INTRODUCTION

The Baby Banana fruit (*Musa acuminata*) is cultivated in Colombia, Ecuador, Costa Rica, Venezuela, Costa Rica and Kenya, however Colombia is the main country in Latin-American which has developed the marketing for exporting fruit to Europe and USA. Colombia exports not only Baby Banana but also other tropical fruits, e.g. *Physalis peruviana* (known as Uchuva) and *Hylocereus undatus* mentioned as white Pitahaya or dragon fruit, and they are classified as “exotic fruits".

The tropical fruit is collected from different regions of Colombia and during the last years increasing experience has been gained to maintain the postharvest quality during exporting the fruit. Since climatic changes influence rainfalls, temperature and water availability, the traders of baby banana in Colombia confirmed the presence of an irregular ripening in several Baby Banana peels during the post-harvest stage that they called “hyperpigmentation” (HP). The occurrence of visible small green bands in the peels during the ripening showed that degradation of chlorophyll was abnormal in comparison with control bananas. Consequently, those Baby Banana fruits with hyperpigmentation were not suitable for export because they did not meet the overall standards of quality and both farmers and traders were affected economically. Farmers strictly classified the fruits during the postharvest stage before offering them for export and the fruit traders contacted the researcher in order to explore the phenomenon of hyperpigmentation in Baby Banana fruits.

Thus, two groups of Baby Banana (with hyperpigmentation and without hyperpigmentation) have been studied under the hypothesis that the degradation of chlorophylls could play a role in the occurrence of the abnormal green bands on Baby bananas peels with hyperpigmentation.

Therefore, the main objective of this study was the analysis of the hyperpigmentation phenomenon in relation with the chlorophyll degradation in Baby Banana peel. Thus, the three specific objectives were: (i) the isolation of chlorophyll and derivatives by means of High Speed Countercurrent Chromatography (HSCCC); (ii) the comparison between Baby Banana peel without hyperpigmentation (control) vs. Baby Banana with hyperpigmentation by application of HSCCC; and (iii) to set up a phytochemical profile of Baby Banana peel.

The optimization of a solvent system for the separation of chlorophylls by means of High-Speed Countercurrent Chromatography (HSCCC) was performed and the proposal of using additional samples such as grass and spinach during the trials could enable the understanding of the role of chlorophylls in plants and fruits.

Finally, a scale-up by means of Spiral Coil-Low Speed Rotary Countercurrent Chromatography (Spiral Coil-LSRCCC) is presented which has proven to be a suitable system for large-scale fractionations of extracts obtained from Baby Banana peel with hyperpigmentation.

1. GENERAL CONCEPTS

1.1 Baby Banana (*Musa acuminata* AA Simmonds cv. Bocadillo)

1.1.1 Taxonomic Classification of Bananas (*Musa acuminata*)

The first edition of Linnaeus' Species Plantarum in 1753 established the botanical nomenclature of genus *Musa*, familiy *Musaceae* and divided bananas and plantains (*Musa* ssp.) according to the consumption, in dessert bananas (*Musa sapientum* L.) being eaten raw, and plantain (*Musa Paradisiaca* L.) being processed by cooking before consumption. APG II system (Angiosperm Phylogeny Group II system) in 2003, assigned *Musaceae* to the order *Zingiberales* in the clade *Commelinids* in monoctos[1]. A third version of APG (The angiosperm Phylogeny Group) in 2009, established an additional criteria of classification based on the genome.[2]

Bananas and plantains have originated in Southeast Asia where they were domesticated by selection from wild species more than 5.000 years ago and now are known as hybrids of polyploids progenies of two wild banana species- *Musa acuminata* and *Musa balbisiana*- according to a genome-based system introduced by Ernest Cheesman, Norman Simmonds, and Ken Sherpherd, which indicates the degree of genetic inheritance from the two wild parents and the number of chromosomes (ploidy). Therefore, Linnaeus' *Musa sapientum* is now identified to be the hbrid Latundan cultivar (M. x *paradisiaca* AAB Group 'Silk'), *Musa paradisiaca* AAB, *Musa balbisiana* ABB and *Musa acuminata* AA and AAA.

