Universidad Politécnica de Cartagena

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Obtención de Un Extracto de Cacao y Café con alto contenido de Compuestos Bioactivos

Programa de Doctorado: Técnicas Avanzadas En Investigación y Desarrollo Agrario y Alimentario

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(Cartagena 2021)

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OBTENCIÓN DE UN EXTRACTO DE CACAO Y CAFÉ CON ALTO CONTENIDO DE COMPUESTOS BIOACTIVOS

Tesis doctoral

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Nacional de Chocolates

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Cartagena, Murcia, España 2021

Obtención de un Extracto de Cacao y Café con alto Contenido de Compuestos Bioactivos

Tesis Presentada como requisito parcial para optar al título de:

Doctor en técnicas avanzadas en investigación y desarrollo agrario y alimentario

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AGRADECIMIENTOS

Quiero agradecer primeramente a Dios, un día alguien me preguntó cómo puedes combinar la ciencia con la fe y lo único que se me vino a la mente fue que mientras más estudio, más me doy cuenta que todo lo que existe solo puede venir de la mente de un diseñador perfecto, no creo que todo esto haya venido de una simple explosión o ¿cuándo has tirado una granada sobre un edificio para construirlo?."El dominio de la ciencia es explorar la naturaleza. El dominio de Dios es el mundo espiritual, un reino que no se puede explorar con las herramientas y el lenguaje de la ciencia. Se debe examinar con el corazón, la mente y el alma- y la mente debe encontrar el modo de abrazar ambos reinos".
Francis Collins-Director del Instituto Nacional de Investigación del Genoma Humano

A mis padres Rubén y Yolanda por su apoyo incondicional y por estar conmigo en todos los momentos de mi vida. A mis dos abuelas Margarita y Rubiela y a las familias Acosta y Otálvaro por creer en mí y celebrar conmigo cada meta alcanzada. A mis amigos y compañeros de trabajo. A Compañía Nacional de Chocolates S.A.S por todo el apoyo en este proceso de formación y en la financiación de este proyecto. A los señores Jorge Eusebio Arango, Conrado Mora y Juan Camilo Mazo por su apoyo incondicional y por creer en mî hace unos años atrás cuando esta aventura empezó.

A Wilmar Valencia Gallego por todo su apoyo y asesoría en las técnicas de laboratorio realizadas en Compañía Nacional de Chocolates S.A.S. A Industria Colombiana de Café S.A.S. por suplir el extracto de café analizado en esta tesis y a Mónica Quintero por su apoyo en los análisis realizados en el laboratorio de Colcafé.

A mi directora Cristina García- Viguera por sus aportes y direccionamiento en la construcción de este proyecto, a Raul Dominguez- Perles y Maria Eugenia Romero por su apoyo en la experimentación realizada durante mi pasantía en el CEBAS-CSIC (Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas).

PRODUCCIÓN CIENTÍFICA: PUBLICACIONES EN REVISTAS CIENTIFICAS

COCOA EXTRACT WITH HIGH CONTENT OF FLAVAN 3-OLS, PROCYANIDINS AND METHYLXANTHINES. Acosta-Otálvaro, E., Valencia-Gallego, W., Mazo-Rivas, J.C., García-Viguera, C. J Food Sci Technol (2021). https://doi.org/10.1007/s13197-021-05119-z

BIOAVAILABILITY AND RADICAL SCAVENGING POWER OF PHENOLIC COMPOUNDS OF COCOA AND COFFEE MIXTURES. Elly Acosta-Otálvaro, Raúl Domínguez-Perles, Juan Camilo Mazo-Rivas, Cristina García-Viguera (2020). Food Science and Technology International (Aceptado)

STABILITY AND QUALITY OF A COCOA EXTRACT AND A COCOA-COFFEE MIXTURE. Elly Acosta-Otálvaro, Wilmar Valencia-Gallego, Juan Camilo Mazo-Rivas, Cristina García-Viguera (2021) (En preparación)

PRODUCCIÓN CIENTÍFICA: PUBLICACIONES EN CONGRESOS

Prediction of total polyphenols content of cocoa extracs from the CIElab color space. E. Acosta-Otálvaro, J.C. Mazo-Rivas, C. García-Viguera (2020). 9h Workshop on Agri-food Research for young researchers. Cartagena, Spain. 11-12 May 2020.

El cacao y el café son dos fuentes de compuestos fenólicos, el cacao contiene flavan 3-oles (catequina y epicatequina) y metilxantinas (teobromina y cafeína), mientras el café contiene ácidos clorogénicos o cafeoilquínicos, compuestos bioactivos con demostrado beneficio sobre la salud humana. En base a esta premisa, el presente trabajo tuvo como objetivo la formulación de un alimento con alto valor nutricional, basado en una mezcla con alto contenido de compuestos dichos bioactivos (flavan 3-oles y ácidos clorogénicos), presentes en extractos de cacao y café. Este proceso se llevó a cabo en cuatro etapas experimentales: la primera enfocada en la obtención y caracterización del extracto de cacao en términos de su contenido en flavan 3-oles, procianidinas, metilxantinas y polifenoles totales; la segunda etapa fue la obtención de la mezcla de dicho extracto de cacao y un extracto de café de Industria Colombiana de Café rico en ácidos clorogénicos y derivados; en la tercera etapa se realizó una evaluación de la calidad y la estabilidad en el tiempo del extracto de cacao obtenido y la mezcla de cacao y café. Para la obtención de una formulación óptima se evaluaron diferentes proporciones de ambos productos (0:100, 25:75, 50:50, 75:25, 100:0 proporción porcentual de cacao:café), basada en su potencial antioxidante. Además, se estudió el transporte transepitelial de los compuestos fenólicos a través del modelo de barrera intestinal células Caco-2 con el fin de determinar la biodisponibilidad de estos compuestos. La calidad se evaluó a partir de la caracterización sensorial, su potencial para la fortificación de alimentos de acuerdo al contenido de minerales de ambos extractos y su seguridad para el consumo a través de un análisis de citotoxicidad mediante la técnica MTT en la línea celular CHO-K1. Por otro lado, se analizó la estabilidad de los compuestos fenólicos presentes en ambos extractos.

