass spectrometry.

7.3. Materials and methods

7.3.1. Project location. All analyses were carried out at the "Institute of Food Science and Biotechnology" of the University of Hohenheim, Stuttgart, Germany, from October 2012 until May 2013.

7.3.2. Plant material. Fruits from three *P. sapota* genotypes were sampled from the collection of the Tropical Agricultural Research and Higher Education Center, CATIE (Turrialba, Costa Rica). The analyzed genotypes were 11163, 11129 and 8747; 11163 produced fruits with a yellow-orange flesh color, while the others bore fruits with a red-orange flesh color. Only fruits with evidently mature flesh color, as evaluated by making a little scratch on the skin, were collected. Three fresh fruits from the 11163 genotype and four fruits each from the other genotypes were transported by plane to the University of Hohenheim, Stuttgart, Germany and stored wrapped in newspapers at 23 °C until fruit maturity.

7.3.3. Color measurements. Color was determined for the three genotypes with a colorimeter (Minolta Cr-300, Osaka, Japan). A puree was prepared from different sections of each fruit; measurements were done by triplicate and results are expressed as CIE-L*a*b* color values.

7.3.4. Carotenoid extraction. The extraction procedure was performed using acetone as extraction solvent, according to previous trials explained in chapter III. Shortly, samples were thawed and homogenized with a porcelain mortar, 1.00 ± 0.01 g of the macerate was taken and placed in a tube with 0.1 g of $NaHCO_3$ and acetone containing 0.1 g/L 2,6-di-*tert*-butyl-*p*-cresol (BHT). Extraction was performed with a probe ultrasonicator (Sonopuls HD 3100, Germany), using a MS 72 probe for 30 s at 75% amplitude. After each extraction, the supernatant was collected in a separate tube. The extraction was repeated at least 5 times until a white residue was left. A last extraction step with n-hexane was done to ensure that all carotenoids were extracted. Phase separation was achieved by adding 4 mL diethyl

ether/n-hexane (1:1 v/v, containing 0.1% w/v BHT) to the tube with collected supernatant and by washing twice with deionized water. The upper fraction, corresponding to the organic phase, was collected, evaporated under a gentle nitrogen stream and stored at -80 °C until saponification or direct HPLC analyses. For saponification, the dry extract was re-dissolved in 3 mL diethyl ether, and 3 mL KOH (30 % MeOH) was added and left in agitation for 3 h. After saponification, samples were washed twice with deionized water, the upper phase (organic) was collected, evaporated under nitrogen atmosphere and stored at -80 °C until analysis.

7.3.5. HPLC-DAD-MS. Carotenoid separation was achieved using a 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a G1379A degasser, a G1312A binary gradient pump, a G1313A autosampler, a G1316A column oven, and a G1315B diode array detector. The column was operated at 40 °C and consisted of an analytical scale YMC C_{30} reverse phase column (3 µm particle size, 150 x 3.0 mm i.d, YMC Europe, Dinslaken, Germany) protected by a YMC C_{30} guard column (10 x 30 i.d., 3 µm particle size, YMC Europe). The HPLC system was coupled on-line to a Bruker 3000+ ion trap mass spectrometer (Bruker, Bremen, Germany) operating in positive mode and an APCI source. The mobile phase used was MeOH/H2O (90:10 v/v, eluent A) and MeOH/TBME/ H2O (20:78:2, v/v/v, eluent B), both containing 1.5 g/L ammonium acetate. The gradient was set as follows: isocratic 100% A for 5 min, from 100 to 25% A in 73 min, from 25% A to 0% A in 4 min and from 0% A to 100% A in 8 min. Total run time was 90 min at a flow rate of 0.8 mL/min. The carotenoids were monitored at 450 nm, and UV-Vis spectra was recorded in the range of 200–600 nm. All LC/MS analyses were carried out as described by Schweiggert *et al.* (2005). The identification of carotenoids was accomplished by comparison of retention times, UV-Vis absorption and mass spectra with those of authentic standards. Standards were available for β-carotene, lutein, zeaxanthin, neoxanthin, and violaxanthin purchased from CaroteNature (Lupsingen, Switzerland) and Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). When standards were unavailable, pigments were tentatively identified by comparing their UV-Vis absorption spectra and mass spectral behavior with previously published data. Due to the lack of commercially available standards for most compounds, quantification of all carotenoids was carried out using linear external calibration curves of β-carotene, except for

neoxanthin and violaxanthin, which were quantitated by a violaxanthin calibration. Molecular weight correction factors (MWCF) were used when necessary, representing the ratio of the molecular weight of the compound to be quantitated and the molecular weight of β-carotene. Unknown compounds were quantitated using the β-carotene calibration curve. Retinol activity equivalents (RAE) were determined considering that 12 μg of β-carotene and 24 μg for other provitamin A carotenoids corresponds to 1 μg of retinol, equivalent to 1 RAE (National Research Council, 2001).

7.3.6. Statistical analysis. A one-way analysis of variance (ANOVA) test was conducted to determine significant differences in carotenoid concentrations between mamey sapote genotypes. Shapiro-Wilk's test was conducted to test normality of the data, and homogeneity of variances was assessed by Levene's test. For non-parametric samples a Kruskall-Wallis test was conducted. When differences between samples were found a Tukey test was applied. All analysis were performed with the program SAS JMP 8 (SAS Institute Inc. Cary, NC, USA).

7.4. Results and discussion

7.4.1. Visual appearance and CIE-L*a*b color values

Photographs of the studied two genotypes with red-orange flesh (8747, 11129) and the yellow-orange fleshed one (11163) are shown in Figure 7.1. Color measurements based on the CIE L*a*b* system showed no significant difference ($p > 0.05$) between the three genotypes for L* and b* values (Table 7.1). On the other hand, significant differences ($p < 0.05$) between the red-orange fleshed fruits and the yellow-orange fruits regarding a* values were observed. Genotype 11163 presented lower a* values (red), which could also be visually confirmed (Fig. 7.1). Lightness (L*) in mamey sapote fruits have been associated with phenolic compounds responsible for flesh darkening during ripening (Alia-Tejacal *et al.*, 2002; Díaz-Perez *et al.*, 2000), while high a* and b* values are associated with the presence of carotenoids. Differences within a* values can be explained with the carotenoid profile and contents, which will be discussed later.

Figure 7.1. Mamey sapote fruits from the genotypes studied. Bar corresponds to 5 cm.

Table 7.1. Color measurement of different genotypes of mamey sapote fruits using the CIE L*, a*, b* scale. Different letters represent significant differences ($p < 0.05$) on the same row.

Parameter	Mamey sapote genotype		
	8747	**11129**	**11163**
L*	58.66 ± 2.20^{a}	58.90 ± 2.36^{a}	63.07 ± 2.01^{a}
a*	34.48 ± 4.00^{a}	29.38 ± 4.13^{a}	16.28 ± 4.97^{b}
b*	41.66 ± 2.21^{a}	37.63 ± 1.10^{a}	40.25 ± 3.71^{a}

In order to identify sapote carotenoids, both saponified and non-saponified sample extracts for each genotype were analyzed by HPLC-DAD-MS^2. Chromatographic separation of carotenoids from saponified and non saponified samples corresponding to the genotypes with red-orange and yellow-orange flesh are displayed in figures 7.2 and 7.3. In Table 7.2, all identified pigments are listed with their corresponding UV-Vis spectra and MS/MS^2 data.

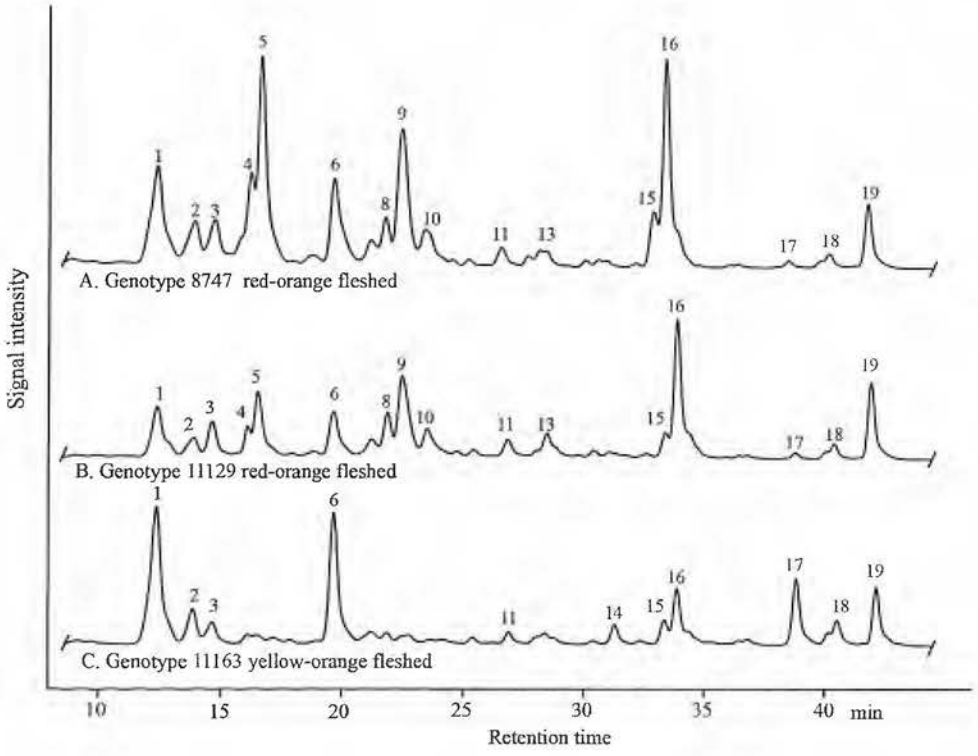

Figure 7.2. HPLC-DAD separation of carotenoids in saponified extracts from different mamey sapote genotypes, monitored at 450 nm. See Table 7.2 for peak assignment. Signal intensity of the different traces are in the same scale.

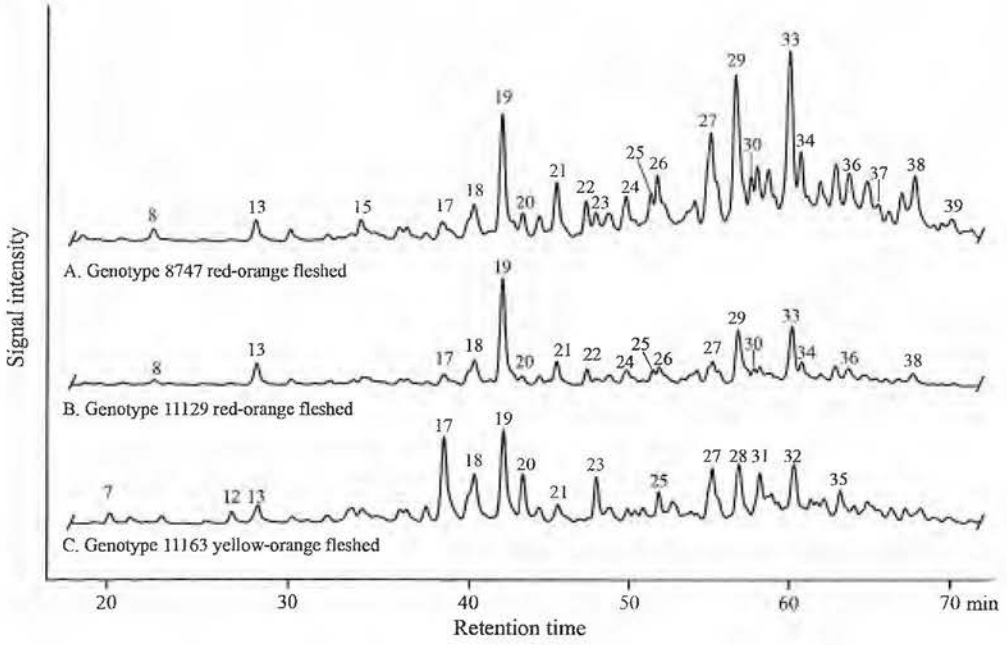

Figure 7.3. HPLC-DAD separation of carotenoids and carotenoid esters in non saponified extracts from different mamey sapote genotypes, monitored at 450 nm. See table 7.2 for peak assignment. Signal intensity of the different traces are in the same scale.

Table 7.2. UV-Vis spectra and MS data of carotenoids from the mamey sapote genotypes analyzed.

n.i.: not identified

Peak n°	Retention time (min)	Tentative identification	HPLC-DAD UV-Vis maxima (nm)	$[M-H]^+$ m/z	HPLC/APCI (+) MS^2		
1	12.2	Neoxanthin	416/440/470	601	583	565	547
2	13.6	Violaxanthin	416/440/470	601	583	565	
3	14.2	n.i.	400/424/448	583	565	547	
4	15.9	n.i.	400/422/450	601	583	565	547
5	16.3	Capsoneoxanthin	472	583	565	495	
6	19.5	β-cryptoxanthin-5,6,5',6'-diepoxide	414/438/468	585	567	549	
7	20.1	n.i.	416/440/470	585	567		
8	21.7	β-cryptoxanthin-5,6, 5',8'-diepoxide	400/420//446	585	567		
9	22.5	Capsorubin	470	601	583	565	495
10	23.3	3'-deoxycapsorubin	472	585	567		
11	26.8	3,3'-dideoxycapsorubin	484	569			
12	26.9	n.i.	416/440/470	569	536	465	
13	28	n.i.	400/422/448	569	551	533	
14	31.3	β-cryptoxanthin-5,6-epoxide	418/442/472	569	551	535	
15	33.2	β-cryptoxanthin-5,8-epoxide	402/424/460	569	551		
16	33.7	Cryptocapsin	474	569	551		
17	38.5	β-carotene-5,6-epoxide	422/446/474	553	535		
18	40.3	β-carotene-5,8-epoxide	406/430/454	553	535		
19	41.8	Sapotexanthin	474	553	535		
20	43	n.i	416/440/470	767	743	567	549
21	44.9	β-cryptoxanthin-5,6-5',8'-diepoxide laurate	400/422/448	767	749	567	549
22	46.7	Cryptocapsin-5,6-epoxide laurate	470	767	749	567	549
23	47	n.i	416/442/470	877	859		
24	49.1	n.i	400/420/450	851	833	595	573
25	50.5	3'deoxycapsorubin myristate	468	795	777	567	549
26	50.7	β-cryptoxanthin-5,6-epoxide stearate	418/442/472	853	835		
27	53.5	Neoxanthin ester	418/442/468		565		
28	54.9	Neoxanthin ester	418/442/472		565		
29	55.2	Cryptocapsin laurate	474	751	733	551	533
30	56.16	Capsorubin/capsoneoxanthin diester (laurate-laurate)	470	965	765	565	
31	56.18	Neoxanthin diester (myristate-myristate)	418/442/472	1003	775	757	547
32	58	n.i	416/440/470	775	551		
33	58.2	Cryptocapsin myristate	472	779	551		
34	58.9	Capsorubin/capsoneoxanthin diester (laurate-myristate)	470	993	975	765	565
35	60.6	n.i	416/440/470	779	551		
36	61.5	Cryptocapsin palmitate	474	807	551		
37	63.1	Capsorubin/capsoneoxanthin diester (myristate-myristate)	470	1021	793	775	565
38	65.3	Cryptocapsin stearate	474	835	551		
39	67.1	Capsorubin/capsoneoxanthin diester (myristate- stearate)	470	1077	849	831	565

Neoxanthin and violaxanthin (peaks 1 and 2) were tentatively identified by comparison with authentic standards in terms of their retention times, UV-Vis-spectra and MS/MS fragmentation. Masses found corresponded to m/z 601 $[M + H]^+$ and dehydrated ions at m/z 583 $[M + H - H_2O]^+$ and 565 $[M + H - 2x\ H_2O]^+$, fragments mass is according to previously reported by Ornelas-Paz *et al.* (2007).

Capsoneoxanthin (peak 5) was identified by UV-Vis and typical fragment profile as described by Deli *et al.* (2000): m/z 583 $[M + H - H_2O]^+$, m/z 565 $[M + H - 2x\ H_2O]^+$ and m/z 495 $[M + H - 106]^+$. The β-cryptocapsin epoxides (peaks 6, 8, 14 and 15), were identified by comparing UV-Vis spectra and MS data from previous studies in mamey sapote fruits (Murillo *et al.*, 2013). For the diepoxides (peaks 6 and 8), fragments were obtained at m/z 585 $[M + H]^+$ and m/z 567 $[M + H - H_2O]^+$, while for epoxides (peaks 14 and 15) the product ions were obtained at m/z 569 $[M + H]^+$ and m/z 551 $[M + H - H_2O]^+$.