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[1] APG II 2003. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. The Angiosperm Phylogeny Group. *Botanical Journal of the Linnean Society*, 2003, 141, 399-436.
[2] APG III 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. The Angiosperm Phylogeny Group. *Botanical Journal of the Linnean Society,* 2009, 161, 105-121.

Actually, banana cultivars or clones are named e.g. *Musa* AAB *Simmonds*, clon Cachaco Cardeñosa, a species from Colombia and in case of Baby Banana from Colombia, its official designation is *Musa acuminata* AA Simmonds, cv. "Bocadillo".[3]

The Baby Banana is known in English as “Lady Finger”, “Singer bananas”, “Date banana”, “Fig banana” or “Sucrier”, depending on the region where it is cultivated.

1.1.2 Plant morphology

The banana plant is the largest herbaceous flowering plant. Cultivated bananas are parthenocarpic, fruits in which the flesh swells and ripens without its seeds being fertilized and developing. The propagation is vegetative due to the lacking of viable seeds and farmers remove and transplant part of the underground stem (corm) from which all the above-ground part of the banana plant grow. The external part of the plant consists in a vertical shoot that develops from the base of the banana plant called "pseudostem" or " false stem". The edges of the sheath meet when it is first produced, make it look tubular. As new growth occurs in the centre of the pseudostem the edges are forced apart. Cultivated banana plants vary in height depending on the variety and growing conditions between 5 to 7 m. Leaves are spirally arranged and grow 2.5 metres long and 50 cm wide (Robinson 1996; Simmonds 1995; Ortiz et al. 1995) (Zeller 2005).

[3] Belalcázar, C., S., 1991. *Manual de asistencia técnica N° 50: El cultivo del plátano en el trópico.* ICA, CIID, INIBAP y Comité de Cafeteros del Quindío. Armenia, Quindío, p.56.

After 10 to 15 months the banana plant is mature and the corm stops producing new leaves. Then a stem develops which grows up inside the pseudostem, carrying the immature inflorescence until eventually it emerges at top. This inflorescence is known as "banana heart". The flowers appear in group (hands) along the stem and are covered by purplish bracts which roll back and shed as the fruit stem develops. The first hands to appear contain female flowers which will develop into bananas. The female flower appears in rows and varies from a few to more than 10, further up the steam from the rows of male flowers that are in the bottom of the inflorescence. The ovary is inferior, meaning that the tiny petals and other flowers parts appear at the tip of the ovary. Male and female flowers are morphologically indistinguishable until the inflorescence is about 12 cm long. At this point the ovary in the male flower fails to develop any further (Simmonds, 1953a). Flowers have a 3-lobed stigma and style and an inferior ovary fused from 3 loculi. Each loculus of a female flower contains two rows of ovules embedded in a strip of mucilage (Simmonds, 1953). There are 5 stamens in male flowers; these are reduced to staminodes in female flowers (Simmonds, 1953a).

Each fruit is a berry and is known as a "finger". Each cluster of fruits at a node is known as a "hand" and the entire collections of hands are known as a "bunch". The number of hands varies with species and cultivar. The outer protective layer of each fruit, known as the "skin" or "peel", is a fusion of the hypanthium (florar receptacle) and outer layer (exocarp) of the pericarp (fruit wall derived from the ovary wall). This peel is easily removed from the fleshy pulp that originates mainly from the endocarp (innermost layer of the pericarp), (Simmonds, 1953). During the development of the fruit from the ovary, the tepals, style and staminodes abscise leaving a characteristic calloused scar at the tip of the fruit. Color, size, texture and flavour of common cultivated *Musa* fruits vary with cultivar (Australian Government, 2008).

Figure 1-1 depicts the cultivation of Baby banana in Colombia. The fruits are berries between 8-13 cm in length and 1.5 to 1.3 cm in width. Fruits turn from green to yellow during the ripening. The flesh, ivory-white is firm, astringent, even gummy with latex when is unripe, turning tender and slippery, soft and mellow, rather dry, mealy or starchy when ripe **(Figure 1-2).**

Figure 1-1. Baby Banana cultivation in Cundinamarca, a central region of Colombia (South America).