Los resultados en la primera etapa mostraron que deshidratar los granos de cacao sin fermentar a 45°C, inmediatamente tras ser cosechados, conserva siete veces más los compuestos bioactivos, como la (-)-epicatequina,

que los granos secados al sol y fermentados convencionalmente. Además, la extracción hidroalcohólica aumenta la (-)-epicatequina tres veces más en base al pretratamiento de deshidratación. Compuestos como las procianidinas y metilxantinas también presentan un incremento significativo a partir de estos tratamientos. En la segunda etapa se encontró que el cacao presenta una capacidad antioxidante (CA) más alta que el café, manteniéndose esa capacidad de forma proporcional, antagónica o sinérgica, en las mezclas. Lo cual se calculó mediante el índice de combinación (IC), con el fin de establecer el tipo de interacción en las mezclas de cacao:café, obteniendo que la mezcla 25:75 era moderadamente antagónica, 50:50 casi aditiva, y 75:25 ligeramente sinérgica. La eficiencia de absorción de los compuestos fenólicos del cacao estuvo alrededor del 90%, mientras que fue del 100% para los del café, variando dicha biodisponibilidad, en las mezclas, en función de la proporción de los ingredientes. Los resultados, obtenidos hasta esta etapa, permitieron concluir que los compuestos fenólicos presentes en el extracto de cacao y de café, se caracterizan por una alta biodisponibilidad y una alta capacidad antioxidante, si bien no se ha encontrado un patrón en el tipo de interacción de las mezclas que permita evidenciar un claro beneficio para utilizarlos de forma combinada.

Los resultados en la tercera etapa demostraron que los compuestos más estables en el extracto de cacao son (+)-catequina y cafeína, mientras que en la mezcla es la (+)-catequina. Aunque los demás compuestos presentaron una alta variabilidad, no se encontraron diferencias estadísticamente significativas, manteniéndose estables durante el periodo de conservación (12 semanas). En cuanto al perfil sensorial las notas de sabor más predominantes fueron las astringentes, amargas y verdosas, típicas de los compuestos fenólicos y los granos de cacao y café sin fermentar. Además, se encontró que ambos ingredientes tienen un alto potencial para la fortificación de alimentos con minerales, como B, Cu, Fe, Mn, Rb, Si and Zn. Por otro lado, se comprobó que ninguno de los extractos, de cacao o café utilizado en la mezcla, son citotóxicos. Previamente a este estudio, el ex-

tracto de café fue reconocido como GRAS (Generally Recognized as Safe) y el extracto de cacao no presentó ninguna citotoxicidad en el análisis MTT realizado en este estudio. De acuerdo a lo anterior, es posible concluir de que se obtuvo un extracto de cacao y una mezcla de cacao y café con alto contenido de compuestos bioactivos y con un alto potencial para el desarrollo de alimentos funcionales.

Palabras Clave: Flavan 3-oles; metilxantinas; ácidos cafeoilquínicos; *Theobroma cacao L; Coffea.*

Cocoa and coffee are two sources of phenolic compounds. Cocoa contains flavan-3-ols (catechin and epicatechin) and methylxanthines (theobromine and caffeine), while coffee contains chlorogenic acids. Several studies have reported health benefits from the consumption of these compounds. This research aimed to design a functional mixture with a high content of bioactive compounds (flavan-3-ols and chlorogenic acids) present in cocoa and coffee extracts. This process was carried out in four experimental stages. In the first stage, a cocoa extract was obtained. Also, we characterized the content of flavan 3-ols, procyanidins, methylxanthines, and total polyphenol content (TPC). The second stage was to get a mixture of cocoa extract and a coffee extract from Industria Colombiana de Café S.A.S. This stage aimed to uncover the radical scavenging activity (AC) of blends of cocoa and coffee extracts, and the bioavailability of their phenolic compounds, resorting to in vitro models: DPPH and ORAC antioxidant methods, and the characterization of the trans-epithelial transport of cocoa and coffee phenolic compounds through Caco-2 cells monolayer model of the intestinal barrier to determine the bioavailability of these compounds. In the third stage was carried out, an evaluation of the quality and stability over time of the cocoa extract and the mixture with the synergies evidenced during the second stage. The quality was evaluated in terms of sensory characterization, the potential for food fortification according to the mineral content of both extracts, and its safety for consumption through a cytotoxicity analysis using the MTT technique with a CHO-K1 cell line. Also, we analyzed the stability over time of the phenolic compounds present in both extracts under environmental conditions (25°C) and extreme storage conditions (40°C).

The results in the first stage showed that dehydrating unfermented cocoa beans at 45°C immediately the harvest time preserves compounds such as (-)-epicatechin. This value is seven times more than conventionally sun-dried and fermented beans. The hydroalcoholic extraction increased the

(-)-epicatechin, three times more based on dehydration pretreatment. Compounds such as procyanidins and methylxanthines also show a significant increase from these treatments. In the second stage, the cocoa displayed a higher AC than the coffee regarding both DPPH and ORAC assays, while the mixtures increased in parallel with the percentage of cocoa in the blends. The combination index was calculated to set up the type of interaction in the cocoa-coffee mixtures, obtaining that the mixture 25:75 was moderately antagonistic, 50:50 nearly additive, and 75:25 slightly synergistic. The absorption efficiency of the cocoa phenolic compounds was 90% approximately, in the coffee compounds was 100%. The mixtures varied according to the proportion of cocoa and coffee. The results obtained allowed concluding that the phenolic compounds present in cocoa and coffee, respectively, are featured by high bioavailability and a valuable antioxidant capacity, while no interaction pattern was found in the mixtures concerning the real benefit of using them combined.

The results obtained in in the third stage, showed that the most stable compounds in cocoa extract were (+)-catechin and caffeine. In the mix it was (+)-catechin. Although the other compounds showed high variability, no statistically significant differences were found between weeks 0 and 12. This result demonstrates the stability of the phenolic compounds present in both extracts over time. As for the sensory profile, the most predominant flavor notes were astringent, bitter, and green taste typical of phenolic compounds and unfermented cocoa and coffee beans. Additionally, the ingredients have a high potential for food fortification about minerals such as B, Cu, Fe, Mn, Rb, Si, and Zn. Regarding cytotoxicity, the cocoa extract was not cytotoxic, and the coffee extract used in the mixture, before this study, was recognized as GRAS (Generally Recognized as Safe). Based on the above, it is possible to conclude from this study that a cocoa extract and a mixture of cocoa and coffee with a high content of bioactive compounds and with a high potential for the development of functional foods were obtained.