Capsorubin, 3'-deoxycapsorubin and 3,3'-dideoxycapsorubin (peaks 9, 10 and 11) were identified by comparison with previous UV-Vis spectra and MS data from Murillo *et al.* (2012). These pigments appear to be present in their esterified form, since they were only observed in saponified samples. An ester of 3'-deoxycapsorubin could also be identified by UV-Vis, and MS data. Protonated ions were detected at m/z 567 $[M + H - H_2O$ - fatty acid$]^+$ and m/z 795 [M + H + myristic acid]$^+$ with corresponding dehydrated ion at m/z 777.

Capsorubin and capsoneoxanthin are believed to be esterified since the peaks appear only after saponification. Some diesters were identified corresponding to the peaks 30, 34, 37 and 39; they could correspond to capsorubin or capsoneoxanthin diesters since they have the same mass m/z 601 $[M + H]^+$ and similar UV-Vis spectra. Each peak presented deacylated products ions at m/z 565 $[M + H - 2x\ H_2O$ - fatty acid$]^+$. Further fragmentation allowed the identification of each diester at m/z 965 [M + H + 2x lauric acid]$^+$, m/z 765 [M + H - 1x lauric acid]$^+$; m/z 993 [M + H + 1x lauric acid+ 1x myristic acid]$^+$ and m/z 765 [M + H - 1x lauric acid]$^+$; m/z 1021 [M + H + 2x myristic acid]$^+$ and m/z 765 [M + H - 1x myristic acid]$^+$; m/z 1077 [M + H + 1x myristic acid + 1x stearic acid]$^+$, m/z 849 [M + H - 1x stearic acid]$^+$.

β-carotene epoxides, sapotexanthin and cryptocapsin (peaks 16, 17, 18 and 19) UV-Vis spectra and MS values were compared to those reported by Murillo *et al.* (2013). After saponification of the samples, a high peak corresponding to cryptocapsin appeared, confirming that cryptocapsin is present in its esterified form in mamey sapote fruits. Fatty acids corresponding to C12:0, C14:0, C16:0 and C18:0 were associated to cryptocapsin (peaks 29, 33, 36 and 38). MS^2 experiments confirm the presence of m/z 551 $[M + H - H_2O$ - fatty acid$]^+$ and further ions at m/z 751 (laurate), 779 (myristate), 807 (palmitate) and 835 (stearate).

Compounds 27 and 28 were tentatively identified as neoxanthin esters. These peaks present UV-Vis spectra and MS data that could match neoxanthin esters, for example, they all presented the mass m/z 601 and 565 $[M + H - H_2O]^+$. Additionally, they disappear after saponification. In the case of the peak 31, fragments at m/z 1003 $[M + H + 2x$ myristic acid - $H_2O]^+$, 775 $[M + H-$ 1xmyristic acid- $H_2O]^+$ and 547 $[M +H -2x$ myristic acid - $H_2O]^+$ allowed its identification as neoxanthin myristate.

Similar UV-Vis spectra (416/440/470) was found for compounds 32 and 35, indicating the presence of violaxanthin or neoxanthin. As the compounds described above, these are likely to be carotenoid esters, since they disappeared after saponification. However, their identity could not be unambiguously elucidated by MS/MS data due to their low concentration (Table 7.2). Since violaxanthin was identified in saponified samples, we hypothesize these compounds could correspond to violaxanthin esters.

The carotenoid profile of sapote fruit is unique due to the presence of numerous ketocarotenoids, which were previously found in fruits of *Capsicum* sp. Examples of these carotenoids found in pepper are capsanthin and capsorubin, both representing hydroxylated ketocarotenoids (Schweiggert *et al.*, 2005). Major ketocarotenoids in sapote are sapotexanthin (non-hydroxylated) and cryptocapsin (hydroxylated) as shown in Fig. 7.4. Unlike ketocarotenoids in red pepper, sapotexanthin and cryptocapsin, according to their structure, are both potential provitamin A precursors, which makes them even more interesting for studying their potential health benefits.

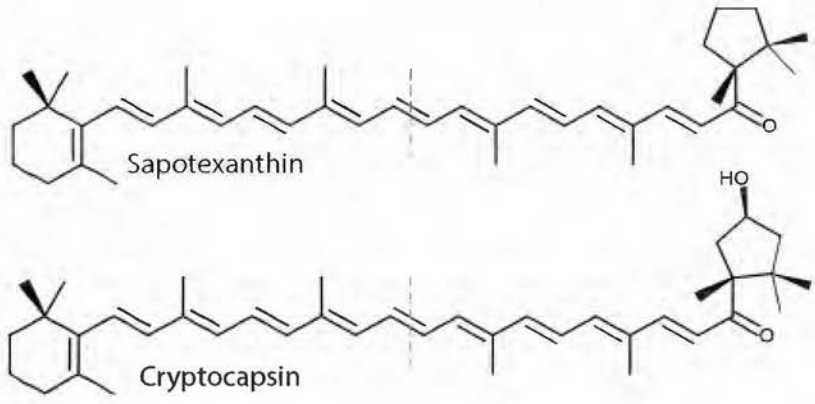

Figure 7.4. Chemical structure of ketocarotenoids sapotexanthin and cryptocapsin. Structures on the left of the dashed line indicates potential provitamin A activity.

The main compounds of yellow-orange fleshed sapote were neoxanthin esters (23 % of total carotenoids), β-cryptoxanthin epoxides (24 %), β-carotene epoxides (8 %) and sapotexanthin (6 %). In contrast, red-orange fleshed sapote fruits showed a higher variation in the carotenoid profile, with abundant epoxides and ketocarotenoids including capsoneoxanthin esters (10-14 %), capsorubin esters (11 %) and sapotexanthin (3- 8 %), some of them also present in the yello-orange fleshed fruit. The main pigments in red-orange fleshed sapote genotypes (8747-11129) were cryptocapsin esters (up to 14.8 %, Table 7.3).

The sapotexanthin concentration of the analyzed genotypes ranged from 289 to 371 ug/100 g of FW, no significant differences ($p > 0.05$) were found between the genotypes; however, there was variation regarding other carotenoids (Table 7.3). As shown in Figure 7.3 and Table 7.3, cryptocapsin esters were predominant in red-orange fleshed sapote fruits. It should be noted that quantification of cryptocapsin esters may include some other pigments that could not be separated. In addition, within fruits from the same genotype great variations in levels of some ketocarotenoids was observed (Table 7.3).

When comparing the total carotenoid contents of sapote with other fruits, genotype 8747 is similar to the papaya Pococí hybrid, with 6496 ± 753 μg/100 g FW (Schweiggert *et al.*, 2012) and has higher values than those of mango, with approximately 3800 ± 770 μg/100 g FW to 5500 ± 500 μg/100 g FW in cv. Keitt from different cultivars (Mercadante and Rodríguez-Amaya, 1998). Carotenoid content in 11163 is similar to that in mango cv. Keitt,

and in different varieties of yellow fleshed apricot fruit, which were between 2694 ± 584 µg/100 g FW and 4 907 ± 191 µg/100 g FW (Ruiz *et al.*, 2005).

Additionally, retinol equivalents in 8747 genotype correspond to half of what has been reported for papaya Pococí hybrid (197 ± 34) (Schweiggert *et al.*, 2012). In papaya the high value of RAE is achieved by the presence of β-carotene and β-cryptoxanthin, while in mamey sapote fruits high concentrations of sapotexanthin and cryptocapsin esters, both with potential provitamin A activity are found. However, even though the structure of sapotexanthin and cryptocapsin indicates that they could have provitamin A activity, no studies have been made to determine if these compounds can be absorbed by the human body and finally converted into vitamin A.

Table 7.3. Content of major carotenoids present in different mamey sapote genotypes.

Sample type	Peak n°	Compound (Tentative identification)	Carotenoid content in sapote genotypes in µg/100 g FW		
			Red-fleshed	**Red-fleshed**	**Yellow-fleshed**
			8747	**11129**	**11163**
Saponified	1	Neoxanthin **	1024.42 ± 63.50^{b}	453.28 ± 126.95^{c}	1500.07 ± 228.38^{a}
	2	Violaxanthin**	310.40 ± 92.62^{a}	163.92 ± 57.46^{b}	360.39 ± 85.46^{a}
	5	Capsoneoxanthin*	926.58 ± 379.82^{a}	348.95 ± 124.13^{b}	n.d.
	9	Capsorubin*	612.74 ±193.63^{a}	346.61 ± 120.16^{b}	n.d.
	14	β-cryptoxanthin-5,6-epoxide	n.d.	n.d.	96.07 ± 30.42
	16	Cryptocapsin*	1052.33 ± 449.75^{a}	356.05 ± 125.03^{b}	243.55 ± 49.86^{b}
Non saponified	17	β-carotene-5,6-epoxide	71.78 ± 16.39^{b}	35.67 ± 14.06^{b}	462.09 ± 143.54^{a}
	18	β-caroten-5,8-epoxide	129.56 ± 30.61^{b}	86.53 ± 35.21^{b}	291.40 ± 48.82^{a}
	19	Sapotexanthin	360.88 ± 107.15^{a}	289.21 ± 112.88^{a}	371.07 ± 42.61^{a}
	29	Cryptocapsin laurate	686.38 ± 187.31^{a}	217.17 ± 100.51^{b}	n.d.
	33	Cryptocapsin myristate	639.76 ± 135.31^{a}	158.76 ± 74.38^{b}	n.d.
	36	Cryptocapsin palmitate	299.13 ± 68.58	n.d.	n.d.
	38	Cryptocapsin stearate	269.34 ± 67.37^{a}	33.69 ± 18.28^{b}	n.d.
	Total carotenoid content		6533.41 ± 1617.01^{a}	1672.09 ± 800.22^{c}	4839.11 ± 1103.47^{b}
	Retinol equivalents		93.98 ± 23.57^{a}	30.60 ± 13.34^{b}	34.72 ± 7.76^{b}

Quantitation by β-carotene calibration with respective mass correction . n.d.: not detected. *: from saponified samples and quantitation by β carotene with respective mass correction **: from saponified samples and quantitation by violaxanthin calibration. Different letters indicate significant difference of means ($p < 0.05$). See table 4.2 for identification data.

As shown in Table 7.3., the yellow-fleshed fruits contained intermediate total carotenoid levels in between those of the red fleshed genotypes. Therefore, the total carotenoid content cannot be solely related to differences in flesh color. The presence of ketocarotenoids could

explain in part the color difference between the genotypes. Cryptocapsin, capsoneoxanthin and capsorubin are red pigments found in higher concentrations in red fleshed fruits than in the yellow fleshed ones. Comparison of relative abundance of the main pigments may allow to better understand how carotenoids determine the flesh color in mamey sapote fruits. In red fleshed fruits, major red pigments account for a 45 % of the total carotenoids and only around 26 % corresponds to yellow pigments. In yellow-fleshed fruits, the only ketocarotenoids present are cryptocapsin and sapotexanthin, which corresponds to 13 % of total carotenoid content, while main yellow pigments reach up to 61 %. In agreement, the contribution of cryptocapsin to total carotenoid levels was low (7 %) and, moreover, cryptocapsin esters could not be detected in the yellow-orange fleshed sapote fruits. In contrast, red-orange fleshed fruits contained up to 14 % cryptocapsin, 12 % capsoneoxanthin and 11 % capsorubin, all esterified. The carotenoid profile of yellow-orange fleshed fruits was dominated by high concentrations of neoxanthin esters (23 %), β-cryptoxanthin diepoxide and epoxide esters (24 %) and, possibly, also violaxanthin esters (5.5 %) with absorption maxima in the high energy range of the visible electromagnetic spectrum, thus appearing more yellowish. Hence, yellow pigments have a relative higher abundance in yellow-orange fleshed fruits explaining their color, while presence of cryptocapsin and other red pigments esters, like cryptocapsin, capsoneoxanthin and capsorubin, seems to be responsible for the flesh color in red-orange mamey sapote genotypes.

As mentioned before, no significant differences were found between the color CIE L*, a*, b* values when comparing the two red-fleshed genotypes. However, total carotenoid contents were ca. 4-fold higher in genotype 8747 as compared to 11129 (Table 7.3). Even though the carotenoid content was evidently different, the relative proportion in which red-yellow carotenoids were present was similar. Previously, Schweiggert *et al.* (2011) observed a non-linear relationship between CIE-a* (redness) values of papaya fruit flesh and the respective carotenoid level. Two-fold differences of the total carotenoid content in papaya were associated with highly similar CIE-a* values. Once reaching a certain pigment level, color perception seems to be widely saturated and only slowly increasing with carotenoid concentration.

Since all fruits were collected from the same site, environmental conditions were the same for all plants bearing the collected samples; therefore, differences in carotenoid content are likely associated with the genotype and, possibly, with different developmental stages. Other studies have reported wide variations in carotenoid contents when accessing different genotypes under the same cultivation conditions (De Rosso and Mercadante, 2005; Farnham and Kopsell, 2009; Yoo *et al.*, 2012).

In general, the biosynthetic carotenoid pathway is regulated by a series of enzymes responsible for synthetizing present carotenoids and their precursors (Figure 7.5). Differences in the genetic background can affect carotenoid biosynthesis regulation at different levels. Phytoene synthase (PSY), catalyzing the conversion of geranylgeranyl pyrophosphate into phytoene, has been described as a key rate limiting enzyme of carotenoid synthesis (Cazzonelli and Pogson, 2010; Shumskaya and Wurtzel, 2013). For example, in marigold flowers (*Tagetes erecta*), petal color vary from pale yellow to orange due to carotenoid content differences. Pale yellow flower presented a lower expression level of PSY and DXS (1-deoxy-D-xylulose 5-phosphate synthase) genes compared to orange colored ones (Moehs *et al.*, 2001). Changes in the expression of the gene encoding for PSY enzyme can have great repercussion in the total carotenoid content (Li and Yuan, 2013). Several studies have obtained good results by enhancing the expression of PSY enzyme encoding gene to increase carotenoid content in tomato plants (Ducreux *et al.*, 2005; Fraser *et al.*, 2002; Kiano *et al.*, 2003).

Activity of PSY enzyme can also be post-translationally regulated leading to differences in the total carotenoid content. In daffodil flower (*Narcissus pseudonarcissus*) chromoplasts, two forms of phytoene synthase are present, one that is bound to the stroma (soluble) and the other bound to the membrane. The enzyme present in the stroma is inactive, while the membrane bound enzyme is active (Schledz *et al.*, 1996). Therefore, even though the enzyme is present, it can be inactive and could not synthesize carotenoids, causing differences in carotenoid content.