Figure 1-2. Baby Bananas (*Musa acuminata AA Simmonds cv*." Bocadillo") from Colombia at different stages of ripening after being harvested.

1.1.3 Pre-harvest

Baby Banana is cultivated in Colombia in different regions whose height oscillate between 1.000 and 2.000 meters above sea level with a temperature between 16-30 °C. The average rainfall of the area changes between 1.800 and 2.800 mm. The main growing areas are in western Antioquia department, known as Uraba, in eastern Santander department and in the Central part of Colombia where the major of department are located in a mountainous area.[4] **(Figure 1-3)**

The cultivation of Baby Banana in Colombia regularly belong to farmers more than to multi-national giant corporation like today´s Chiquita Brands International and Dole. The first trader for Baby banana fruit export begun in the 80´s.

Banana represents 244 US $ millions of global exportations of Colombia and currently ranks third in the exportation in comparison with coffee and flowers in first and second ranking, respectively. The major countries in Europe that receive banana are Belgium, United Kingdom and Germany.[5] **(Figure 1-4)**

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[4] Belalcázar, C., S., 1991. *Manual de assistência técnica N° 50: El cultivo del plátano en el trópico.* ICA, CIID, INIBAP y Comité de Cafeteros del Quindío. Armenia. Quindío. p.56
[5] Ministerio de Comercio, Industria y Turismo de Colombia. Estadísticas e Informes. –DANE-DIAN. 2013 (http://www.minicit.gov.co/publicaciones.php?id=15815)

Figure 1-3. Commercial Baby Banana growing areas in Colombia, in western Antioquia Department, in central territory, and in the eastern Santander Department (Belalcázar, 1991).

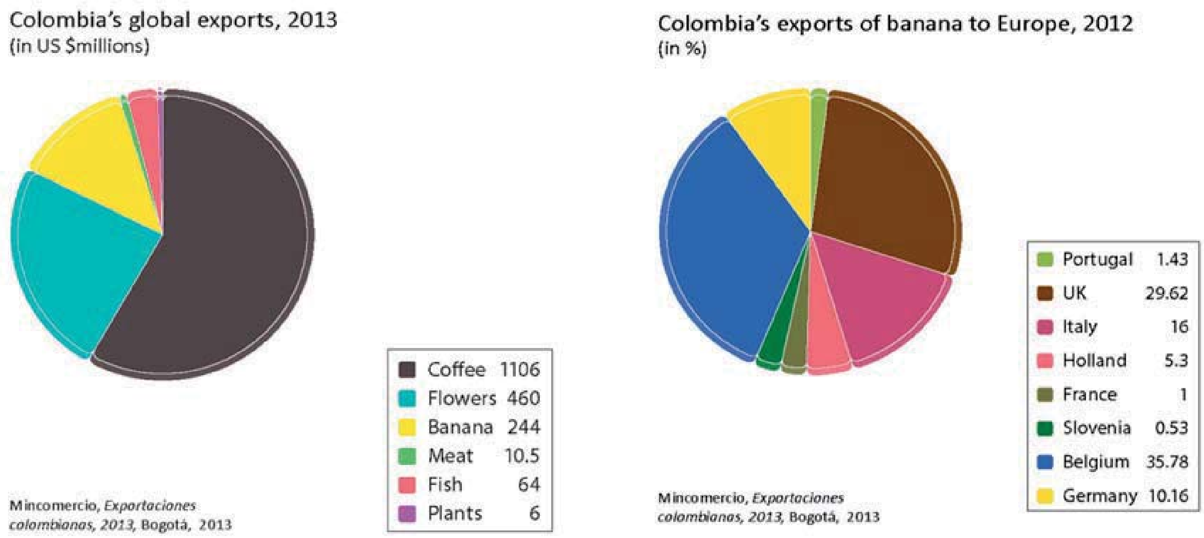


Figure 1-4. Exportations from Colombia ranking in US $ (millions) for 2013 (left) and receiving countries in Europe (right).