Key words: Flavan 3-ols; methylxanthines; chlorogenic acids; *Theobroma cacao L; Coffea.*

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CAPITULO I:
INTRODUCCIÓN

1. INTRODUCCIÓN

En la actualidad existe una tendencia creciente asociada a nuevos hábitos de alimentación debido al aumento de diferentes patologías como la obesidad y algunas enfermedades no transmisibles (ENT) tales como las cardiovasculares, diferentes formas de cáncer y diabetes tipo 2, las cuales representan el 71% de las muertes a nivel mundial. Los expertos recomiendan cambios en el estilo de vida, consumo de dietas saludables y actividad física como prevención y tratamiento a las ENT (OMS, 2015). En concordancia con esta tendencia, la industria alimentaria ha venido desarrollando diferentes estrategias para ofrecer al consumidor productos que contribuyan a la prevención de las ENT y se ha trabajado en una búsqueda de ingredientes saludables para el desarrollo de alimentos funcionales (Ribeiro, Leitão, Ramalho, & Lidon, 2014). Muchos de esos ingredientes funcionales, están basados sus compuestos bioactivos, los cuales son sustancias presentes en las plantas, que tienen beneficios en la salud. Dentro de los compuestos bioactivos, más comunes, se encuentran los compuestos fenólicos, presentes en todas las plantas superiores (Serrano, López, & Espuñes, 2006). Existen diferentes estudios destinados al esclarecimiento de la actividad biológica y las propiedades saludables de los polifenoles presentes en matrices alimentarias tales como: frutas, cacao, aceite de oliva, café, té, soya, entre otros, los cuales se declaran como antioxidantes y son altamente valorados por el consumidor (Tomé-carneiro & Visioli, 2016).

Una de las industrias con mayores retos en términos de su aporte a la salud, en el campo alimentario, es el sector de la confitería por el uso en sus formulaciones de algunos restrictores de consumo tales como la sacarosa y las grasas. En términos económicos se estima que el sector mundial confitero crecerá de 193.2 mil millones de dólares en 2019 a 219.5 mil millones en 2024, registrando una tasa compuesta anual del 2,6% durante 2019-2024. Dentro de este mercado la categoría de chocolate proyecta los volúmenes más altos y un comportamiento entre 2019-2024 entre 55% y 48.4%, respectivamente. Esta disminución en 2024 se será debida a la percepción negativa de los consumidores de que este tipo de productos no son saludables, por la presencia de niveles excesivos de azúcar, como

se mencionó anteriormente. Actualmente los productos de confitería con atributos de salud y bienestar representaron en 2019 el 16.3% de las ventas generales del sector a nivel mundial, y se espera que dicho porcentaje pueda incrementarse (Global Data, 2020).

En 2019 la ANDI (Asociación Nacional de Industriales) en Colombia, mencionó que el sector de chocolatería en el país crece de manera sostenible, las hectáreas de cultivo de cacao han aumentado un 24.2% en el total de toneladas nacionales. Hoy Colombia exporta chocolates, cacao, licor de cacao, entre otros, a 74 países en los cinco continentes (Andi, 2019). Una de las industrias líderes en este sector en Colombia, es el Negocio de Chocolates Nutresa (Compañía Nacional de Chocolates S.A.S-CNCH), con un porcentaje de participación en el mercado nacional del 69.3% en la categoría de golosinas de chocolate, 56.6% en chocolates de mesa y 28.4% en modificadores de leche (Grupo Nutresa, 2019).

Con este contexto es posible mencionar el interés de CNCH en desarrollar alimentos a base de cacao que puedan contribuir a la salud del consumidor a través de declaraciones nutricionales que permitan expresar las bondades saludables de un producto. Para el caso de alimentos a base de cacao, existen diferentes estudios que validan los efectos benéficos sobre la salud, los cuales se asocian al contenido de antioxidantes de esta matriz. Algunos de esos beneficios del cacao recaen sobre la salud cardiovascular, diabetes, presión arterial, salud cognitiva y cerebral (Balzer et al., 2008; Pokorney & Piccini, 2017; Sokolov, Pavlova, Klosterhalfen, & Enck, 2013). En la normativa colombiana no existe una regulación que establezca las cantidades mínimas requeridas para la realización de una declaración nutricional en relación al contenido de antioxidantes en un producto alimentario, como ocurre con la EFSA. Sin embargo en el año 2012 la EFSA aprobó la solicitud realizada por la empresa Barry Callebaut, donde se presentaron las evidencias científicas del efecto de consumir 200 mg de flavanoles de cacao para lo cual se presentó el siguiente claim: “los flavanoles del cacao ayudan al mantenimiento de la vasodilatación dependiente del endotelio, lo cual contribuye al flujo sanguíneo normal” (EFSA, 2012).

En los últimos años, en el mercado local e internacional de chocolates, es común encontrar productos que declaran un alto contenido de antioxidantes basado en su contenido de sólidos de cacao, tales como Cocoa Fusión (Tumaco), Pacarí (Ecuador), Amazonía (Ecuador) y Special Dark de Hersheys (USA), entre otros (Vinson & Motisi, 2015). No obstante, en el mercado internacional ya se encuentran chocolates suplementados con extractos de polifenoles desarrollados por la empresa Barry Callebaut (Hadleigh Maid Dark Chocolate Cranberries), con el sello ActicoaTM en los que se declara un alto contenido de antioxidantes y el siguiente claim: "los antioxidantes ayudan a neutralizar los radicales libres del cuerpo" (Ramsey, 2009).