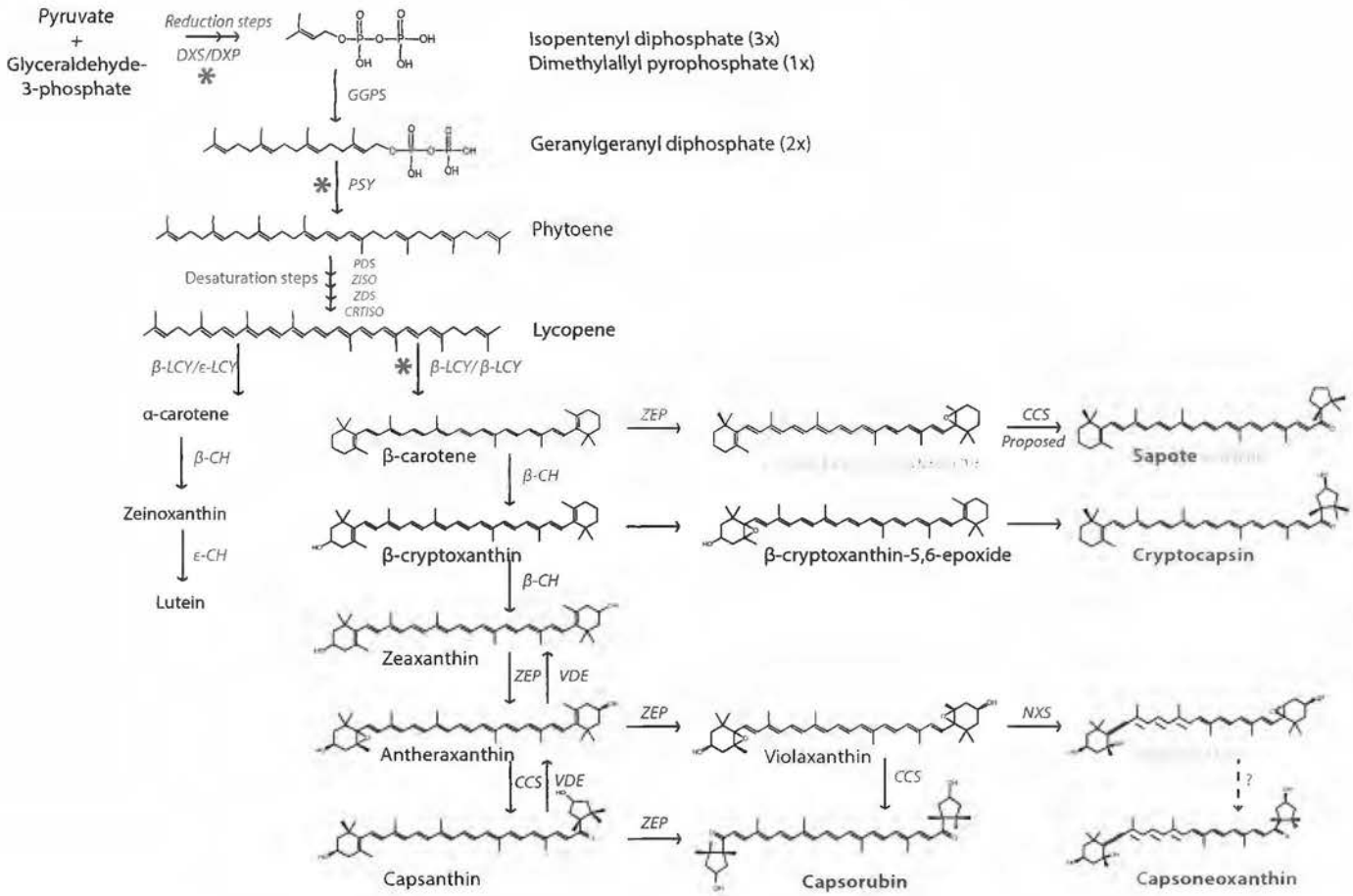

Figure 7.5. Biosynthetic pathway of carotenoids to obtain the ketocarotenoids capsanthin, capsorubin and cryptocapsin. GGPS : Geranylgeranyl pyrophosphate synthase; PSY: Phytoene synthase; PDS: Phytoene desaturase, ZISO: *cis*-ζ-carotene isomerase, ZDS: ζ-carotene desaturase, CRTISO: Carotenoid isomerase, β-LCY: lycopene- β-cyclase; ε- LCY: lycopene- ε-cyclase; β-CH: β-carotene hydroxylase cyclase; ε- CH: ε-carotene hydroxylase cyclase; ZEP: Zeaxanthin epoxydase; VDE: Violaxanthin de-epoxidase; CCS: Capsanthin-capsorubin synthase; NXS: Neoxanthin synthase . Key regulation steps are marked with a red asterisk (*). Carotenoid names highlighted in yellow correspond to pigments present in yellow-orange fleshed mamey sapote, while orange highlighted names refer to carotenoids present in red-orange genotypes. Sapotexanthin was present in both genotypes and hence it is highlited in both yellow and orange.

Lastly, carotenoid synthesis has also been associated with chromoplast biogenesis. Since chromoplasts are the site of carotenoid biosynthesis and storage, an increase in size and number of chromoplasts will probably imply higher carotenoid contents (López *et al.*, 2008). Experiments in transgenic potatoes demonstrated that an increase in the amount of chromoplasts can lead to a increment in total carotenoid content (Lu *et al.*, 2006).

In mamey sapote, total carotenoid contents could be also linked to regulation of PSY, as exposed above, causing the differences between the three genotypes analyzed, or it could also be associated to chromoplasts biogenesis. The level at which carotenoid contents is regulated in this species is still unknown as carotenoid biosynthesis pathways have not been elucidated yet.

Differences were also present in the carotenoid profiles of the genotypes of red-orange fleshed and yellow-orange fleshed fruits. Red-orange fleshed genotypes showed lower amounts of β-carotene-5,6-epoxide (peak 17), than the yellow-orange fleshed sapote analyzed. The β-carotene-5,6-epoxide was proposed to be a biosynthetic precursor of sapotexanthin by Murillo *et al.* (2011). The possible pathway for this ketocarotenoid involves the enzyme CCS (capsorubin-capsanthin synthase), where a β-ring is converted into a κ-ring in sapotexanthin (Fig. 7.6). The enzyme CCS was originally found in red peppers where it drives the conversion of antheraxanthin and violaxanthin into capsanthin or capsorubin, respectively (Ha *et al.*, 2007). These latter pigments are ketocarotenoids similar to sapotexanthin. Figure 7.6 shows the mechanism through which a 3-OH β-ring is converted into a 3-OH κ-ring, as described for red peppers. At full maturity, red peppers acquire their characteristic red coloration due to the synthesis of both capsanthin and capsorubin.

Although differences were found in the concentration of the proposed precursor of sapotexanthin, sapotexanthin levels for the three genotypes were similar (Table 7.3). If β-carotene-5,6-epoxide was the precursor of sapotexanthin and it is being accumulated in the yellow-orange fleshed fruits, it would be expected to be related with lower levels of sapotexanthin. One explanation might be differences in the genes encoding for CCS or the enzyme in charge of the synthesis of sapotexanthin, so different activity levels are present.

Therefore, even if the precursor is present in high concentrations, the efficiency of conversion is low. In Guzmán *et al.* (2010), *Lcyb* gene sequences, encoding for lycopene-β-cyclase (β-LCY) from orange and red peppers, differed, resulting in a different carotenoid content. However, the enzyme in orange peppers was still functional, since β-carotene was biosynthesized. It was suggested that this enzyme could be less effective than the one reported for red peppers, resulting in lower concentrations of β-carotene and, furthermore, lower amount of capsorubin and capsanthin.

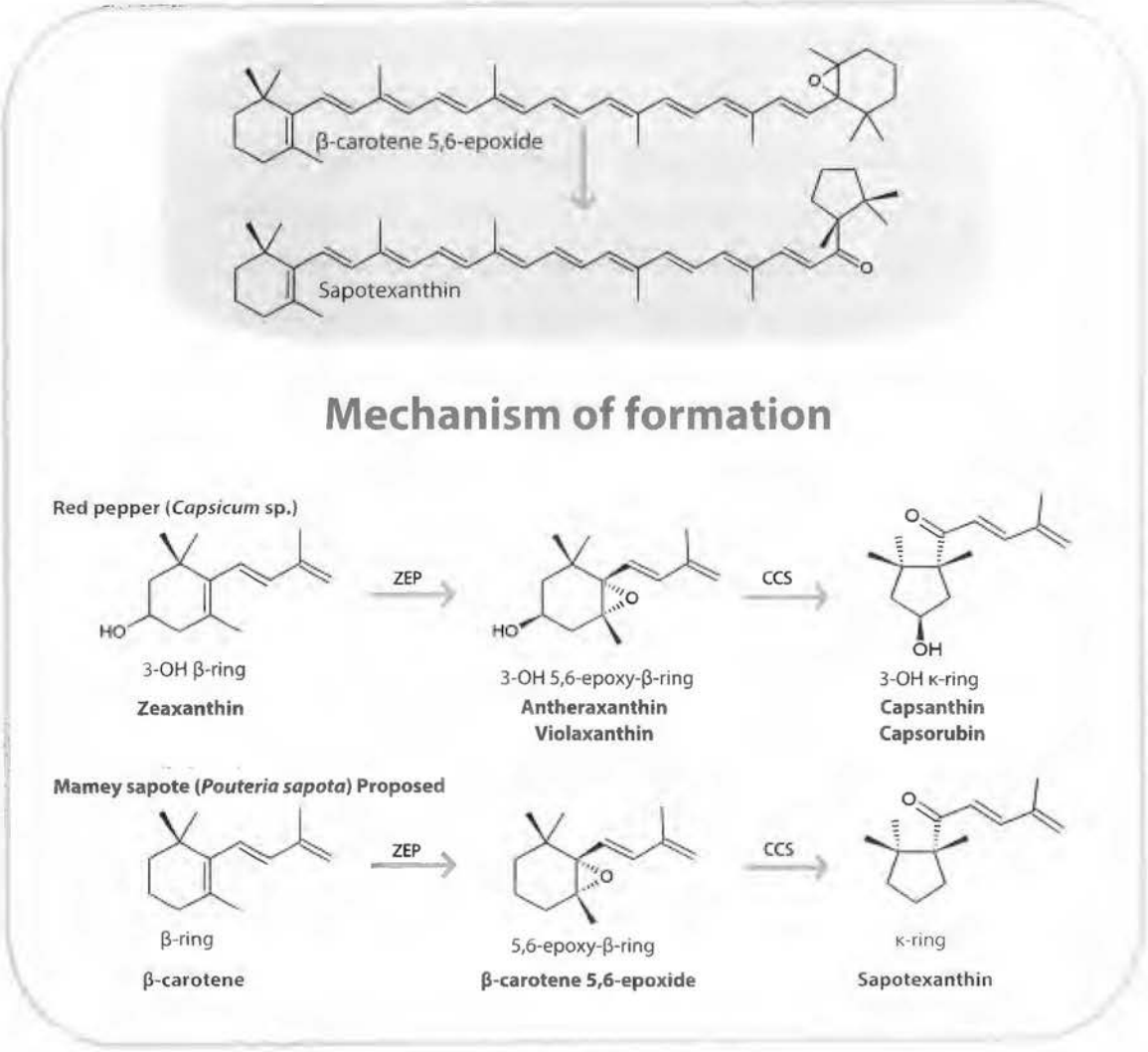

Figure 7.6. Mechanism proposed by Murillo *et al.* (2011) for formation of the κ-ring in sapotexanthin and comparison to the one reported for red pepper. ZEP: Zeaxanthin epoxidase, CCS: Capsorubin Capsanthin Synthase.

A similar case involving two LCY enzymes has been described in red- and yellow-fleshed papaya fruits (Devitt *et al.*, 2010). They presented highly similar carotenoid patterns, except for the near absence of β-carotene precursor, lycopene, in the latter. In papaya, such differences were attributed to one functional and one dysfunctional lycopene-cyclase (LCY) for the red type, whereas two functional LCYs are present in yellow papaya. In the case of dysfunctional LCY, lycopene was accumulated in high amounts. When the functional LCY was present, the major proportion of lycopene was converted to typical papaya carotenoids. In sapote, two CCS may be present. While one of them is assumed to be either dysfunctional or inhibited in the yellow-orange-fleshed genotype (11163), both of them could be functional in the red-orange-fleshed variants. This would explain the significantly higher accumulation of proposed precursor β-carotene-5,6-epoxide mainly in one of the genotypes. If β-carotene-5,6-epoxide is the precursor of sapotexanthin as proposed by Murillo *et al.* (2011), a lower concentration of sapotexanthin would be expected in yellow-orange fleshed fruits. The presence of another , yet unknown, precursor is suggested to explain why sapotexanthin is accumulated to the same level in all genotypes.

Interestingly, there are other carotenoids showing a similar behavior, apparently accumulating precursors, and since presence of ketocarotenoids seems to be the main difference between the genotypes with different flesh color they should also be considered. Ketocarotenoids present mainly in red-orange fleshed mamey sapotes like capsorubin, capsoneoxanthin and cryptocapsin, are synthetized in a similar reaction by pinacol rearrangement of a 3-OH-5,6-epoxy end group that produces the κ-ring, neoxanthin and cryptocapsin epoxide, respectively. As mentioned before, in red pepper the enzyme CCS is responsible for the conversion of violaxanthin into capsorubin, but enzymes involved in the conversion of the other ketocarotenoids have not been described yet.

It has been demonstrated that alteration in the CCS enzyme in peppers is responsible for the absence of the ketocarotenoids capsanthin and capsorubin in yellow and orange peppers (Li *et al.*, 2013; Popovsky and Paran, 2000). In yellow peppers, there is a linkage between the absence or modification of the CCS and, since this is not functional, capsanthin and capsorubin cannot be synthetized (Rodríguez-Uribe *et al.*, 2012). Several molecular studies have proposed different explanations for the coloration in yellow and even orange peppers.

It has been demonstrated that deletion of the CCS in yellow peppers, deletion of the amino terminus of CCS in orange peppers or even a premature translation termination stop due to base pair deletion that causes a frame shift in Fogo variety (orange) are responsible for the different color and carotenoid profile (Guzmán *et al.*, 2010; Lefebvre *et al.*, 1998). These yellow and orange peppers at ripe stage accumulate the precursors violaxanthin or zeaxanthin, respectively (de Azevedo-Meleiro and Rodríguez-Amaya, 2009). Therefore, absence of capsorubin in yellow-orange fleshed genotype could be the result of CCS regulation.

In saponified samples from yellow-orange fleshed sapote, it was noted that a larger amount of neoxanthin was present compared to the other two red-orange fleshed genotypes. One of the pigments, tentatively identified as capsorubin (peak 9), was not detected in yellow-orange fleshed fruit, only in the red-orange fleshed ones. Violaxanthin is the precursor of capsorubin, as shown in Figure 7.5, but violaxanthin can also be converted into neoxanthin (via neoxanthin synthase). Apparently, in yellow-orange fleshed fruits the conversion from violaxanthin to neoxanthin is favored, rather than the biosynthesis of capsorubin with CCS. This is in agreement with capsorubin only been found in red-orange fleshed fruits (Table 7.3).

A similar behavior is observed with capsoneoxanthin (peak 5) that is present in both red-orange fleshed genotypes but not in the yellow-orange one. This carotenoid was previously described as a minor carotenoid in *Asparagus falcatus* (Deli *et al.*, 2000) and later in mamey sapote fruits (Turcsi *et al.*, 2010). Two mechanisms for its synthesis have been proposed, the first and most accepted one by conversion of neoxanthin by a pinacol rearrangement to capsoneoxanthin; and the second one with capsanthin 5,6-epoxide as precursor (Deli *et al.*, 2000). The first mechanisms is more probable for mamey sapote fruits since neoxanthin is present in all genotypes and it appears that many biosynthetic reactions occurring in mamey sapote fruits include pinacol rearrangement. Additionally, in yellow-orange genotype, capsoneoxanthin was not found and instead high concentrations of neoxanthin were present. Somehow, the biosynthesis of capsoneoxanthin in yellow-orange fleshed fruits may be negatively affected, which can be reflected in the accumulation of the precursor neoxanthin.

Another pigment that was present in higher concentration in saponified samples of 8747 red-orange fleshed genotype was cryptocapsin (peak 16) (Fig. 7.2) (Table 7.3). This pigment has been found previously in red pepper in low amounts (Almela *et al.*, 1991; Mínguez-Mosquera and Hornero-Méndez, 1993; Rodríguez-Amaya *et al.*, 2007) and it is believed to be synthetized via β-carotene – β-cryptoxanthin with the formation of β-cryptoxanthin epoxides (Almela *et al.*, 1996). β-cryptoxanthin epoxides (peaks 6, 8 and 15) and cryptocapsin esters that were present in all three genotypes, but there was a significant difference in their concentration between the genotypes. In red-orange fleshed fruits, higher amounts of cryptocapsin esters were found (14 %), while it only represented around a 7 % of total carotenoid content in yellow-fleshed genotype. As for β-cryptoxanthin epoxides and diepoxides, in red-orange fleshed fruit it constituted a 12,5 % of the total carotenoid content and up to 24 % in yellow-orange fleshed fruits, this includes β-cryptoxanthin 5,6-epoxide (peak 14) only found in in yellow-orange fleshed fruits. Therefore, the accumulation of the cryptocapsin precursors in yellow-orange fleshed fruits is evident, similar to the case of capsoneoxanthin and capsorubin discussed above.

7.5. Conclusions

Both the qualitative and quantitative carotenoid composition varied between the genotypes analyzed. Red-orange fleshed fruits presented higher concentrations of cryptocapsin esters and other ketocarotenoids, like capsoneoxanthin and capsorubin, while in yellow-orange fleshed fruits genotypes, neoxanthin and other epoxides appear to be the main pigments. Sapotexanthin was present in similar concentrations in the all three genotypes; however, yellow-orange fleshed fruits appear to accumulate higher amounts of β-carotene-5,6-epoxide, its proposed precursor. Regulatory mechanisms involving inactivation or less efficient activity of the enzymes involved in sapotexanthin biosynthesis are proposed. Total carotenoid content varied significantly between the two red-orange fleshed genotypes despite the similarity of their color. This suggests that flesh color measurements in mamey sapote should not be used for the estimation of total carotenoid contents in red-orange fleshed fruits. Therefore, more detailed studies on carotenoid contents are recommended for assessing the nutritional potential of fruits with similar flesh color.

Further studies are needed to reveal the mechanisms responsible for the differences found in mamey sapote carotenoids from red-orange and yellow-orange fleshed genotypes. In order to elucidate these mechanisms by which carotenoid biosynthesis is regulated, genetic analyses are necessary. The first step will be to determine the presence of key genes like PSY and CCS and their expression level, as well as the variations between fruits with different flesh color. Additionally, activity assays could determine if all enzymes present are active and at which level. Since provitamin A carotenoids were found, further studies to establish their absorption in humans would be interesting. Sapotexanthin bioavailability in sapote fruits remains unknown to date. Furthermore, breeding programs looking for sapote fruits with higher content of the provitamin A carotenoids, sapotexanthin and cryptocapsin, are encouraged.