1.1.4 Post-harvest

The mature bunch is harvested after one year of planting by vegetative propagations. Since then plant produce a bunch each four months. Therefore, the growers implement farm management for obtaining harvests in sequence during the year. In this way they can adequate the marketing according to the supply and demand. The period between flowering and harvest varies from 6 to 10 weeks depending on the stage of ripening that is necessary according to the marketing either national or international.[6] In most commercial operations, the banana bunches are covered in plastic or cloth bags to prevent blemishes from mechanical and bird/plying and sugar glider damage.

Commercially, harvesting takes place when the fruit on the upper hands are just changing to light green and during the post-harvest it is monitored with a color chart **(Figure 1-5).** Because of the ripening process could be halted due to an inadequate temperature, the fruit should kept between 13.5 and 15 °C.

Figure 1-5. Post-harvest of Baby Bananas in green stage before being exported (left). Control color chart of Bananas used during the post-harvest marketing.

[6] Corporación Colombiana Internacional, Departamento de Planeación. *Análisis internacional del sector hortifrutícula para Colombia* (1994).

The critical temperature of storage is 12 °C at 70% humidity and the optimal time is two weeks for prolonging four week of shelf-life of fruits.[7] When the Baby Banana fruit is exposed to 2 °C and 7 °C damage occurs due to variation in the respiratory activity with a low level of carboxylic acids and sugars in comparison with a control at 20 °C. The peel shows black spots and a high contamination by fungi. The organoleptic characteristics as flavour and color are altered.[8]

Short treatment post-harvest (15% CO_2, 2% O_2, 83% N_2) were applied during 48 h to Baby Banana fruits after harvesting, and the climateric peak was delayed by 21 days without chemical and sensorial changes. Extraction and enzymatic activity of chlorophyllase was performed in Baby Banana peels both with treatment and without treatment (control) and the apparent Michaelis-Menten kinectics (Km) was reported. The substrate concentration at which the reaction rate is half of V_{max} (361 µM-Chl *a*/min/mg) was 0,034 µM-Chl *a* at 37 °C and pH 7.0. The results depict a stable enzyme-substrate complex with a high affinity for chlorophyll *a.* SDS-PAGE experiments were applied to protein extracts from Baby Banana peel and molecular mass between 12 and 35 kD were reported for chlorophyllase.[9]

[7] Díaz, R., Porras, G., *Determinación de la temperatura crítica de almacenamiento del banano bocadillo* (*Musa paradisiaca* L.). Trabajo de grado. Santafé de Bogotá. Universidad Nacional de Colombia. Departamento de Química (1998).
[8] Bustos, Y., *Estudio preliminar del efecto de las bajas temperaturas en el almacenamiento del banano bocadillo* (*Musa paradisiaca* L.). Trabajo de grado. Santafé de Bogotá. Universidad Nacional de Colombia. Departamento de Química (1995).
[9] Castro, B. M., *Efectos de tratamientos postcosecha en la actividad enzimática de la clorofilasa del Banano Bocadillo* (*Musa acuminata*). Trabajo de grado. Mención meritoria. Título Maestría. Santafé de Bogotá. Universidad Nacional de Colombia. Departamento de Química. Departamento de Agronomía (2001).

1.1.4.1 Hyperpigmentation phenomenon

The hyperpigmentation phenomenon in Baby Banana peel was observed for first time in Colombia during the recollection of fruits for export around the 90´s and this fact was confirmed when the sanity control in the European Union was applied to the fruits due to the occurrence of green band in the peels **(Figure 1-6).** The fruits were rejected as a consequence that fruit with hyperpigmentation were excluded into the international marketing. The farmers learned to do a strict classification during the post-harvest stage and fruits with hyperpigmentation were addressed to the local marketing. The label of "hyperpigmentation" was assigned by the traders to identify the fruit with the quality problem.

Figure 1-6. Baby Banana with hyperpigmentation (HP) (left), and without hyperpigmentation (Control), (right).