Dentro de esta búsqueda de ingredientes con beneficios para la salud, el Grupo Nutresa en Colombia cuenta también con el Negocio de Café (Industria Colombiana de Café S.A.S.-Colcafé) quienes son líderes en el país y tienen una participación en el mercado nacional del 49.1% en la categoría de café molido y el 39.9% en café soluble. Colcafé viene desde hace unos años atrás desarrollando un extracto de café verde con un alto contenido de ácidos clorogénicos sobre el cual recibieron su patente de invención en el año 2015 (WO2015/170283A3) y demostraron que los antioxidantes del café inhiben la peroxidación lipídica del colesterol LDL y tienen un papel fundamental en la modulación del estrés oxidativo, lo que reduce el riesgo de ateroesclerosis (Agudelo-ochoa, Pulgar, Vel, Duque-ram, & Naranjo-cano, 2016; Lara-guzmán, Álvarez-quintero, & Osorio, 2016; Tabares-Guevara et al., 2017; Gallo & Naranjo, 2015)

De acuerdo a lo mencionado anteriormente se propuso dentro del Centro de investigación, desarrollo y calidad CIDCA de Compañía Nacional de Chocolates S.A.S. liderar una investigación que permitiera desarrollar un extracto rico en compuestos bioactivos de cacao y a su vez evaluar la posibilidad de obtener una mezcla de dicho de extracto con el desarrollado por el Negocio de Café Colcafé, con el fin de obtener un ingrediente que permita su incorporación en diferentes tipos de productos y la posibilidad de realizar declaraciones de salud asociadas a ambas matrices. Para el caso de productos a base de cacao y café se han encontrado diferentes estudios

sobre los efectos benéficos de cada uno sobre la salud, los cuales se asocian al contenido de antioxidantes de ambas matrices. Algunos reportan beneficios del cacao sobre la salud cardiovascular, diabetes, presión arterial, salud cognitiva y cerebral. Para el caso de café se destacan beneficios sobre enfermedades antiinflamatorias, antimutagénicas, cardiovasculares y reumatológicas (Budryn, Zaczynska, & Oracz, 2016). Sin embargo, no se han encontrado muchos estudios que evaluaran la potencialidad de ambas matrices juntas, solamente Çelik y Gökmen (2018) realizaron un estudio en el que evaluaron las interacciones en la capacidad antioxidante de diferentes infusiones de café y los principales antioxidantes cacao: (+)-catequina y (-)-epicatequina, así como las interacciones entre diferentes cafés e infusiones de chocolate negro, encontrando, en algunas mezclas, comportamientos sinérgicos, aditivos y antagónicos.

Tanto el cacao como el café se consideran fuentes de compuestos fenólicos. El cacao contiene principalmente de flavanoles, los cuales se clasifican en tres grupos: catequinas o flavan-3-oles, antocianos y proantocianidinas (Schinella et al., 2010a). La forma principal de los monómeros de flavanoles de cacao son: (-)-epicatequina y (+)-catequina. La procianidina está compuesta por oligómeros y/o polímeros de catequina y epicatequina (Hu, Kim, & Baik, 2016; Rabadán-Chávez et al., 2016). Los ácidos cafeoilquínicos o clorogénicos son los compuestos fenólicos más abundantes presentes en el café y comprenden varios ésteres de ácidos quínico y cafeico. Los granos verdes de café son la principal fuente de estos compuestos, suponiendo aproximadamente entre el 7 y 10% de su materia seca, siendo el más importante el 5-O-cafeoilquínico (Budryn et al., 2016).

El alcance sobre el beneficio para la salud de estas matrices depende en gran medida de su capacidad antioxidante y su biodisponibilidad (Ern et al. 2003; Sotelo, Alvis and Arrazola, 2015). El objetivo de esta investigación fue la obtención de un extracto de cacao alto en compuestos bioactivos y el entendimiento de la posible funcionalidad de este en combinación con un extracto de café verde, para establecer sus sinergias o antagonismo a partir de la determinación de su capacidad antioxidante y la evaluación de su biodisponibilidad a través del transporte transepitelial por células

Caco-2 de los flavanoles, procianidinas y ácidos clorogénicos presentes en diferentes mezclas de cacao y café.

1.1. COMPUESTOS BIOACTIVOS PRESENTES EN EL CACAO Y CAFÉ

En la naturaleza existe una amplia variedad de compuestos que tienen una estructura molecular caracterizada por la presencia de uno o varios anillos fenólicos. Estos compuestos se denominan ácidos fenólicos y polifenoles, los cuales se originan principalmente en las plantas, como producto de su metabolismo secundario (Quiñones & Aleixandre, 2012). Diferentes estudios científicos demuestran que el consumo de estos compuestos, contribuyen a la disminución del riesgo de diferentes patologías, presentando tanto el cacao como el café un alto contenido de dichos compuestos (Cienfuegos-Jovellanos Fernández, 2016; Vega, De León, & Reyes, 2017).

Los compuestos fenólicos comprenden una gran variedad de moléculas que tienen una estructura polifenólica (varios anillos fenólicos aromáticos) pero también moléculas con un solo anillo fenólico. Los principales grupos de polifenoles y ácidos fenólicos son: ácidos fenólicos, flavonoides, estilbenos y liganos (Belščak-Cvitanović et al., 2015; Ignat, Volf, & Popa, 2011; Lucci, Saurina, & Núñez, 2017). Los principales compuestos fenólicos del cacao se encuentran dentro del grupo de flavonoides y los del café se encuentran dentro de los ácidos fenólicos como se muestra en la Figura 1.1.

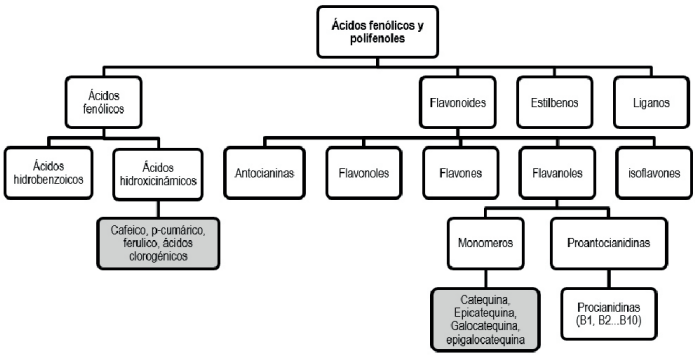
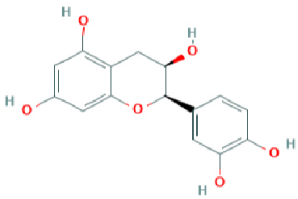


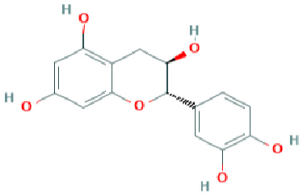
Figura 1.1 Clasificación de ácidos fenólicos y polifenoles (Belščak-Cvitanović et al. 2015; Lucci et al. 2017).