7.6. References: Chapter IV

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8. Chapter V: *In vitro* bioaccessibility of carotenoids present in mamey sapote fruits: effect of processing and chromoplast ultrastructure, comparison with other ketocarotenoid sources

8.1. Abstract

Vitamin A deficiency is a health problem affecting mainly developing or poor countries. Numerous efforts have been made to study new plant sources of carotenoids that have provitamin A activity and are more accessible and less expensive in production than alternative known animal sources. Mamey sapote fruits are one of these promising plant foods, since it contains high concentrations of two ketocarotenoids with potential provitamin A activity, sapotexanthin and cryptocapsin. Most other known common ketocarotenoids, like capsorubin, capsanthin and astaxanthin, are not considered precursors of vitamin A for humans due to their structure. In order to define the nutritional value of the carotenoids present in mamey sapote fruits, the determination of their bioaccessibility is a prerequisite for subsequent human intervention trials. Therefore, the goal of this study was to assess the *in vitro* bioaccessibility of mamey sapote fruits and other known sources of ketocarotenoids, such as red bell pepper and salmon. In general, relative bioaccessibility was low (< 4 %) for all samples, being capsanthin from red bell pepper the most bioaccessible (4 %), followed by sapotexanthin (1 %), cryptocapsin esters (0.1%) and finally astaxanthin (1.5 %). Thermal treatment and fat addition enhanced bioaccessibility of almost all carotenoids studied, increasing up to 8 % (capsanthin). Since chromoplast ultrastructure was previously shown to exert an enormous impact on carotenoid bioaccessibility, the carotenoid bearing elements of sapote chromoplasts were elucidated using light and transmission electron microscopy. Mainly, globular-tubular chromoplasts were found to contain large lipid globules and fibrillar accumulations (tubules), presumably containing lipid-dissolved and liquid-crystalline carotenoids. Such carotenoid deposition forms are expected to be highly bioavailable; however, a further human study is required to prove this hypothesis.

8.2. Introduction

Fruits and vegetables are known sources of a wide array of micronutrients, including the yellow, orange, and red pigment class called carotenoids, with some of them known for their provitamin A activity. Deficiency of this vitamin has been reported as a moderate to severe health problem in at least 122 countries (FAO/WHO, 2002). Therefore, there has been increasing investigations targeting at the identification and quantification of carotenoids from new vegetable sources, which are more accessible than other alternative known sources. Besides of their provitamin A activity, carotenoids have been also associated with numerous health benefits like boosting the immune system and acting as antioxidants (Deming *et al.*, 2002). In order to exert these benefits carotenoids need to be absorbed by human body, i.e., they need to be bioavailable. Bioavailability refers to the "fraction of an oral dose of a parent compound or active metabolite that reaches the systemic circulation" (Schumann *et al.*, 1997). Despite the existence of over 700 different carotenoids, only few have been found in human plasma including β-carotene, β-cryptoxanthin, lycopene, lutein, α-carotene and zeaxanthin (Faulks and Southon, 2005).

Determination of bioavailability requires human clinical trials; however, these studies are expensive, often invasive and time consuming. Therefore, alternative *in vitro* digestion models have been proposed for the prediction of bioavailability (Fernández-García *et al.*, 2012; Rodríguez-Amaya, 2010). These models can evaluate the so-called bioaccessibility of compounds, which corresponds to the capacity of the digestion process to liberate metabolites from the food matrix (Goñi *et al.*, 2006). *In vitro* models have the advantage of being less expensive, faster and that they allow the evaluation of several factors in one single study (Fernández-García *et al.*, 2009). Carotenoids go through a simulation of the digestion process and, at the end of this process, a micellized fraction is recovered. Carotenoids are lipophilic compounds, therefore, they are incorporated into the human body via lipid absorption. Carotenoids that go through the digestion process are incorporated into micelles. Micellization of these lipophilic micronutrients is a prerequisite for their absorption by the enterocytic cells (Parker, 1996). Several carotenoid sources have been evaluated using *in vitro* models to determine bioaccessible-micellar fractions (Fleshman *et al.*, 2011; Garrett *et al.*, 1999; Granado-Lorencio *et al.*, 2007; Schweiggert *et al.*, 2012).

Carotenoid bioaccessibility and bioavailability can be affected by diverse factors summed up in the mnemonic "SLAMENGHI" (West and Castenmiller, 1998). For bioaccessibility, the first four are important; this includes, species of carotenoids, molecular linkage, amount of carotenoids, matrix and effector of absorption. Recent studies have also remarked the influence of plant ultrastructure and carotenoid localization, specifically chromoplasts structure, on bioaccessibility of pigments in different fruits (Schweiggert *et al.*, 2012).

There is a series of carotenoids whose bioaccessibility and bioavailability has been assessed, i.e., β-carotene, β-cryptoxanthin, lycopene and some xanthophylls have been evaluated (Chitchumroonchokchai and Failla, 2006; Goñi *et al.*, 2006; Hornero-Méndez and Mínguez-Mosquera, 2007). Mamey sapote fruits have a high content of ketocarotenoids, including sapotexanthin and cryptocapsin esters (Chapter IV). Ketocarotenoids are commonly found in other food sources like red pepper and salmon, capsanthin and astaxanthin (Curl, 1962; Storebakken *et al.*, 1987) , respectively. According to their structure, ketocarotenoids present in mamey sapote could have human provitamin A activity in contrast to those in red pepper and salmon (Fig. 8.1).

In order to be considered provitamin A active, carotenoids need to have at least one unsubstituted β-ionone ring. For example, in β-carotene, the molecule can be enzymatically cleaved into two identical retinal structures, which will be subsequently converted into retinol (Vitamin A_1) and retinol esters for storage (Woollard, 2012) (Fig. 8.2). Despite not being precursors for provitamin A, capsanthin and astaxanthin can also provide health benefits due to their antioxidant activity (Matsufuji *et al.*, 1998; Naguib, 2000). Additionally, presence of sapotexanthin or cryptocapsin in plasma have not been reported in literature, contrary to capsanthin and astaxanthin for which *in vivo* bioavailability assays have confirmed their absorption in human body (Etoh *et al.*, 2000; Østerlie *et al.*, 2000). Since ketocarotenoids are rare in edible foods, comparison of carotenoids in mamey sapote fruits with other sources can be difficult, particularly since the ketocarotenoid sapotexanthin has only been described for mamey sapote fruits so far (Murillo *et al.*, 2011). Additionally, no information is available on mamey sapote ketocarotenoids bioaccessibility or bioavailability.

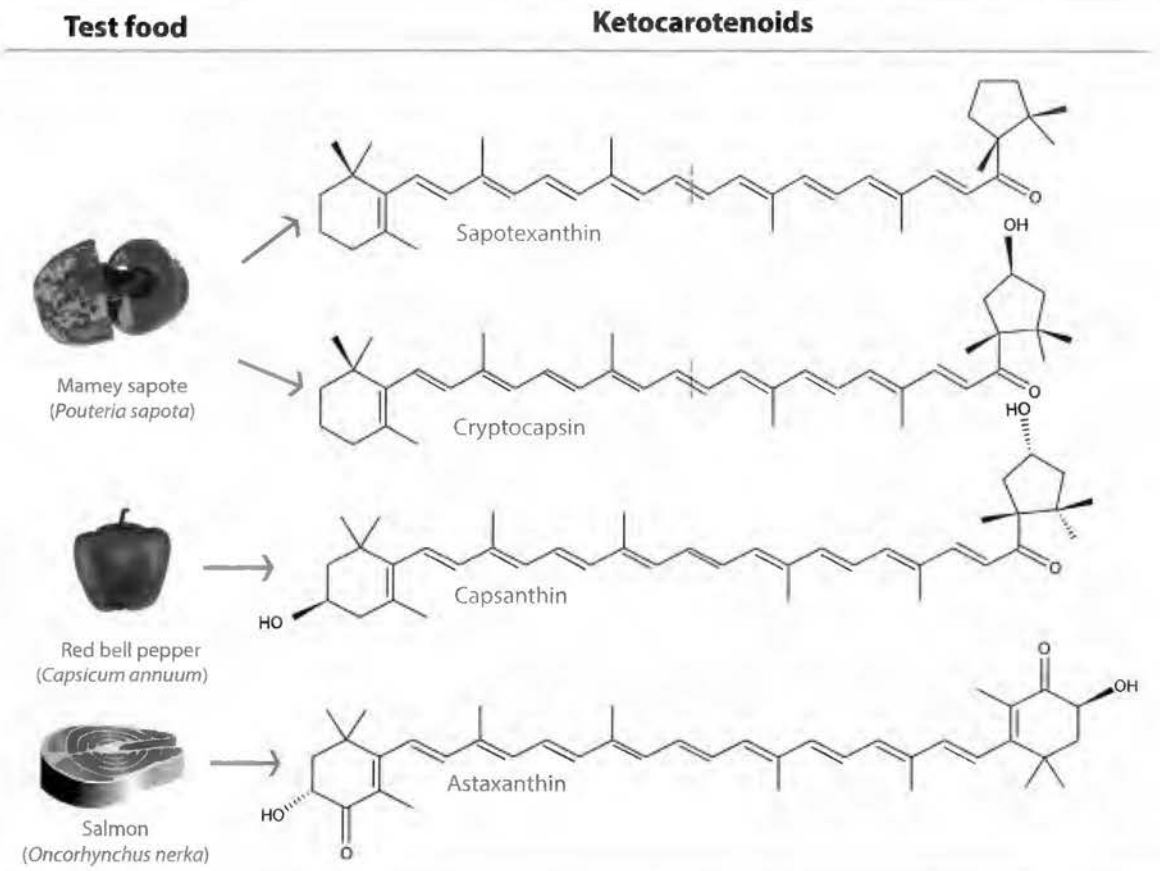

Figure 8.1. Structure of ketocarotenoids present in test foods mamey sapote, red bell pepper and salmon. Orange dashed lines mark eventual cleavage to obtain vitamin A.

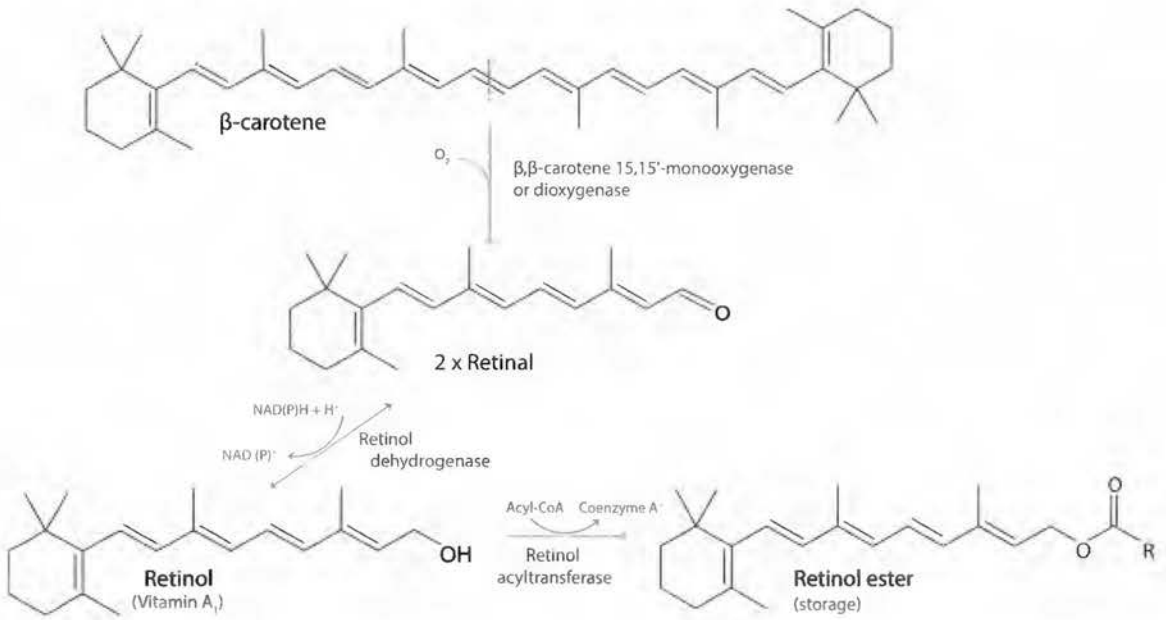

Figure 8.2. Conversion of β-carotene to retinal, retinol and retinol esters.

Therefore, the goal of this study was to determine the bioaccessibility of mamey sapote carotenoids using an *in vitro* digestion model. The fruits were digested raw and after cooking them with 1% (w/w) oil. The bioaccessibility was compared to that of two other known ketocarotenoid sources, salmon and red pepper. Additionally, chromoplast ultrastructure in mamey sapote fruits was evaluated and compared with previously reported data on chromoplasts from red pepper and carotenoid distribution in salmon flesh.

8.3. Materials and methods

8.3.1. Samples and sample processing. Test foods to evaluate bioaccessibility consisted of red-orange fleshed mamey sapote fruits, red bell pepper fruits (*Capsicum annum* L.) and salmon filet (*Oncorhynchus ner*ka) (Sockeye Wildlachs, Suempol, Germany). Red pepper and salmon were obtained at a local market in Stuttgart in 2013 and stored at 7 °C until analysis. Mamey sapote fruits were sampled from the collection of the Tropical Agricultural Research and Higher Education Center, CATIE (Turrialba, Costa Rica). The fruits were transported by plane to the University of Hohenheim, Stuttgart, Germany, and stored wrapped with newspapers at 23 °C until fruit maturity. Mamey sapote fruit pulp was cut into small cubes, frozen with liquid nitrogen, packed airtight and stored at -80 °C. Samples from different fruits were pooled to obtain a homogeneous sample. Salmon and red bell pepper samples, including flesh and peel for the latter, were cut into small squares on the day of the bioaccessibility assay. One fraction of each test food was used for the bioaccessibility assay of raw samples and the other fraction was cooked for 30 min at 90 °C (water bath) with 1% (w/w) soybean oil (Sojola, Germany) to evaluate the effect of heat and lipid addition. Heat-treated samples were frozen and stored at -80 °C for further bioaccessibility analysis. A fraction of each test food raw and cooked was stored at -80 °C for further carotenoid analysis.

8.3.2. In vitro bioaccessibility assay. Bioaccessibility assays were performed based on the method of Garrett et al. (1999) and modified according to Schweiggert et al. (2012). The employed in vitro digestion model involved oral, gastric and intestinal phases (Fig. 8.3). All samples were homogenized with a mortar for 45 sec simulating chewing process prior to in vitro digestion. The initial sample weight for test food was 10 g for red pepper and salmon,

while initial volume of mamey sapote samples was doubled due to low recovery. Volume for the digestion was adjusted according to the weight. After homogenization, 10 g (20 g from mamey sapote) were transferred to an amber glass bottle and 10 mL of artificial saliva solution (50 mM NaCl, 10 mM KH_2PO_4, 2 mM $CaCl_2 \cdot 6H_2O$, 40 mM $NaHCO_3$) were added and the pH was adjusted to 6.9 with 1N NaOH or 1 M HCl. Subsequently, 100 µL of an α-amylase solution (25 U) were poured into the mixture and each flask was gently shaken for homogenization. For the gastric phase, 3 mL of gastric solution (51 mM NaCl, 14.7 mM KCl, 10 mM $CaCl_2 \cdot 2H_2O$, 3.7 mM KH_2PO_4, 3.4 $MgCl_2 \cdot 6H_2O$) were added and the pH was adjusted to 4 with 1 M HCl. Subsequently, 2 mL of a porcine pepsin solution (40 mg/mL in 0,1 M HCl) were added and pH was re-adjusted to 2 with 1 M HCl. Headspace of each flask was flushed with nitrogen and samples were kept in a shaking bath at 37 °C for 1 h at 95 rpm. After this, pH was adjusted to 5.3 with 0.9 M NaHCO3 and 9 mL of bile extract/pancreatin solution (12 mg/mL porcine bile extract and 2 mg/mL pancreatin in aqueous 0.1 M $NaHCO_3$ solution) were added. Additionally, 174 µl of a cholesterol esterase solution (5 U in 0.1 M Na_2HPO_4 buffer, pH 7) were included and pH was readjusted to 7.5 with 1 N NaOH. Headspace of each flask was flushed again with nitrogen and samples were kept in a shaking bath at 37 °C for 2 h at 95 rpm. After in vitro digestion, samples were made up with deionized water to 50 mL for red pepper and salmon and to 100 mL for mamey sapote pulp. Samples were centrifuged for 60 min at 10 °C in a JA-25.50 rotor at 75 000x g for 60 min (Avanti J-26 XP/XPI, Beckman Coulter, Krefeld, Germany) in order to separate the precipitate and the aqueous phase containing the liberated carotenoid and micellar fraction. Half of the aqueous phase was stored at -80 °C and the other half was filtered through a 0.2 µm syringe filter (cellulose acetate based, Klaus Ziemer, Mannheim, Germany) to separate the micellar fraction. The filtrate was stored at -80 °C until carotenoid analyses.