The green bands are visible when the fruit is in the green stage (1-3 point according to the control color chart), more than in yellow stage. Even though this fact does not mean that fruit develops a normal stage of ripening. The effect of the hyperpigmentation was monitored during 10 days postharvest in fruit, hence the influence of the phenomenon on the peels could be observed. The green bands could be related to the degradation of chlorophyll since the hyperpigmentation shows green veins through the peels especially in the posterior view that could depict chlorophyll accumulation on the peels. The control usually turn on green to yellow on the peels without intermediate stage as it is illustrated in the control color chart, except for stage 5 where only the upper and button area maintain a green color **(Figure 1-7.)**

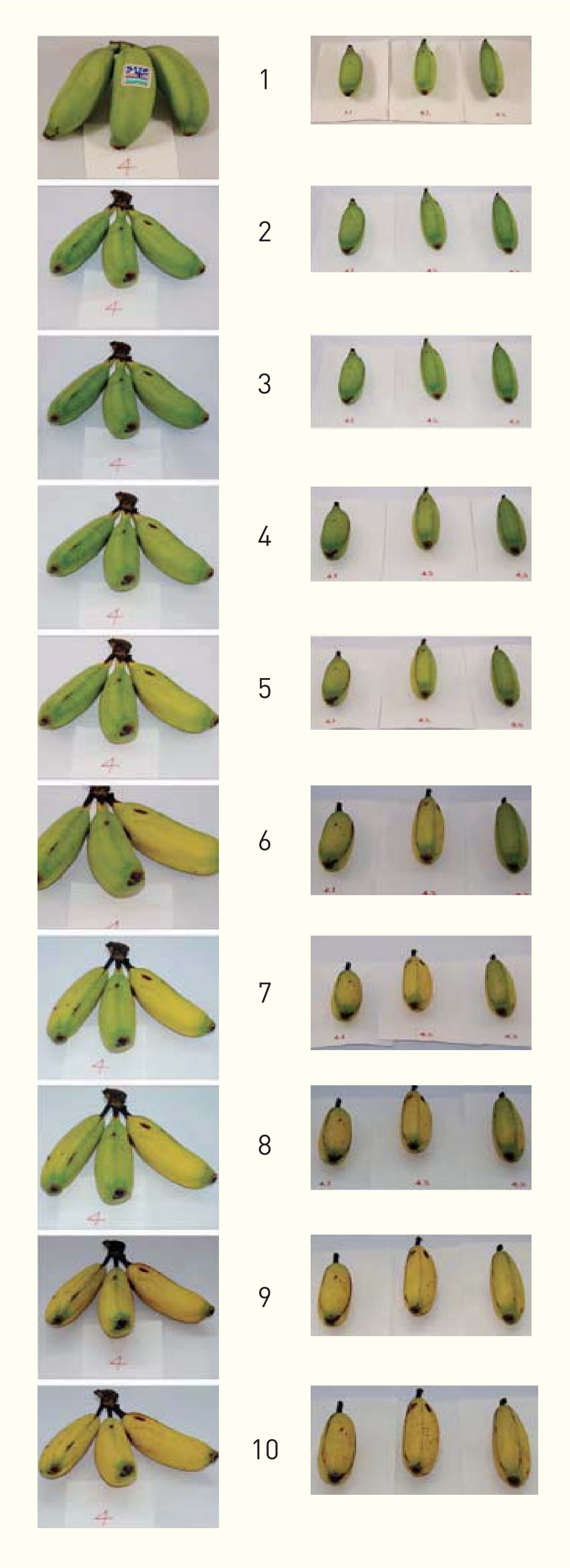


Figure 1-7. Baby Banana with hyperpigmentation at different stages of ripening during 10 days after being harvested.