1.1.1. Compuestos bioactivos presentes en el cacao.

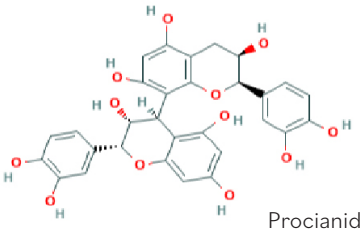
El cacao es considerado como una fuente de compuestos fenólicos principalmente de flavanoles, los cuales se clasifican en tres grupos: 37% catequinas o flavan 3-oles, 4% antocianos y 58% proantocianidinas (cantidades reportadas en granos de cacao crudos no fermentados). Es importante considerar que este contenido de compuestos fenólicos puede variar significativamente entre los granos de cacao de acuerdo a su origen, genética y condiciones de cultivo (Martín & Ramos, 2016). La forma principal de los monómeros de flavanol de cacao son: (-)-epicatequina y (+)-catequina (Hu et al. 2016a; Rabadán-Chávez et al. 2016). La procianidina está compuesta por oligómeros y/o polímeros de catequina y epicatequina, que son miembros de la familia de la proantocianidina, en el cacao la más predominante es la procianidina B2 (Kothe, Zimmermann, & Galensa, 2013). La estructura de estos compuestos se presenta en la Figura 1.2.



(-)-Epicatequina



(+)-Catequina



Procianidina B2

Figura 1.2 Estructuras químicas de los principales flavanoles presentes en el cacao (National Center for Biotechnology Information, 2021)

1.1.2. Compuestos bioactivos presentes en el café.

Los ácidos clorogénicos son los compuestos fenólicos más abundantes presentes en el café. Estructuralmente se forman por la esterificación de los ácidos hidroxinámicos tales como el cafeico, ácidos ferúlicos y p-cumáricos, con ácido quínico para formar diferentes estructuras conjugadas - ácidos clorogénicos (Lara-Guzmán, Álvarez-Quintero, Osorio, Naranjo-Cano, & Muñoz-Durango, 2016). Los granos verdes de café son la principal fuente de estos compuestos, ya que se constituyen aproximadamente entre el 7 y 10% de su materia seca. El ácido clorogénico más importante es el 5-O-cafeoilquínico (Budryn, Zaczynska, & Oracz, 2016). En la Figura 1.3 se presentan las estructuras químicas de dichos compuestos.

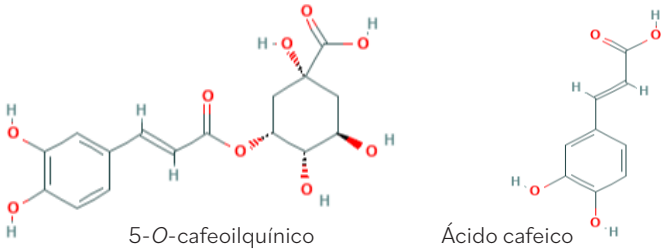


Figura 1.3 Estructura química de los princ ipales ácidos fenólicos del café (National Center for Biotechnolog y Information, 2021)

1.2. Beneficios nutricionales de los compuestos bioactivos presentes en el cacao y café.

Una de las razones principales por las cuales se añaden compuestos bioactivos a diferentes matrices alimentarias, es porque pueden servir para enriquecer dichos alimentos, con constituyentes diferentes a los propios o incrementar la concentración de algunos presentes en ellos, los cuales pueden deteriorarse por algunos efectos físicos o químicos del procesado. Incorporar estos compuestos en una matriz alimentaria permite realizar declaraciones nutricionales, si se demuestra científicamente, con el fin otorgar al consumidor un beneficio que contribuya a su salud. Para el caso de productos que contienen café y cacao se han encontrado diferentes estudios asociados a los efectos benéficos de cada uno de ellos.

1.2.1. Beneficios del consumo de compuestos bioactivos de cacao

Actualmente en productos a base de cacao solo se declara el contenido de antioxidantes en aquellos con un alto contenido de sólidos de cacao (entre 50-70%) y en su gran mayoría, no se puede realizar una declaración nutricional por falta de evidencias científicas que respalden un beneficio funcional (Vinson & Motisi, 2015). Algunos estudios reportan los beneficios del cacao sobre la salud cardiovascular, diabetes, presión arterial, salud cognitiva y cerebral (Balzer et al., 2008; Pokorney & Piccini, 2017; Sokolov et al., 2013).

En la normatividad colombiana no existe una regulación que establezca las cantidades mínimas requeridas para la realización de una declaración nutricional, en relación al contenido de antioxidantes en un producto alimentario. Sin embargo, en el año 2012 la EFSA aprobó la solicitud realizada por la empresa Barry Callebaut, presentanto las evidencias científicas del efecto de consumir 200mg de flavanoles de cacao sobre la vasodilatación y su contribución a mantener el flujo sanguíneo (EFSA, 2012). En este sentido, Taubert et al., (2007) mencionaron que el consumo de 30mg/ día de flavanoles reduce la presión sanguínea en humanos. Por otro lado Vlachojannis et al., (2016) estudiaron el efecto de los flavanoles de cacao en la salud cardiovascular determinando la importancia de la dosis, encontrando que los efectos están dados principalmente con un aumento de la vasodilatación, lo cual mejora el flujo sanguíneo. Además, pusieron de manifiesto que el consumo de estos compuestos, reduce la glucosa sanguínea y el metabolismo lipídico. Estos efectos se lograron con el consumo de 100mg de (-)-epicatequina de cacao en una dosis de 900 mg de flavanoles totales de cacao. Ibero-baraibar et al. (2014) presentaron un trabajo donde estudiaron la influencia del consumo de un extracto de cacao dentro de una dieta hipocalórica en sujetos con sobrepeso y obesidad. Los voluntarios recibieron comidas suplementadas con 1,4g de extracto de cacao con 645,3mg de polifenoles, encontrándose que a la cuarta semana de intervención tuvieron una mejoría en la presión arterial y presentaron reducciones en el colesterol LDL oxidado.