Carotenoid extraction Carotenoids from fresh samples were extracted with a modified method from Murillo *et al.* (2011) as follows. The samples were shortly homogenized with a porcelain mortar, 1.0 ± 0.1 g of the macerate was taken and put in a tube with 0.1 g of $NaHCO_3$. The extraction was carried out with an ultrasonic system (Sonopuls HD 3100, Germany) with a MS 72 probe, using acetone (0.1 % BHT). The extraction was repeated at least 4 times until sample residue appeared colorless. A last

extraction was done with n-hexane; subsequent phase separation was achieved with 4 mL d iethyl ether/n-hexane (1:1 v/v, 0.1 % BHT) and washed twice with deionized water. The upper phase containing extracted carotenoids was collected and evaporated under nitrogen atmosphere and stored at -80 °C until carotenoid analysis. Mamey sapote samples were resuspended in TBME/MeOH (1:1, v/v) and filtered with a 0.45 µm pore PTFE for HPLC analysis.

Figure 8.3. Diagram of *in vitro* bioaccessibility methodology to obtain micellar and liberated fraction of carotenoids from test foods.

Carotenoid extraction of the supernatant and filtered fraction of the digestion model was performed as follows. An aliquot of 12 mL of the respective fraction was extracted two times with 3 mL acetone (0.1 % BHT) and 6 mL methanol, ethyl acetate and light petroleum (bp 40-60 °C) (1:1:1 v/v/v) extraction mixture. The upper phases were collected, combined, evaporated under nitrogen atmosphere, and stored at -80 °C. For HPLC analysis, samples were re-suspended in TBME/MeOH (1:1, v/v) and filtered with a 0.45 µm pore PTFE.

8.3.4. HPLC-DAD analysis Carotenoid separation was achieved using a 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a G1379A degasser, a G1312A binary gradient pump, a G1313A autosampler, a G1316A column oven, and a G1315B diode array detector. The column was operated at 40 °C and consisted of an analytical scale YMC C_{30} reverse phase column (3 µm particle size, 150 x 3.0 mm i.d., YMC Europe, Dinslaken, Germany), protected by a YMC C_{30} guard column (10 x 30 i.d., 3 µm particle size, YMC Europe). The mobile phase used was MeOH/H_2O (90:10, v/v/v, eluent A) and MeOH/TBME/ H_2O (20:78:2, v/v/v, eluent B), both containing 1.5 g/L ammonium acetate. The gradient was set as follows: isocratic 100% A for 5 min, from 100 to 25% A in 73 min, from 25% A to 0% A in 4 min and from 0% A to 100% A in 8 min. Total run time was 90 min at a flow rate of 0.8 mL/min. The carotenoids were monitored at 450 nm and additional UV-Vis spectra was recorded in the range of 200–600 nm. When necessary, mass spectrometric analysis was performed by coupling the above-described HPLC system to a Bruker 3000+ ion trap mass spectrometer (Bruker, Bremen, Germany), operating in positive mode and an APCI source. Parameters for analysis were carried out as described by Schweiggert *et al.* (2005). Identification of carotenoids was accomplished by comparison of UV-Vis absorption spectra, retention times and mass spectra with those of authentic standards. When standards were unavailable, pigments were tentatively identified by comparing their UV-Vis absorption spectra and mass spectral behavior with previously published data (See Chapter IV).

8.3.5. Quantitation Quantitation of carotenoids was achieved with a HPLC calibration curve using standards for β-carotene and astaxanthin, purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). When standards were not available, quantitation was performed using the β-carotene calibration curve. Molecular weight correction factors

(MWCF) were used when necessary, representing the ratio of the molecular weight of the compound to be quantitated and the molecular weight of β-carotene. Unknown compounds were quantitated using the β-carotene calibration curve.

Light microscopy Fresh, free-hand sections of mamey sapote, red bell pepper and salmon were cut with razor blades and mounted on glass slides without staining. Slides were observed in an inverted microscope Olympus IX- 51 (Tokyo, Japan).

Transmission electron microscopy Sections of mamey sapote mesocarp of approximately 1 mm^2 were obtained with a razor blade. Samples were immediately fixed in a modified Karnovsky solution (2.5 % w/v glutardialdehyde, 2 % w/v paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.4) for at least 4 h at 4 °C. Samples were then washed three times for 15 min with 0.1 M sodium phosphate buffer. After 2 h post fixation at room temperature with a 2 % w/v osmium tetroxide solution, samples were washed three times for 15 min with water and, subsequently, dehydrated with an acetone series (30 %, 50 %, 70 %, 90 % and 3 x 100 %). Samples were then embedded in Spurr's medium and polymerized at 60 °C for 48 h. Ultrathin sections were obtained with a diamond knife using a Power Tome PC (RMC Products, Arizona, USA) ultramicrotome and collected in cooper grids. Grids were stained with uranyl acetate and lead citrate prior to observation in a Hitachi H-7100 (Tokyo, Japan) transmission electron microscope at 100 kV.

Statistical analysis A one-way analysis of variance (ANOVA) was conducted to determine significant differences between bioaccessible and liberated fractions within the same test food. Shapiro-Wilk's test was conducted to test normality of the data, and homogeneity of variances was assessed by Levene's test. For non-parametric samples, a Kruskall-Wallis test was conducted. All analyses were performed with the program SAS JMP 8 (SAS Institute Inc. Cary, NC, USA). All *in vitro* assays were performed in triplicate and carotenoid extraction in duplicate.

Estimation of *in vitro* carotenoid liberation and bioaccessibility was calculated as a percentage of the carotenoids that were transferred from the test food to the supernatant phase (liberated carotenoids) recovered after ultracentrifugation and to the micellar fraction

(bioaccessible carotenoids) obtained after filtration, as described previously by Bengtsson *et al.* (2009).

8.4. Results and discussion

Food matrix plays an important role in determining carotenoid bioaccessibility. Animal or plant sources have different tissues that could act as barriers to access carotenoids. Plant ultrastructure has been linked with carotenoid bioaccessibility in several studies since it constitutes a barrier to access carotenoids stored inside the cells (Bengtsson *et al.*, 2010; Colle *et al.*, 2010; Jeffery *et al.*, 2012; Schweiggert *et al.*, 2012; Tumuhimbise *et al.*, 2009). Carotenoids location within plant tissue, specifically the type of chromoplast, in which they are embedded, has shown great impact on carotenoid bioaccessibility (Schweiggert *et al.*, 2012).

In mamey sapote fruits, light micrographs of mesocarp cells showed small round-shaped colored structures corresponding to chromoplasts. Some starch granules were also present. No crystalloid structures were observed (Fig.8.4).

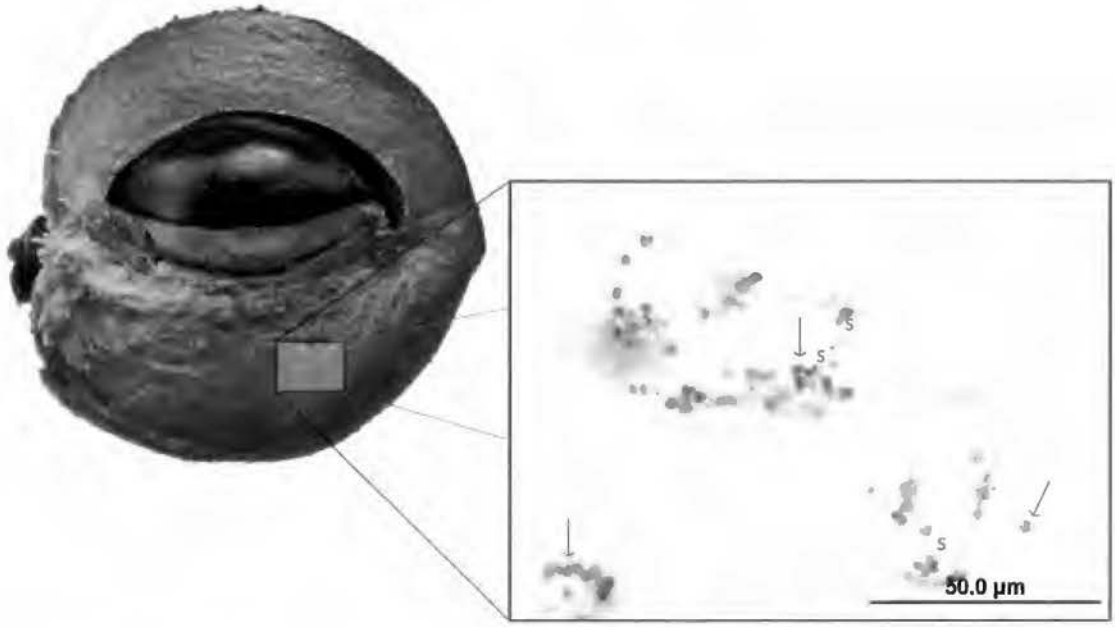

Figure 8.4. Mamey sapote fruit and light micrograph of fruit mesocarp. Arrows mark chromoplasts; some starch grains (S) are also visible.

Transmission electron micrographs allowed detailed elucidation of carotenoid-bearing ultrastructures within chromoplasts of the mamey mesocarp tissue. Chromoplasts containing tubular elements and numerous globules were observed; therefore, they could be categorized as globular-tubular (Fig. 8.5. A., B., C.). This type of chromoplasts have been previously described in mango cv. 'Tommy Atkins' (Vásquez-Caicedo *et al.*, 2006) and in papaya (Schweiggert *et al.*, 2011). Interestingly, uncommonly large globules were observed, reaching a diameter of up to 0.72 µm. These globules are bigger than the ones found in mango chromoplasts (0.1 to 0.5 µm) (Vásquez-Caicedo *et al.*, 2006). The total size of the chromoplasts (diameter) found were around 1.6 – 2.7 µm, similar to the ones reported in mango (approx. 3 µm) and papaya (approx. 2.5 µm) (Schweiggert *et al.*, 2011; Vásquez-Caicedo *et al.*, 2006).

Other forms of chromoplast were also observed in this work, like globular chromoplasts containing some apparent stroma thylakoid remnants (Fig. 8.5 D.) and amylo-chromoplasts that also contained some tubular elements (Fig. 8.5 E.). Amylo-chromoplasts have recently been found in yellow peach palm mesocarp and tissue near the peel (Hempel *et al.*, 2014). Furthermore, such plastids were observed in mango fruits (Vásquez-Caicedo *et al.*, 2006). Amylo-chromoplasts have been previously observed in nectaries of tobacco flowers, where they were described to be an intermediate stage in the conversion of amyloplast into chromoplasts (Horner *et al.*, 2007).

Carotenoids can be stored in other types of chromoplasts or different structures, depending on the matrix in which they are embedded. Figure 8.6 shows a comparison between carotenoid storage structures of the food samples analyzed. In mamey sapote, as described before, carotenoids seem to be stored mainly in globular-tubular chromoplasts. Carotenoids stored in lipid globules are in lipid-dissolved physical state, and they are arranged into spherical shape structures (globules) due to an hydrophobic effect with the aqueous medium surrounding them (Sitte, 1981). Globular chromoplasts containing numerous lipid globules were found in fruits including yellow peppers, being devoid of ketocarotenoids (Evert, 2006). Tubular elements were also observed in red pepper chromoplasts (Frey-Wyssling and Kreutzer, 1958). Tubule assembly was evaluated by Deruère *et al.* (1994) to determine

the composition of these elements; they found that esterified carotenoids were more efficiently assembled into this structure than β-carotene (Deruère *et al.*, 1994). These tubules present an internal apolar core where carotenoids are stored surrounded by a layer of polar lipids and proteins (Sitte, 1981; Winkenbach *et al.*, 1976). Carotenoids in this type of structure are described as nematic liquid crystals and require high concentrations of apolar carotenes or carotenoid esters to be formed (Sitte, 1980, 1981) (Fig.8.6).

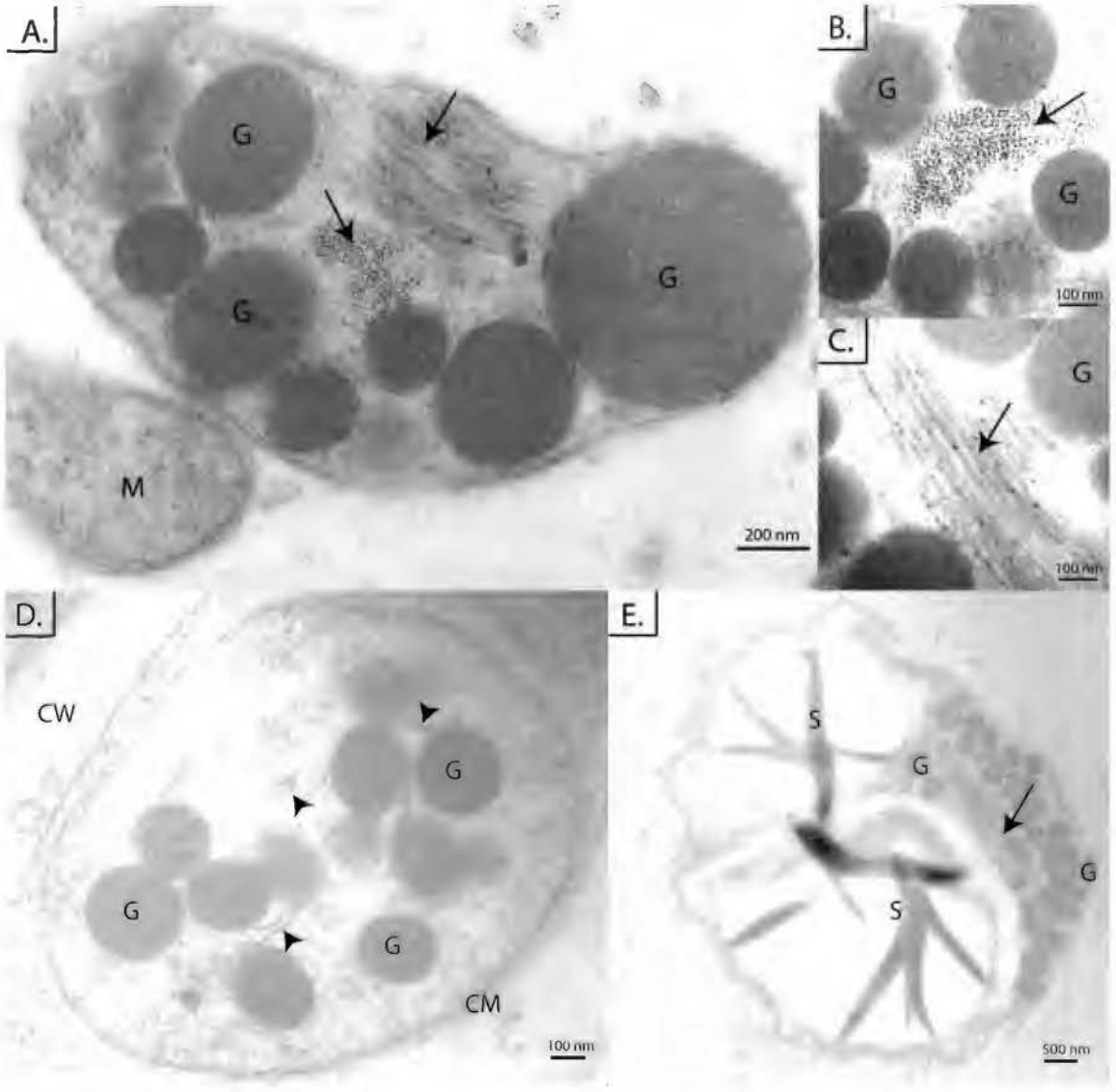

Figure 8.5. A. Electron micrographs of globular-tubular chromoplast in fully ripe red-orange fleshed mamey sapote fruits (A). In B and C, a detailed view of the tubular elements with different orientation is shown. D. Amylo-chromoplast presenting some starch granules (S), as well as tubular elements. E. globular chromoplast containing some apparent stroma thylakoid remnants (➤). Arrows: tubular elements, G: globules, M: mitochondria, CM: chromoplast membrane, CW: cell wall, S: starch.