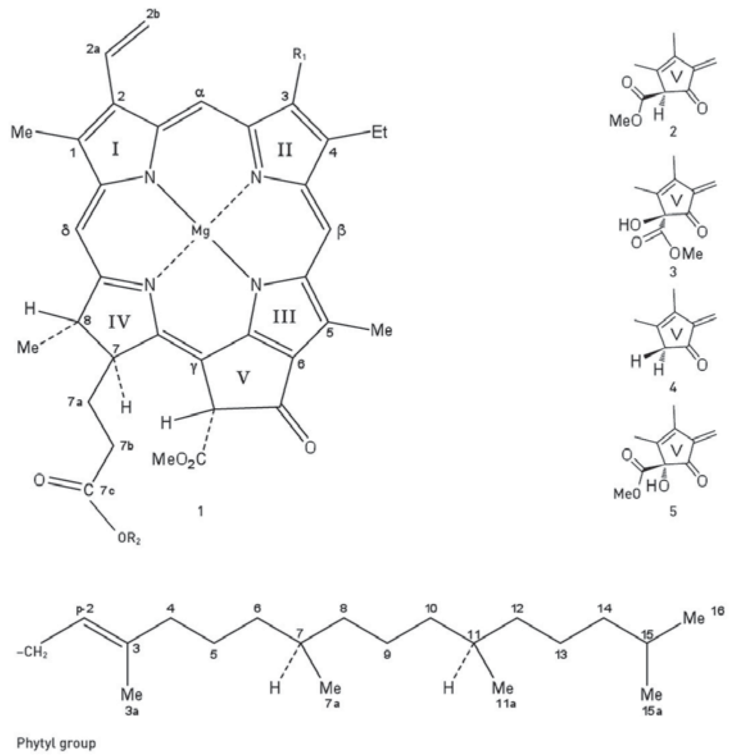
1.2 Chlorophyll degradation

The knowledge on chlorophyll degradation has grown considerably nowadays due to relevant reports about chlorophyll catabolites in higher plants and fruits which reveal a path of chlorophyll breakdown not only in chloroplast but also in both cytosol and vacuole (Kräutler et al. 2012).

The controversy about whether chlorophyll catabolism occurs either *in vivo* via oxidase or peroxidase (Minguez-Mosquera and Garrido-Fernandez 1996; Thomas & Janave 1992) or via the pheophorbide *a* oxigenase (PaO) pathway proposed by Hörtensteiner (1999) and Kräutler at al (1997) has been clarified in favor of the PaO pathway (Pružinská et al. 2005; Berghold et al. 2006; Moser et al. 2012).

The first step in chlorophyll *a* catabolism corresponds to a loss of phytol by chlorophyllase enzyme and subsequently chlorophyll derivatives are formed such as pheophytin *a*, pheophytin *b*, pyropheophytin *a,* pyropheophytin *b*, pheophorbide *a*, pheophorbide *b*, chlorophyllide *a*, chlorophyllide *b*. Structural isomers of chlorophyll *a* and *b*, such as chlorophyll derivatives, are shown in **Figures 1-8, 1-9.**

Chlorophyllase was one of the first plant enzymes described by Willstätter and Stoll in 1913. Studies of biochemistry and molecular biology concluded that the enzyme is a serine lipase-type esterase (Jakob-Wilk et al. 1999) Additional results in Citrus (C*itrus sinensis*) suggest that chlorophyllase functions as a rate-limiting enzyme in chlorophyll catabolism controlled via posttranslational regulation (Harpaz-Saad et al. 2007; Ginsburg 1993).



Compound	Mg*	R_1	R_2	Isocycling Ring (V)
Chlorophyll *a*	+	CH3	Phytyl	1
Chlorophyll *b*	+	CHO	Phytyl	1
Chlorophyll *a*’	+	CH3	Phytyl	2
Chlorophyll *b*’	+	CHO	Phytyl	2
Hydroxypheophytin a	–	CH3	Phytyl	3
Hydroxypheophytin *a*’	–	CH3	Phytyl	5
Pheophytin *a*	–	CH3	Phytyl	1
Pheophytin *a*’	–	CH3	Phytyl	2
Hydroxypheophytin *b*	–	CHO	Phytyl	3
Hydroxypheophytin *b*’	–	CHO	Phytyl	5
Pheophytin *b*	–	CHO	Phytyl	1
Pheophytin *b*’	–	CHO	Phytyl	2
Hydroxychlorophyll *a*	+	CH3	Phytyl	3
Hydroxychlorophyll *b*	+	CHO	Phytyl	3
Pyropheophytin a	–	CH3	Phytyl	4

*Mg is represented by 2H in pheophytins.

Figure 1-8. Structural formulas and nomenclature of chlorophylls and their derivatives (Huang et al. 2008).