1.2.2. Beneficios del consumo de compuestos bioactivos del café

Para el caso de café se indican propiedades antiinflamatorias, antimutagénicas, cardiovasculares y reumatológicas (Budryn et al., 2016). Además se han realizado otras donde se han determinado su potencial para modular la salud mental, plasticidad cerebral, el estado de ánimo, depresión y la cognición (García-blanco, Dávalos, & Visioli, 2016).Otros investigadores como Sokolov et al., 2013 propusieron realizar más estudios contundentes para probar la acción inmediata de los flavanoles del cacao sobre la neurocognición. Agudelo-ochoa et al., (2016) evaluaron la biodisponibilidad de los ácidos clorogénicos de dos extractos de café con 420mg y 780mg de dichos compuestos, además de sus efectos en la función vascular en adultos sanos, encontrando que después de 8 semanas de consumo de las dosis mencionadas vehiculizadas en una bebida, las concentraciones de ácido caféico y ferúlico aumentaron en los grupos que consumieron dicho producto, incrementando la capacidad antioxidante en plasma en ambas poblaciones. Sin embargo, no se encontraron efectos en la función vascular de las personas evaluadas.

En términos generales, como se ha mencionado anteriormente, tanto los compuestos fenólicos presentes tanto en el cacao, como en el café, tienen una relación con la salud cardiovascular y diferentes autores lo demuestran (Tomé-carneiro & Visioli, 2016). Como puede observarse, se han realizado diferentes estudios sobre las propiedades antioxidantes y los efectos relacionados con la salud del café y el cacao, sin embargo, es limitada la información sobre su consumo simultáneo y sus sinergias, si bien Çelik & Gökmen, (2018) propusieron un estudio cuyo objetivo fue investigar las interacción entre las fracciones insolubles de diferentes infusiones de café y los principales antioxidantes libres del cacao: (+)-catequina y (-)-epicatequina, a través de la medición de la capacidad antioxidante de algunas mezclas con diferentes proporciones de café y cacao. El resultado obtenido, por estos investigadores, fue la identificación de sinergias entre las fracciones insolubles de las infusiones de café y las catequinas del cacao.

Adicionalmente encontraron algunas interacciones tanto antagónicas como aditivas para las mezclas de flavanoles con las fracciones insolubles de otras infusiones de café.

1.3. Usos alimentarios de los compuestos bioactivos de cacao y café.

En la actualidad existe un gran número de alimentos en los cuales se combinan el cacao y café. A continuación, se presenta una revisión de diferentes aplicaciones alimentarias reportadas en la literatura y algunas bases de datos, donde fue posible identificar como ambas matrices son incorporadas de manera conjunta en diferentes tipos de productos alimentarios, con el fin de realizar declaraciones nutricionales o simplemente para tener atributos de sabor diferenciados.

Budryn et al. (2016) propusieron la incorporación de ácidos clorogénicos del café en forma libre o nanoencapsulados en pan, galletas, rellenos de nuez, rellenos de carne y un caramelo de queso cottage. Ribeiro et al. (2014) describieron una bebida con 94% de café en polvo tostado, 3% de cacao en polvo, 2% café silverskin y 1% café verde mínimamente procesado, el autor menciona que esta mezcla tiene una importante capacidad antioxidante y se consideró sensorialmente aceptable. Barry Callebaut obtuvo la patente de un método para preparar una mezcla de cacao y café molido puro, el cacao y el café se tuestan por separado y posteriormente se mezclan. Esta mezcla se considera una materia prima para ser incorporada en la elaboración de galletas o bebidas con un fin sensorial, por la aceptación que tienen ambos sabores juntos (Bernaert & Allegaert, 2013). Perlman (2011) obtuvo una patente que protege la elaboración de una bebida tipo infusión, rica en antioxidantes de té, café, cacao, camelia y otros, cuando ésta se sumerge en agua caliente, queda disponible el contenido de compuestos fenólicos presentes en la porción de la bebida. Ese producto está diseñado para que la cantidad de compuestos antioxidantes se mantenga en su mayor proporción, a lo largo de la vida útil del producto. Un concepto similar es el propuesto por la empresa Javazen quienes presentan una bolsa que contiene café premium, té y cacao donde se declaran las bondades de este producto en función de sus beneficios energéticos.

CAPITULO II:

PLANTEAMIENTO DEL PROBLEMA, JUSTIFICACIÓN, OBJETIVOS Y PLAN DE TRABAJO

2.1. Planteamiento del problema

Las enfermedades cardiovasculares (EVC) son la principal causa de mortalidad en el mundo occidental. Cada vez se implementan más las medidas preventivas para evitarlas, las cuales promueven la reducción del consumo de sal, la actividad física y en general un estilo de vida adecuado. Adicionalmente el tratamiento farmacológico de las EVC, aunque ha sido exitoso, tiene severos efectos secundarios, los cuales limitan su eficacia. En la actualidad se están desarrollando numerosos productos para contribuir a la mejora de estas patologías con un enfoque nutracéutico y funcional, como tratamiento complementario de las EVC o para prevenirlas. Ejemplo de ello son los diferentes estudios para esclarecer la actividad biológica y las propiedades saludables de los polifenoles presentes en diferentes matrices alimentarias tales como: frutas, cacao, aceite de oliva, café, té, soya, entre otros, los cuales se declaran como antioxidantes y son altamente valorados por el consumidor (Tomé-carneiro & Visioli, 2016).

La mayoría de los estudios indican que los beneficios de la salud atribuidos al cacao y al café dependen de los polifenoles y las metilxantinas (teobromina y cafeína) presentes en ellos. Sin embargo, el contenido de polifenoles del cacao y café se reduce desde etapas tempranas de la industrialización de los granos como lo son la fermentación y tostado, lo que no permite mantener las concentraciones indicadas para la realización de una declaración nutricional en un producto final (Schinella et al., 2010ª; Hu, 2016; Çelik & Gökmen, 2018). Adicionalmente, el uso de fuentes de antioxidantes para el enriquecimiento de un producto, representa un reto tecnológico desde el punto de vista sensorial, ya que estos compuestos son los responsables del sabor astringente y amargo del cacao y café, lo cual limita su uso en altas concentraciones (Brunetto et al. 2007; Belščak et al. 2009). Este estudio pretende desarrollar una solución para la obtención de un extracto funcional a base de cacao y café rica en compuestos bioactivos que permita su aplicación en estudios futuros y además proporcione un beneficio en la salud cardiovascular del consumidor.