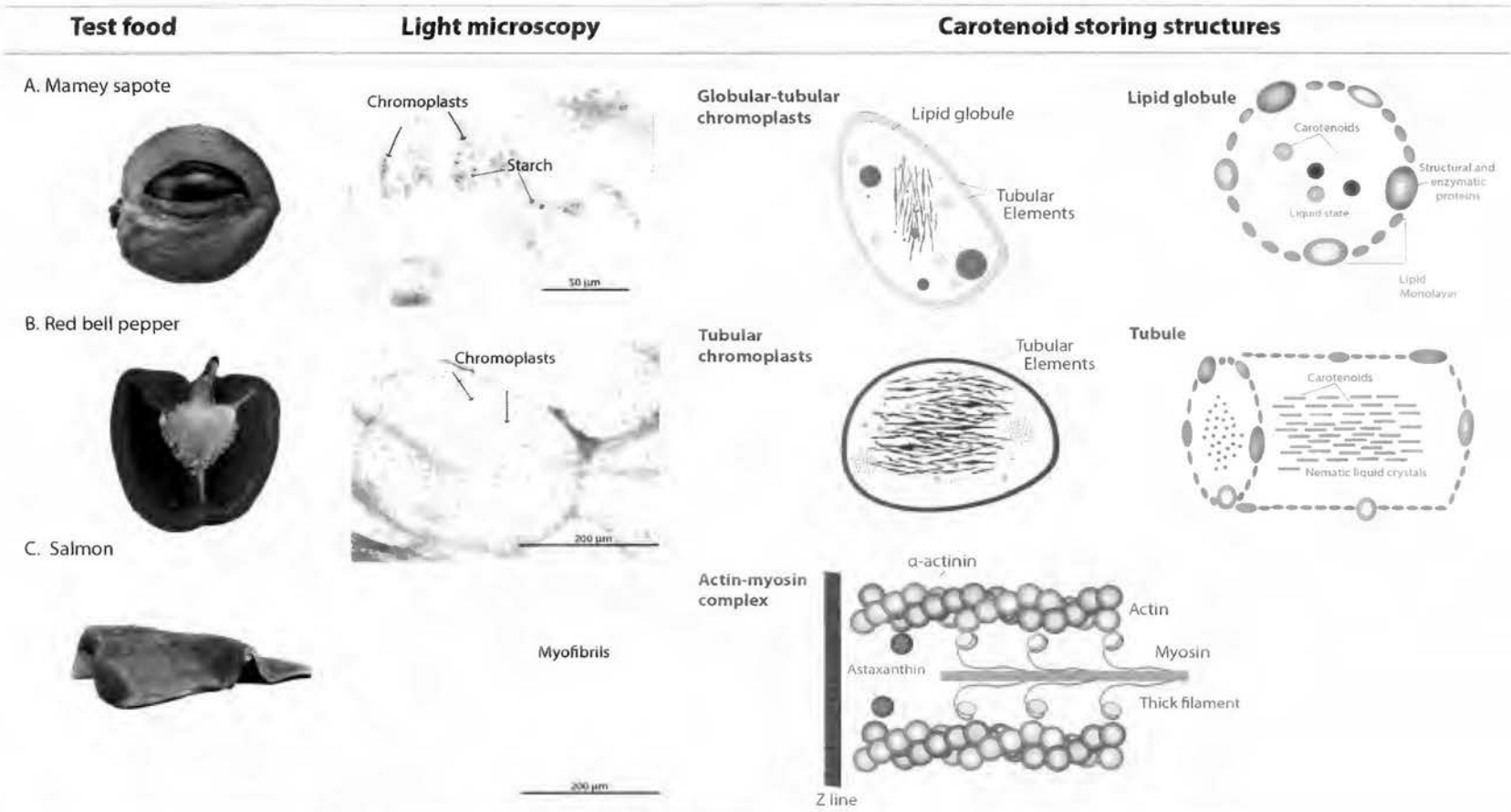

Figure 8.6. Light microscopy images and detailed diagram of carotenoid storage structures in food samples analyzed. A. Mamey sapote fruits in globular-tubular chromoplasts, detailed view of lipid globule. B. Red bell pepper in tubular chromoplasts, detailed view of tubule. C. Salmon associated to α-actinin in the actin-myosin complex inside muscle cells. Models of lipid globules, tubules and astaxanthin bonded to α-actinin based in descriptions by Sitte (1981) and Matthews *et al.* (2006).

Since mamey sapote contains cryptocapsin esters as mayor pigments it could be suggested that these are the main ones deposited in the tubular elements. This in accordance to description and evaluation of tubule assembly conducted by Sitte (1981) and Deruère *et al.* (1994), where esters showed to be more easily arranged into tubules than free carotenoids. While other polar pigments, including sapotexanthin and epoxides, found in mamey sapote fruits could be stored in the globules, which were also abundant. In mamey sapote, both storage forms are found as can be seen in detail in figure 8.5. Carotenoids stored in globular or globular–tubular chromoplasts appear to be more bioaccessible than those in crystal forms. Schweiggert *et al.* (2012) described a higher bioaccessibility of β-carotene deposited inside globular-tubular chromoplasts from mango and papaya than from crystalloid chromoplasts in carrot.

On the other hand, in salmon, carotenoids are stored within the muscle tissue, where astaxanthin is deposited in the myotome (muscle fibers). At first, it was believed that astaxanthin stablished weak unspecific hydrophobic bonds in the actin and myosin protein complex (Henmi *et al.*, 1989). However, a study by Matthews *et al.* (2006) determined that this carotenoid was bound to the hydrophobic core of an specific protein within the actin-myosin complex, called α-actinin (Fig. 8.6). Astaxanthin presents both hydroxyl and a keto group that allows a stronger binding strength than other carotenoids present in salmon (Henmi *et al.*, 1989).

8.4.2 Absolute carotenoid levels in red foods

Differences in total carotenoid contents from the test foods were evident. Red bell pepper contained the highest total carotenoids levels (32.6 – 50.0 mg/100 g of FW), followed by salmon (4.3-5.3 mg/100 g of FW) and then mamey sapote (1.7 mg/100 g of FW) with the lowest concentration (Table 8.1). Values for mamey sapote had not been reported yet and salmon values vary depending on the fish diet (Storebakken *et al.*, 1987). While, red bell pepper carotenoid content was similar to that reported in literature (Marín *et al.*, 2004).

Table 8.1. Carotenoid content in test foods (raw and cooked +1 % soybean oil) for the *in vitro* digestion assays.

Compound	Carotenoid content in micellar fraction of test food in µg per 100g fresh weight					
	Mamey Sapote		Red bell pepper		Salmon	
	Raw	Cooked + oil	Raw	Cooked + oil	Raw	Cooked + oil
Astaxanthin	-	-	-	-	**5 359.76 ± 279.25**	**4358.48 ± 96.53**
Capsanthin	-	-	1482.18 ± 530.22	833.60 ± 203.80	-	-
Capsanthin esters	-	-	13 992.64 ± 5113.25	7451.91 ± 1519.68	-	-
Sapotexanthin	249.61 ± 6.48	234.22 ± 33.07	-	-	-	-
Cryptocapsin laurate	188.67 ± 4.00	157.71 ± 46.21	-	-	-	-
Cryptocapsin myristate	133.16 ± 8.65	221.03 ± 51.00	-	-	-	-
Total carotenoid content	**1719.29 ± 29.24**	**1690.70 ± 405.04**	**50 025.11 ± 12 425.84**	**32 555.73 ± 14 968.99**	**5 359.76 ± 279.25**	**4358.48 ± 96.53**

Carotenoids must be released from the food matrix to be bioaccessible. In fruits and vegetables, these hydrophobic pigments are usually in an aqueous environment and need to be incorporated into micelles to be absorbed. The supernatant fraction obtained after ultracentrifugation corresponds to the carotenoids that were liberated from the food matrix, while micellar or bioaccessible fractions were recovered after microfiltration of the supernatant.

In general, carotenoid levels in the liberated and micellized fractions from all test foods were low. Carotenoids released from mamey sapote samples were mainly sapotexanthin (2.19 - 6.27 µg/100 g of FW) and cryptocapsin esters (0.28 - 3.24 µg/100 g of FW), other compounds were also detected in lower amounts, like some epoxides and traces of free cryptocapsin. In red pepper, aside from capsanthin and its esters (557.14 - 676 µg/100 g of FW), β-carotene, zeaxanthin and β-cryptoxanthin were also found, but focus will remain in capsanthin bioaccessibility. As for salmon samples, the only carotenoid present is astaxanthin (54.43 – 80.44 µg/100 g of FW) and it was recovered from liberated and bioaccessible fractions (Table 8.2, Fig. 8.7).

Release of ketocarotenoids from raw samples was higher in red bell pepper (9 %) than for the other sources evaluated, sapote (1.8 - 3 %) and salmon (2 %). After liberation, carotenoids need to be incorporated into mixed micelles to be called bioaccessible. The amount of micellized ketocarotenoids was again higher from red pepper (3.6 %) than from salmon (1.5 %) and mamey sapote (0.1 - 0.9 %).

Table 8.2. Carotenoid content in micellar fraction of test foods obtained after *in vitro* digestion assays.

Compound	Carotenoid content in micellar fraction of test food in µg per 100g fresh weight					
	Mamey Sapote		**Red bell pepper**		**Salmon**	
	Raw	Cooked + oil	Raw	Cooked + oil	Raw	Cooked + oil
Astaxanthin	-	-	-	-	80.44 ± 34.78	51.43 ± 21.12
Capsanthin	-	-	557.14 ± 21.35	304.53 ± 12.35	-	-
Capsanthin esters	-	-	-	372.32 ± 59.80	-	-
Sapotexanthin	2.19 ± 0.29	6.27 ± 1.69	-	-	-	-
Cryptocapsin laurate	0.47 ± 0.15	3.24 ± 0.75	-	-	-	-
Cryptocapsin myristate	0.28 ± 0.13	3.10 ± 0.84	-	-	-	-

In general, carotenoids from raw samples are difficult to release, since cell walls have a more solid structure. This also occurs in fish meat since carotenoids can be trapped within the dense fiber net of muscular structures (Johnston *et al.*, 2000; Kotake-Nara and Nagao, 2011). Additionally, even though carotenoids are released from the matrix, the incorporation into mixed micelles could be affected by the carotenoid polarity (O'Connell *et al.*, 2007). Apolar carotenoids are generally incorporated into the triglyceride (TAG)-rich core of lipid droplets. While polar carotenoids, like capsanthin, are preferably located in the surface facilitating the transference into mixed micelles. Carotenoid esters behave in a similar way as apolar carotenoids, like β-carotene: they need to be hydrolyzed from the TAGs before they can be incorporated into the micelles (Borel *et al.*, 1996). According to their polarity, the ketocarotenoids from the tested foods can be arranged from more to less polar as follows: astaxanthin, capsanthin, sapotexanthin, capsanthin esters and cryptocapsin esters. The percentage of carotenoids being micellized was according to their polarity, except for astaxanthin in salmon. As it can be observed in figure 8.7, the liberation of astaxanthin from salmon muscle was low and similar to the micellized fraction indicating that matrix effects could be involved.

In raw red bell pepper samples, it was observed, that only capsanthin in its free form was recovered from the micellar fraction (Table 8.2), despite the presence of capsanthin esters in the liberated fraction. Capsanthin is present mainly in its esterified form (28 % from total carotenoid content); however, it can also be found, in a lower proportion, as free capsanthin (3 %) (Table 8.1). Therefore, the possibilities are that only capsanthin in its free form was

incorporated into the micelles and the capsanthin esters were hydrolyzed during the simulated digestion phase by the cholesterol esterase and later incorporated as free form into the micelles. Previous *in vitro* bioaccessibility assays with papaya (Schweiggert *et al.*, 2012), showed a high cleavage rate of β-cryptoxanthin esters after digestion, resulting in an increased level of free β-cryptoxanthin.

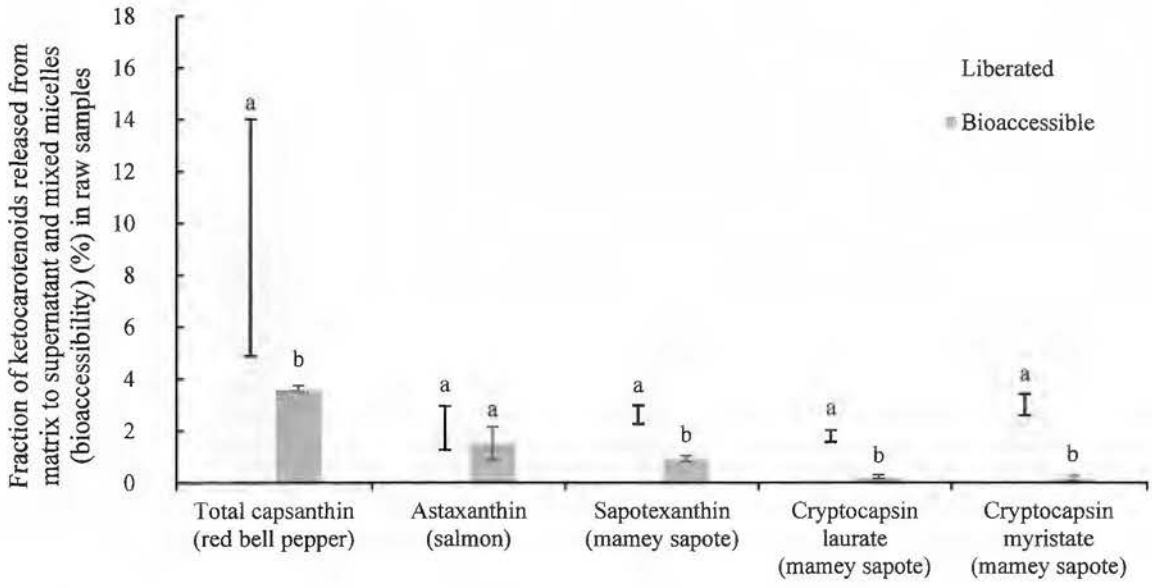

Figure 8.7. Fraction of ketocarotenoids recovered from liberated and micellar fraction of raw test foods. Liberated carotenoids correspond to the fraction recovered from supernatant after centrifugation. Bioaccessible carotenoids or micellar fraction corresponds to the phase obtained after supernatant filtration. Different letters indicate significant differences ($p < 0.05$) of the means between liberated and bioaccessible fractions of the corresponding compound.

Sapotexanthin in mamey sapote is only present in its free from and unlike capsanthin does not present hydroxyl groups in its structure (Fig. 8.1). Carotenoids with higher number of hydroxyl and keto groups were shown to be generally more bioaccessible, since they are more hydrophilic and consequently easily incorporated into micelles (Borel *et al.*, 1996). Considering this, and leaving aside food matrix effect, sapotexanthin bioaccessibility is expected to be lower than that of capsanthin. According to this, cryptocapsin should also have a higher incorporation into micelles in comparison to sapotexanthin; however, as observed in figure 8.7 and table 8.2, this is not the case. Micellar fraction of cryptocapsin esters was around 0.2 %, while from sapotexanthin was a little higher, 0.91 %. The difference lays in the esterification of cryptocapsin: esterified carotenoids have a lower

polarity leading to less efficient incorporation into micelles (Borel *et al.*, 1996). A study on bioaccessibility of carotenoid from citrus juices demonstrated that the recovered fraction, both liberated and micellized, of free β-cryptoxanthin was higher than that of β-carotene and β-cryptoxanthin esters (Dhuique-Mayer *et al.*, 2007).

Since ketocarotenoids in red bell pepper and mamey sapote are mainly present in its esterified forms and carotenoids are cleaved before incorporation into micelles, enzymatic hydrolysis should be considered. Cryptocapsin esters presented low liberation and micellized fraction levels while free cryptocapsin was found as traces in both fractions. The question is if the low rates of micellization is due to a low cleavage rate of cryptocapsin esters by the porcine cholesterol esterase used in the model. Enzymatic hydrolysis plays and important role during digestion, since esters are not so readily incorporated into micelles, unlike free forms of carotenoids that are easily incorporated. As showed by Breithaupt *et al.* (2002) ester cleavage rate can be different depending on the source of the enzymes used. It was determined that hydrolysis of xanthophyll esters, including capsanthin, was low when using porcine pancreatin and cholesterol esterase. The low efficiency was especially noticeable when cleaving capsanthin diesters. This will also explain the low bioaccessibility of capsanthin from red bell pepper that was mainly present in its esterified form.

Astaxanthin from salmon behaved differently than ketocarotenoids from mamey sapote and red bell pepper, both with higher liberated and micellized fractions. No difference was observed between the liberated and micellized fractions. Astaxanthin has two hydroxyl and keto groups and is found in its free form in salmon. Therefore, high levels of both liberated and micellized fractions were expected. A previous study by Sy *et al.* (2012) evaluated the physicochemical properties of carotenoids and their bioaccessibility, including commercially obtained astaxanthin and from Norwegian smoked salmon. It was observed, that even though pure astaxanthin was efficiently incorporated into mixed micelles, astaxanthin from smoked salmon was seven times less bioaccessible. This indicates that matrix plays an essential role in carotenoid release and incorporation into mixed micelles, as will be discussed in the next sections.