2.2. Justificación

En el año 2012 el Negocio de Chocolates Nutresa realizó su primer estudio de prospectiva, el cual fue revisado en el año 2015, realizándose una actualización del mismo. Una de las declaraciones estratégicas realizadas en la imagen de futuro para el año 2035 fue la siguiente: *"Nuestra razón de ser está fundamentada en brindar Nutrición, Salud y Bienestar a nuestros consumidores y clientes. Nos apalancamos en uno de nuestros principales ingredientes ¡el cacao!, una súper fruta con beneficios antioxidantes, y en otras materias primas que proveen propiedades Funcionales, que contribuyen al sistema inmunológico y a la función cognitiva y cerebral"* (Prospectiva Chocolates Nutresa 2035). Este proyecto propone presentar una solución para este reto de futuro propuesto.

Adicionalmente, el consumidor actual quiere conocer el origen y los beneficios de los alimentos que consume, valora la procedencia de todo lo natural y quiere sentir que se nutre de forma integral. Este proyecto le permitirá al Negocio de Chocolates Nutresa y a la Línea de Investigación en Chocolates y Snacks en salud, la posibilidad de satisfacer la necesidad de este consumidor actual, al obtener un extracto funcional a base de cacao y café con un alto contenido de compuestos bioactivos con la posibilidad de comunicar sus beneficios en salud en aplicaciones futuras.

2.3. OBJETIVOS

2.3.1. Objetivo general: Diseñar una mezcla funcional con alto contenido de compuestos bioactivos (flavan 3-oles y ácidos cl rogénicos) presentes en extractos de cacao y café.

2.3.2. Objetivos específicos:

- Obtener un extracto de cacao con alto contenido de flavan 3-oles, procianidinas y metilxantinas.
- Desarrollar una mezcla funcional con alto contenido de com puestos bioactivos de cacao y café (flavan 3-oles y ácidos clor ogénicos) y establecer sus posibles sinergias.
- Evaluar el potencial del extracto de cacao y la mezcla de cacao y café para la elaboración de alimentos funcionales.

2.4. PLAN DE TRABAJO

Este trabajo se desarrolló en cuatro etapas experimentales como se describen en la Tabla 1.

Tabla 2.1. Etapas experimentales de este proyecto.

Etapa	Descripción	Capítulo
Etapa I: Identificación y exploración y selección de tecnologías para la obtención de un extracto de cacao con alto contenido de compuestos bioactivos.	Esta primera etapa consistió en realizar una revisión del estado del arte de los diferentes métodos y tecnologías para la conservación y extracción de compuestos bioactivos presentes en el cacao. Además de una exploración de dichas tecnologías para establecer el uso de aquellas con las que se pudieran alcanzar los mayores contenidos de los compuestos de interés, en este caso se definió como variable de referencia la (+)-epicatequina, ya que es el principal compuesto referenciado en la literatura con el mayor beneficio funcional.	III
Etapa II: Obtención de un extracto de cacao con alto contenido de compuestos bioactivos (flavan 3-oles, procianidinas y metilxantinas)	Con los métodos de conservación de compuestos fenólicos y la mejor tecnología de extracción, seleccionadas en la fase anterior de este estudio, se procedió a realizar un diseño experimental para obtener un extracto de cacao con un alto contenido de dichos compuestos.	IV

Etapa	Descripción	Capítulo
Etapa III: Obtención de una mezcla de extractos de cacao de café con alto contenido de compuestos bioactivos (flavan 3-oles, procianidinas y metilxantinas y ácidos clorogénicos)	En esta etapa se realizaron diferentes mezclas del extracto de cacao obtenido en la fase anterior de este estudio y un extracto de café obtenido por Industria Colombiana de Café S.A.S. Para la evaluación de dichas mezclas se definieron variables tales como la capacidad antioxidante y la biodisponibilidad a través de células Caco-2, con el fin de establecer el sinergismo o antagonismo de estos compuestos.	V
Etapa IV: Evaluación de la calidad y estabilidad del extracto de cacao obtenido y una mezcla de cacao y café.	En esta última etapa se evaluó la calidad tanto del extracto de cacao obtenido como de la mezcla de cacao y café en términos sensoriales, de su contenido de minerales y la citotoxicidad. Además de la estabilidad en el tiempo en condiciones de almacenamiento de los compuestos bioactivos presentes en cada uno de ellos.	VI

CAPITULO III:

TECNOLOGÍAS PARA LA EXTRACCIÓN DE COMPUESTOS BIOACTIVOS DEL CACAO

3. TECNOLOGÍAS PARA LA EXTRACCIÓN DE COMPUESTOS BIOACTIVOS DEL CACAO

Existe un gran interés de la industria alimentaria en buscar diferentes tecnologías que permitan la extracción y concentración de esos compuestos con el fin de incorporarlos en diferentes productos para ofrecer al consumidor productos que puedan contribuir a su salud y bienestar. Esto se hace necesario ya que el proceso tradicional de beneficio del cacao conduce a pérdidas significativas de estos compuestos biológicamente activos (Todorovic et al., 2015). Los flavanoles del cacao tienen una reducción significativa desde etapas tempranas del proceso de industrialización (fermentación y secado), en las cuales se pierden alrededor del 60% de estos compuestos (Gil, 2012). Es por esta razón que se deben buscar estrategias que permitan mejorar la calidad nutricional del cacao en términos de su actividad biológica, las cuales están enfocadas en realizar diferentes pretratamientos a los granos de cacao después de su cosecha, con el fin de preservar la mayor cantidad posible de estos compuestos. Se tienen evidencias de que realizar pre-tratamientos en los granos de cacao contribuyen con la conservación de los antioxidantes y, por ende, con su calidad nutricional del grano (Carriel, Obrebska, Bru-martínez, & Patiño, 2014; Schinella et al., 2010a; Toro-Uribe, Montero, López-Giraldo, Ibáñez, & Herrero, 2018). Muchas de estas técnicas están acompañadas de otras tecnologías de extracción, las cuales buscan aumentar y concentrar aún más el contenido de estos compuestos, una de las más comunes es a través de solventes, su principio de extracción es sólido-líquido, el cual se da mediante el contacto entre una fase sólida (en este caso el cacao, que contiene la sustancia a extraer (compuestos fenólicos) y un solvente; las sustancias que tienen afinidad por este se solubilizan y pasan de la fase sólida a la fase líquida (Ariel, 2014). Algunos de los solventes más comunes son: agua, etanol, solventes orgánicos, acetona, isopropanol o mezclas de los mismos (Mrabet et al., 2015; Bortlik & Lainnya, 2007; Hoving et al., 2000, Steffan at al., 2012).