Matrix composition and ultrastructure plays an important role on carotenoid liberation and incorporation into mixed micelles. In fruits and vegetables, pectin, one main component of plant cell walls, could be an important barrier for carotenoid liberation. Presence of pectin has shown an impact on β-carotene in vitro bioaccessibility in carrots (Lemmens *et al.*, 2009). Pectin in mamey sapote was estimated in 0.77 % FW (Mahattanatawee *et al.*, 2006), and 0.73 % FW for red pepper (López-Hernández *et al.*, 1996). These values are in the range of those reported for other fruits rich in carotenoids like papaya (0.66-1.0 % FW), mango (0.66-1.75 % FW) and a little lower than in carrot (1% FW) (Kaur and Sharma, 2013). Despite previous reports that state the similar pectin content of red pepper and mamey sapote fruits, there was a clear difference in the fraction of carotenoids released from each source. Total liberated capsanthin from red pepper was around 9 %, while only about 1.8 - 3 % of sapotexanthin and cryptocapsin esters from mamey sapote fruits were released. Since pectin content could be similar in both samples, other components should be considered. Total dietary fiber from mamey sapote fruits is considerably higher than from red bell peppers, 7.95 % (Chapter II) and 2.2 % (López-Hernández *et al.*, 1996), respectively. Therefore, the presence of other dietary fibers could be leading to a lower released carotenoid fraction from mamey sapote fruits.

Once liberated carotenoids need to be incorporated into mixed micelles in order to be considered bioaccessible. Relative bioaccessibility refers to the portion of carotenoids that is incorporated into mixed micelles with regard to the initial carotenoid content, expressed as a percentage. In the previous section, it was explained that micellized fractions tend to have lower values than those from liberated carotenoids. Presence of dietary fibers, not only acts as a barrier to access the carotenoids from the matrix but it also has an impact on carotenoid incorporation into mixed micelles. *In vivo* bioavailability assay in humans determined that pectin content could also reduce in more than one-half β-carotene present in plasma (Rock and Swendseid, 1992). Soluble fibers could increase viscosity in the gastrointestinal tract and therefore reduce the volume of the duodenal fluid blocking carotenoid incorporation into mixed micelles. An evaluation of the effect of pectin on bioaccessibility of β-carotene-enriched emulsions, performed by Verrijssen *et al.* (2014), it

was determined that presence of pectin (1 - 2 %) had an effect on oil droplet size and viscosity during digestion, negatively affecting carotenoid bioaccessibility. Carotenoids from mamey sapote were not only less released than capsanthin from red pepper (Fig. 8.8), as explained above, but they were also less bioaccessible (Fig. 8.9). Total capsanthin relative bioaccessibility was at least three times higher than that of ketocarotenoids in mamey sapote.

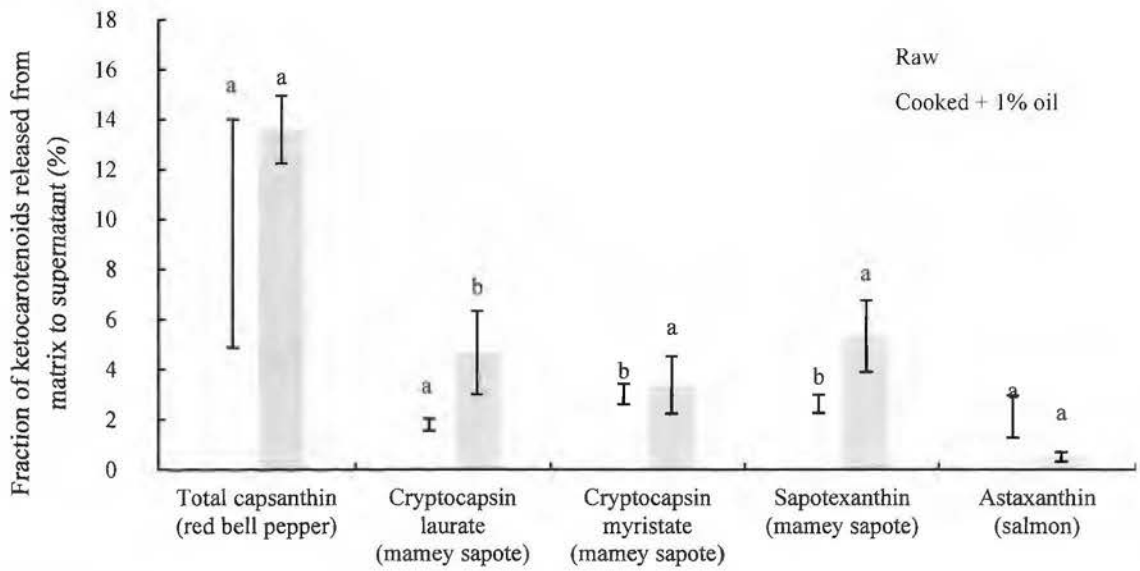

Figure 8.8. Fraction of ketocarotenoids recovered from liberated fractions of raw and cooked test foods. Liberated carotenoids correspond to the fraction recovered from supernatant after centrifugation. Different letters indicate significant differences ($p < 0.05$) of the means between liberated fractions of the corresponding compound.

Food processing has a big impact on bioaccessibility because it usually affects the microstructure in which carotenoids are embedded. As expected, both fractions of liberated and micellized carotenoids were enhanced by addition of a fat source and thermal treatment (Fig. 8.8 and 8.9). Thermal processing helps to release carotenoids from the food matrix by disruption of cell walls, but it also degrades some of these pigments (Maiani *et al.*, 2009). In carrots, it was described that during the first minutes of heating in boiling water tissue firmness is lost, after 6 min changes were observed in pectin as breakdown by β-elimination occurs and tissue is softened (Greve *et al.*, 1994a; Greve *et al.*, 1994b). The thermal treatment for the test foods evaluated in this study had a negative effect in red bell pepper and salmon samples. Total carotenoid contents in red bell pepper and salmon was reduced in approximately 34 % and 18 %, respectively, while in mamey sapote fruits lost was

estimated in less than 2 % (Table 8.1). Aside from differences in food matrix, heat effect on carotenoids could also vary between samples due to differences in carotenoid profile (Mercadante, 2007). Even though heat could degrade up to some degree the carotenoids present in the samples, it also liberates them from the cell structure, allowing an easier incorporation into mixed micelles and positively influencing their absorption. This effect was clear for sapotexanthin and cryptocapsin laurate from mamey sapote fruits, since fraction of liberated carotenoids increased from a 1.8 - 2.6 % up to 4.7 - 5.3 % (Fig. 8.8). This was not the case for red bell peppers, where total capsanthin liberation was similar from raw or cooked samples. As for the bioaccessible fraction, a significant difference between raw and cooked samples was observed (Fig. 8.9). In red pepper and mamey sapote fruits samples, addition of fat and thermal treatment caused an enhancement in relative bioaccessibility of 2.2-fold in capsanthin, 3.3-fold in sapotexanthin and between 10 to 11-fold for cryptocapsin esters (Fig. 8.9).

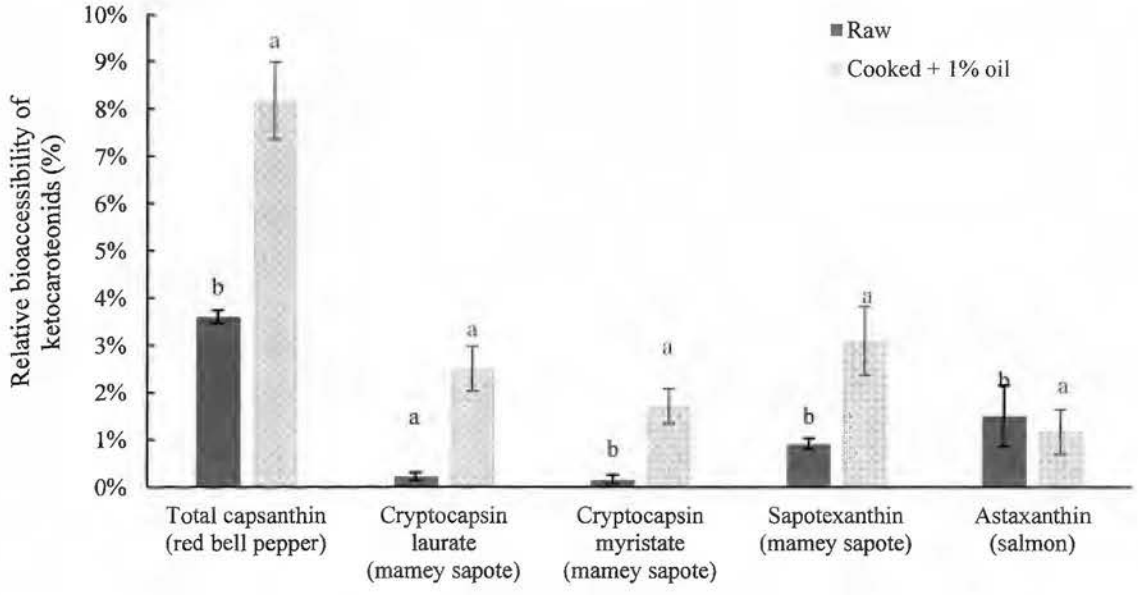

Figure 8.9. Relative bioaccessibility total capsanthin, astaxanthin, sapotexanthin and cryptocapsin esters recovered from micellar fraction of raw and cooked + 1 % oil. Different letters indicate significant differences ($p < 0.05$) of the means between bioaccessible fractions of each compound.

In salmon samples, fraction of released carotenoids was lower in comparison to carotenoids from the other samples analyzed. Additionally, heat treatment had a negative effect on astaxanthin release, as it was lowered from 2.1 % to 0.5 % (Fig. 8.8). Astaxanthin from

salmon could be strongly attached to the muscle and not be release during the digestion process. It is possible that heat alters the food matrix forming new complex structures that entraps this carotenoid. Proteins from the sarcomere tend to coagulate, thermal treatment provokes water lost and general shrinkage since it denaturalizes myosin and produce myofibril collapse (Bell *et al.*, 2001). As seen in figure 8.6, astaxanthin is believed to be located within the actin-myosin complex, shrinkage of this structure due to heat could be in some way encapsulating this carotenoid explaining the reduction on liberated fraction after processing.

The other factor evaluated was fat addition. During digestion, carotenoids are released from their matrix and need to be transferred first to lipid globules, which are later incorporated into mixed micelles. However, low fat content could be a limiting step for inclusion in mixed micelles. Several studies have reported the increase in bioaccessibility when a fat source is added (Gleize *et al.*, 2013; Huo *et al.*, 2007; Ornelas-Paz *et al.*, 2008; Schweiggert *et al.*, 2012). This enhancement makes sense given that natural fat content of most fruits and vegetables is low. Supplementing carotenoid rich foods with a fat source has been recommended, since fat also stimulates release of bile salts and propitiates a lipophilic environment (Pérez-Gálvez and Mínguez-Mosquera, 2005). Recommended dose to efficiently access carotenoids is around 3-5 g per meal (van het Hof *et al.*, 2000). Schweiggert *et al.* (2012) found an enhancement in β-carotene, lycopene and xanthophylls bioaccessibility with a supplement of 1 % sunflower oil.

In mamey sapote fruit pulp, natural fat content is around 0.2 %, and main carotenoids sapotexanthin and cryptocapsin, are found free and esterified, respectively. In red pepper, fat content is also low, approximately 0.3 %, and ketocarotenoids, capsanthin and capsorubin, are commonly found in both free and esterified form. Ketocarotenoid esters in red pepper contain saturated fatty acids like lauric acid, myristic acid and palmitic acid. The same kind of fatty acids are bonded to cryptocapsin in mamey sapote fruits (Table 7.2, Chapter IV). Esterification plays an important role in plants during senescence, fruit ripening, biogenesis of other carotenoids and also influences color storage capacity (Pérez-Gálvez and Mínguez-Mosquera, 2005). Esterified carotenoids have a higher liposolubility without affecting chromophore properties. However, it seems that during digestion these

carotenoids concentrate within the core of lipid emulsions making it harder to be incorporated into micelles (Pérez-Gálvez and Mínguez-Mosquera, 2005). Lipophilic conditions achieved by addition of an external fat source favors micellization of xanthophyll esters. In the previous sections, it was observed that capsanthin esters were recovered in the micellar fraction only after thermal processing and fat addition, while levels of relative bioaccessibility of cryptocapsin esters also increased after processing. However, it is important to note that carotenoid esters have not been reported in plasma, even though they can be micellized they are not present in chylomicrons (Breithaupt *et al.*, 2003; Failla *et al.*, 2008).

In salmon, after the *in vitro* assay, there was no difference in relative bioaccessibility between raw samples and the ones that received the thermal treatment and fat addition. Since salmon already contains a high percentage of fat (around 3.4 %) addition of more fat will most likely not have an evident effect on bioaccessibility of astaxanthin, as it was observed in this study. Liberated fraction was also similar to the micellar one; this could indicate a low release from the food matrix, as discussed above. Similar results were obtained by Sy *et al.* (2012).

Heat treatment and fat addition enhanced the incorporation of carotenoids into mixed micelles by propitiating a more lipophilic environment and disrupting plant tissue barriers resulting in an increase of almost two fold in carotenoid levels. However, recovery of carotenoids in the micellar fraction is still low and other factors aside processing need to be considered. As mentioned before, food matrix plays an important role while determining carotenoid bioaccessibility.

Additionally, it has been suggested that different carotenoids in the same matrix could compete for their incorporation into the mixed micelles (van den Berg, 1999) since competition has been already documented in a later stage of absorption in an *in vitro* Caco-2 model (During *et al.*, 2002). Other fat components found in plants like sterols could also compete for incorporation into mixed micelles, since they are known for reducing cholesterol absorption (Richelle *et al.*, 2004). It has been hypothesized, that due to a greater affinity of sterols to micelles, cholesterol-like compounds, including carotenoids, are

displaced (Ostlund, 2002; Reboul and Borel, 2010; Richelle *et al.*, 2004). This would help to explain low recovery of carotenoids from the micellar fraction.

Another factor that could affect carotenoid release from matrix is related to the chromoplast type. Carotenoids, like β-carotene stored in globular-tubular chromoplasts, have shown to be more bioaccessible than the ones in crystal form (Schweiggert *et al.*, 2012). Apparently, ketocarotenoids from red bell peppers stored in tubular chromoplast could have a better liberation rate than those from globular-tubular chromoplasts in mamey sapote, based on the liberation and micellization rates obtained. However, it is still unknown if this effect is only related to the chromoplast or to other matrix characteristics or even if properties of the different ketocarotenoids are responsible for low liberation rates.

8.5. Conclusions and recommendations

Carotenoids in mamey sapote fruits are mainly stored in globular-tubular chromoplasts, containing lipid-dissolved and liquid-crystalline carotenoid aggregates. Bioaccessibility assays showed low levels of carotenoids in micellar fractions recovered from all test foods (0.2 – 3.6 %). The addition of a lipid source and heat treatment enhanced the relative bioaccessibility of ketocarotenoids present in red bell pepper up to 2.2-fold and in mamey sapote fruits 3.3-fold for sapotexanthin and 10 to 11-fold for cryptocapsin esters. In salmon samples, variation was not that evident and little differences were found between treatments. The level of ketocarotenoids released was higher in red bell pepper (up to 13.5 %) than in mamey sapote fruits (up to 5.3 %); carotenoid liberation could be affected by matrix properties including type of chromoplast, globular-tubular versus tubular. Additionally, the presence of ketocarotenoids in their esterified form could be affecting incorporation into mixed micelles for capsanthin and cryptocapsin esters. The importance of bioaccessibility of sapotexanthin and cryptocapsin is due to their potential provitamin A activity; however, further studies to determine this activity should be performed. Complementation of these studies with *in vivo* bioavailability assays are also necessary to determine if absorption tendency is similar to that of *in vitro* models, since other factors related to the individual human organism could affect absorption of these ketocarotenoids.

8.6. References: Chapter V

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9. Conclusions and recommendations

- Mamey sapote fruits analyzed presented relatively higher levels of dietary fiber and protein than other fruits. Additionally, levels of total soluble solids were considerably high and comparable with those of other members of the Sapotaceae family, particularly with *P. viridis* and *M. sapote*.

- The carotenoid extraction using acetone was a better suitable extraction solvent for carotenoids present in mamey sapote fruits than the MeOH/EtOAc/PB mixture. This is probably mainly due to the presence of numerous xanthophylls in mamey sapote fruits.

- Qualitative and quantitative carotenoid composition varied between the genotypes analyzed. Red-orange fleshed fruits presented higher concentrations of cryptocapsin esters and other ketocarotenoids including capsoneoxanthin and capsorubin, responsible of the reddish coloration. While the yellow pigments, neoxanthin and other epoxides, appear to be the main pigments in yellow-orange fleshed fruits genotypes explaining its characteristic color.

- The ketocarotenoid sapotexanthin was present in similar concentrations in all three genotypes analyzed, however, accumulation of higher amounts of β-carotene-5,6-epoxide, its proposed precursor, was observed only in yellow-orange fleshed fruit. Regulatory mechanisms involving inactive or lower active enzymes involved in sapotexanthin biosynthesis are proposed.

- Total carotenoid contents varied significantly between genotypes. The two red-orange fleshed genotypes had the highest and lowest values, while yellow-orange fleshed fruits contained intermediate total carotenoid levels.

- Similar relative abundance of red and yellow pigments in red-orange fleshed fruits appears to be responsible for the homogeneity of their color measurements despite the significant variation in total carotenoid contents.

- It was determined by observation under light and transmission electron microscopy that carotenoids in mamey sapote fruits are mainly stored in globular-tubular chromoplasts, containing lipid-dissolved and liquid-crystalline carotenoid aggregates.

- Bioaccessibility assays showed low levels of micellar fractions recovered from all test foods. The ketocarotenoids recovered from liberated and micellar fractions corresponded to capsanthin, from red pepper, sapotexanthin and cryptocapsin esters from sapotexanthin, and astaxanthin from salmon.

- Thermal treatment and addition of a lipid source enhanced relative bioaccessibility of ketocarotenoids from red bell pepper and mamey sapote fruits, but not of astaxanthin from salmon. The increase in the bioaccessible fraction of carotenoids from red pepper and mamey sapote fruits may be due to cell wall disruption by heat exposure.

- The level of ketocarotenoids released was higher in red bell pepper than in mamey sapote fruits. Carotenoid liberation could be affected by matrix properties including type of chromoplast, globular-tubular versus tubular.

- Matrix effect appears to be responsible for the low recovery rates of astaxanthin in salmon samples in comparison to the other test foods analyzed. Thermal treatment reduced even more the level of bioaccessible astaxanthin, possibly due to structural changes provoked by heat.

- Further study is needed to reveal the mechanisms responsible for the differences found in mamey sapote carotenoids profile and concentration from red-orange and yellow-orange flesh genotypes. This includes genetic analyses to determine the presence of key genes, like the ones encoding for PSY and CCS, as well as their expression and activity levels in fruits from different genotypes.

- Since sapotexanthin and cryptocapsin, both potentially provitamin A carotenoids were found in mamey sapote fruits, further studies to determine their provitamin A activity

should be performed. Additionally *in vivo* assays to establish their absorption in humans are suggested.

- Furthermore, breeding programs to obtain sapote fruits with higher content of the provitamin A carotenoids sapotexanthin and cryptocapsin are encouraged.

10. Conclusiones y recomendaciones

- Los frutos de zapote analizados presentaron niveles relativamente altos de fibra dietética y proteína en comparación con otros frutos. Adicionalmente, los contenidos de sólidos solubles totales fueron considerablemente altos y comparables con valores encontrados en la literatura para otros miembros de la familia de las sapotáceas, particularmente con *P. viridis* y *M. sapota.*

- El protocolo de extracción de carotenoides utilizando acetona fue más eficiente para el análisis de frutos de zapote que la mezcla de solventes con metanol/acetato de etilo/ benzina de petróleo. Esto probablemente debido a la presencia de numerosas xantofilas en este fruto.

- Se encontró una variación tanto cualitativa como cuantitativa en los carotenoides presentes en los diferentes genotipos de mamey sapote analizados. Los principales pigmentos encontrados en frutos con pulpa de color rojo-anaranjado fueron los ésteres de criptocapsina, así como, otros cetocarotenoides, incluyendo capsoneoxantina y capsorubina, responsables de la coloración rojiza observada. Mientras que en los frutos con pulpa amarillo-anaranjada los principales pigmentos fueron ésteres de neoxantina y otros epóxidos de color amarillo, explicando el color característico de la pulpa.

- El cetocarotenoide sapotexantina fue encontrado en concentraciones similares en los tres genotipos analizados; sin embargo, se encontró que el genotipo con pulpa amarilla-anaranjada acumuló altas concentraciones de β-caroteno-5,6-epóxido, el presunto precursor de sapotexantina. Se propusieron diferentes mecanismos de regulación que incluían menor nivel de actividad o inactividad de las enzimas involucradas en la síntesis de la sapotexantina para explicar este fenómeno.

El contenido de carotenoides totales varió significativamente entre los genotipos analizados. Los que presentaron tanto los mayores como los menores valores fueron los frutos con pulpa roja-anaranjada, mientras que el genotipo con una coloración amarilla-anaranjada presentó niveles intermedios de contenido total de carotenoides.

La similitud en la proporción relativa de pigmentos rojos y amarillos en los frutos con pulpa roja-anaranjada parece ser la responsable de los valores similares obtenidos en las mediciones de color, esto a pesar, de presentar grandes diferencias en el contenido total de carotenoides.

La observación de muestras de pulpa del zapote bajo el microscopio de luz y el microscopio electrónico de trasmisión permitió la identificación de cromoplastos del tipo globular-tubular, donde los carotenoides estaban depositados en agregados disueltos en lípidos o líquido-cristalino.

Los ensayos de bioaccesibilidad mostraron bajos niveles de carotenoides en las fracciones micelares recuperadas de los diferentes alimentos analizados. Los cetocarotenoides recuperados tanto en la fracción micelar como en la de liberados fueron capsantina en chile, sapotexantina y ésteres de criptocapsina en zapote y astaxantina a partir de salmón.

El tratamiento térmico y la adición de lípidos incrementó la fracción de carotenoides bioaccesibles presentes en chile y en zapote al doble, no así para la astaxantina en salmón. El aumento en los niveles de bioaccesibilidad en chile y zapote probablemente se debe a la ruptura de las paredes celulares por la exposición a al calor, lo que ocasiona una mayor liberación de los carotenoides.

El nivel de carotenoides liberados del chile fue mayor al del zapote. La liberación de estos carotenoides pudo ser afectada por propiedades de la matriz, incluyendo el tipo de cromoplasto, en este caso, globular-tubular contra tubular.

La incorporación de los carotenoides en las micelas fue más eficiente para la capsantina en el chile, que para sapotexantina y los ésteres de criptocapsina del zapote; esto probablemente no sólo debido a posibles efectos de la matriz, sino también a la polaridad de los respectivos carotenoides.

- El efecto de la matriz parece ser el responsable de los niveles de carotenoides recuperados a partir de las muestras de salmón en comparación con los de los otros alimentos evaluados. Además el tratamiento térmico redujo aún más el porcentaje de astaxantina bioaccesible, posiblemente por cambios estructurales de la matriz al ser sometidos a calentamiento.

- Es necesaria la realización de otros estudios para determinar los mecanismos responsables de las diferencias encontradas en el perfil de carotenoides entre los genotipos con diferente color de pulpa analizados. Esto incluye estudios genéticos para establecer la presencia de genes clave que codifiquen para las enzimas CCS y PSY, y otras posibles involucradas, así como el nivel de expresión y niveles de actividad enzimática en frutos de los diferentes genotipos.

- Dado que los cetocarotenoides sapotexantina y criptocapsina tienen potencial como provitamina A, se necesitan de nuevos estudios para determinar su actividad. Adicionalmente se recomienda la realización de estudios *in vivo* para establecer si estos pigmentos pueden ser absorbidos por el cuerpo humano.

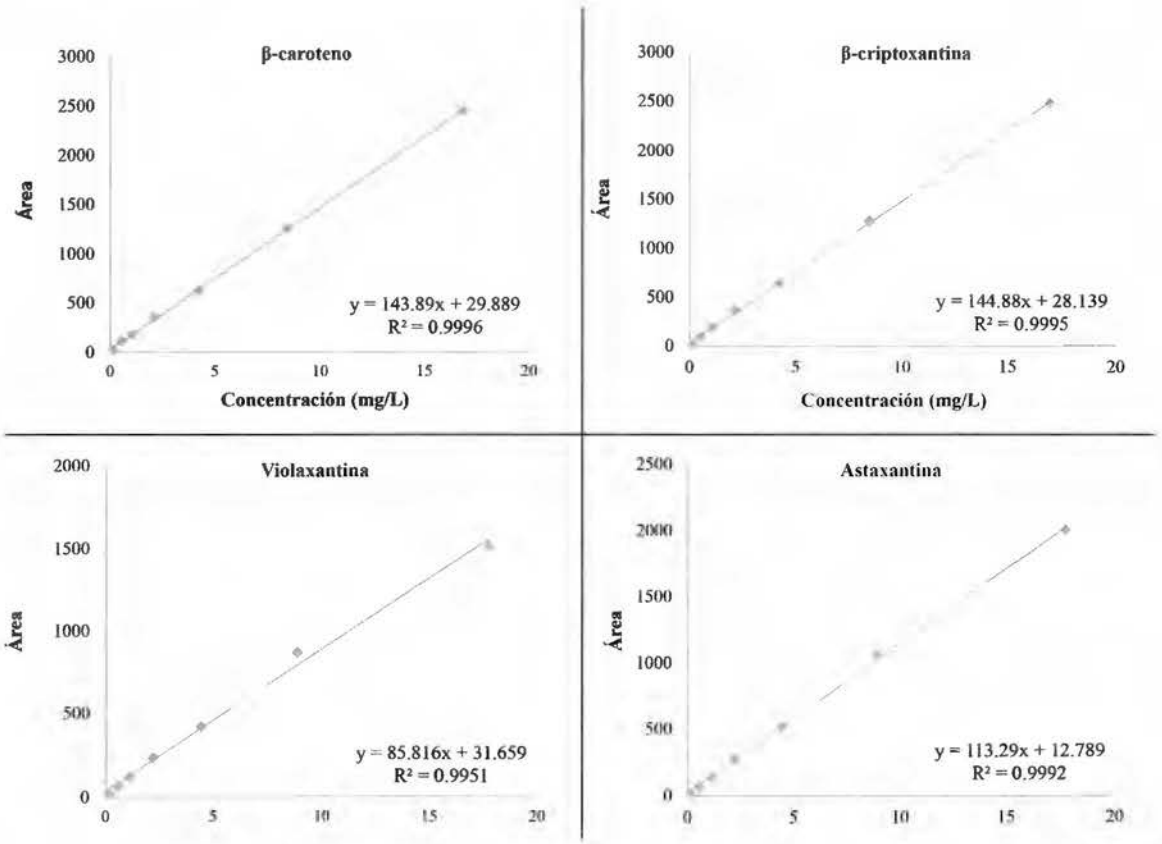
- Además, es necesario incentivar la selección de genotipos promisorios con alto contenido de sapotexantina y criptocapsina por su potencial actividad provitamina A.

11. Annexes

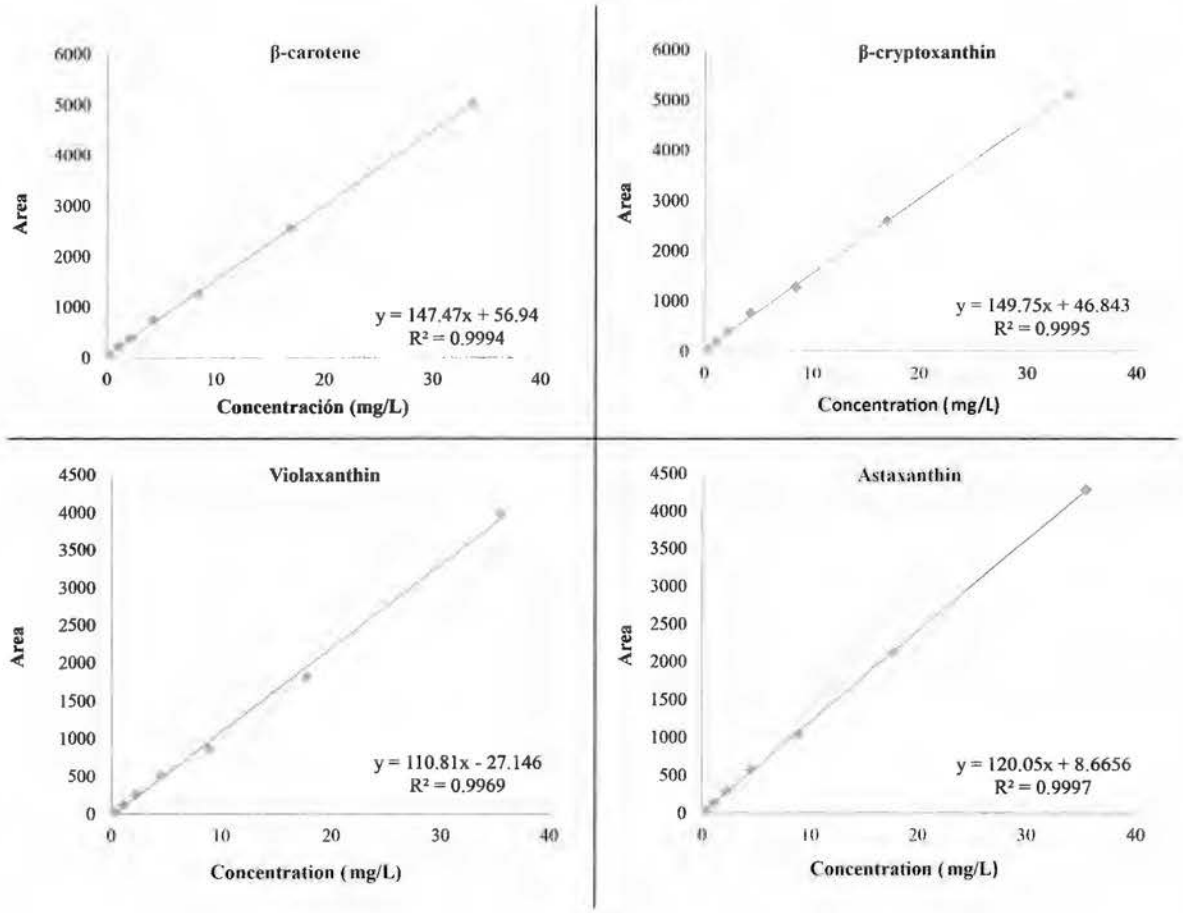
11.1. Physicochemical parameters from the three genotypes of mamey sapote fruits analyzed

Parameter	Mamey sapote genotype		
	8747	**11129**	**11163**
Flesh color	Red-orange	Red-orange	Yellow-orange
Total weigth (g)	478.13 ± 51.20^{a}	671.48 ± 173.36^{a}	408.38 ± 168.38^{a}
Seed weight (g)	53.84 ± 17.77^{a}	61.64 ± 29.448^{a}	41.42 ± 19.34^{a}
Number of seeds	1-3	1-2	1
Lenght (cm)	10.15 ± 1.01^{a}	11.43 ± 1.71^{a}	12.53 ± 2.31^{a}
Diameter (cm)	30.30 ± 1.78^{a}	34.50 ± 3.21^{a}	24.30 ± 2.56^{b}
pH	6.38 ± 0.38^{a}	6.69 ± 0.10^{a}	5.84 ± 0.57^{b}
TSS (° Brix)	27.56 ± 2.51^{a}	29.36 ± 1.80^{a}	27.20 ± 0.87^{a}

11.2. Calibration curves for HPLC-DAD MS system



11.3. Calibration curves for HPLC-DAD Agilent system



11.4. Table for calibration curve preparation for β-carotene, β-cryptoxanthin, violaxanthin and astaxanthin

	Carotenoid standards			
	β-carotene	**β-cryptoxanthin**	**Violaxanthin**	**Astaxanthin**
Solvent	Petroleum benzin	Petroleum benzin	Acetone	Hexane
Wavelenght	450nm	449 nm	442 nm	474 nm
Absorbance	1.089	1.012	1.328	1.271
ε (L/(mol*cm)	138900	131900	144000	128322
Distance (cm)	1	1	1	1
C (mol/L)	7.84E-06	7.67E-06	9.22E-06	9.90E-06
Dilution	10	2	2	3
mol/L in volumetric flask (10 mL)	7.84E-05	1.53E-05	1.84E-05	2.97E-05
Molar Mass (g/mol)	536.87	552.85	600.85	596.85
g/L	4.21E-02	8.48E-03	1.11E-02	1.77E-02
mg/L	4.21E+01	8.48E+00	1.11E+01	1.77E+01
µg/L	4.21E+04	8.48E+03	1.11E+04	1.77E+04
mL taken from 5mL	1	5	4	2.5
Dilution	0.1	0.5	0.4	0.3
mols final	7.84E-08	7.67E-08	7.38E-08	7.43E-08
mol/L (10 mL)	7.84E-06	7.67E-06	7.38E-06	7.43E-06
mg final	4.21E-02	4.24E-02	4.43E-02	4.43E-02
mg/L (10 mL)	4.209	4.242	4.433	4.434