3.1. Tratamiento de granos de cacao para la conservación de compuestos fenólicos

Los flavanoles del cacao se reducen de forma significativa desde etapas tempranas de su pre industrialización como lo son la fermentación y secado, esto ocurre principalmente por reacciones de oxidación catalizadas por la enzima polifenol oxidasa (PPO) (Gil, 2012). Uno de los principales sustratos de la PPO es la (-)-epicatequina, uno de los compuestos más importantes desde el punto de vista de absorción lo que facilita el efecto funcional de este compuesto (Brito et. al, 2002). Las polifenol oxidasas son enzimas que catalizan el pardeamiento enzimático en frutas y verduras. Los sustratos polifenólicos son catalizados y oxidados por las PPO resultando pigmentos marrones que conducen a la pérdida de calidad y en cambios indeseables en el sabor, textura, color, propiedades nutricionales y la vida útil de las frutas y verduras (Qian-Yun, 2020).

Para la inhibición de la PPO existen diferentes métodos químicos y físicos que están enfocados en el eliminar componentes de la reacción tales como el sustrato, cobre, oxígeno, intermedio de reacción (o-quinonas) o la propia enzima (Qian-Yun, 2020). Algunos autores han reportado tratamientos de diferentes matrices alimentarias con el fin de inhibir o reducir el efecto de esta enzima (Tabla 3.1). De esta revisión es posible mencionar que dentro de las técnicas más comunes se encuentran aquellas asociadas a tratamientos térmicos como la congelación, el calentamiento por encima de 90°C y el uso de algunos inhibidores químicos.

3.1.1. Congelación

La congelación es un proceso en el cual se baja la temperatura, en cualquier muestra biológica, lo cual ralentiza el crecimiento microbiano y la actividad enzimática (Seader, Henley, Roper, 2010). Las enzimas como la PPO en las células vegetales existen tanto en estado libre como unido (Zhu, Luo, & Sun, 2020). Estos dos estados de las enzimas pueden cambiar después de la congelación, ya que la congelación puede inactivar parcialmente las enzimas en estado libre, mientras que parte de las enzimas en estado

unido en la pared celular y otros orgánulos se pueden liberar y convertir al estado libre debido a la congelación (Zhu et al., 2020).

Tabla 3.1 Metodologías para inhibición de la PPO

Matriz alimentaria	Pre tratamiento de la muestra	Mecanismo de inhibición de la PPO	Post tratamiento de las muestras	Referencia
Granos de cacao verdes sin fermentar	Almacenamiento inmediato después de la cosecha a -20°C	Los granos de cacao congelados en una solución de ácido ascórbico 70 mM / L-cisteína (1: 1 v / v) y Procesamiento térmico de los granos a 96 ° C durante 6,4 minutos, seguido de enfriamiento inmediato en hielo durante 30 minutos.	Secado de la muestra a 70°C durante 3 h (Humedad final <4% agua p / p). La muestra seca se molió y se almacenó a 20°C	(Toro-Uribe et al., 2018
Granos de mazorcas de cacao maduras (6 a 7 meses)	Se lavaron los granos y se almacenaron a -20°C	-	Secado a 45°durante 24 h. Posteriormente la muestra fue molida	(Gil, 2012)
Granos de cacao CCN51	-	Los granos fueron tratados con vapor de agua a 95°C durante 5 min y posteriormente un choque térmico para inhibir la PPO	Secado a 45°C hasta alcanzar una humedad del 7%. Las muestras fueron descascarilladas, molidas, y desgrasadas mediante una prensa hasta lograr una masa con un 12% de grasa. Esta mezcla se trató en una autoclave 121 ° C durante 1 min	(Schinella et al., 2010a)
Granos de cacao CCN51	-	Los granos fueron tratados con vapor de agua a 95°C durante 5 min y posteriormente un choque térmico para inhibir la PPO	Secado a 45°C hasta alcanzar una humedad del 7%. Las muestras fueron descascarilladas, molidas, y desgrasadas mediante una prensa hasta lograr una masa con un 12% de grasa. Esta mezcla se trató en una autoclave 121 ° C durante 1 min	(Schinella et al., 2010a)

Matriz alimentaria	Pre tratamiento de la muestra	Mecanismo de inhibición de la PPO	Post tratamiento de las muestras	Referencia
Baya de goji	-	Congelación nitrógeno líquido a -80°C	Para el análisis, la baya de goji congelada se descongeló a 2°C durante 12h en el frigorífico.	(Zhu et al., 2020)
Granos de cacao	Almacenamiento en congelación	Los granos de cacao congelados en una solución de ácido ascórbico 70 mM / L-cisteína (1: 1 v / v) y Procesamiento térmico de los granos a 96 ° C durante 6,4 minutos, seguido de enfriamiento inmediato en hielo durante 30 minutos.	Almacenamiento a -20°C después del proceso de inhibición de PPO	(Toro-Uribe et al., 2018)
Membrana aislada y extraída de Manzanas Granny Smith	-	Aplicación sobre las muestras de los siguientes inhibidores: ácido ascórbico, L-cisteína, glutatión y ácido cítrico; todos 0.05 M	-	(Qian-Yun, 2020)
Agua de coco	-	Microondas a 93°C	Enfriamiento rápido con nitrógeno líquido y almacenado a -80°C	(Matsui, Granado, Oliveira, & Tadini, 2007)
Granos de cacao	-	Inhibición por microondas (105°C durante 5min)	-	(Kim & Kenney, 1984)

3.1.2. Calentamiento

La PPO es conocida por su alta resistencia térmica, se han encontrado evidencias de actividad de la enzima en alimentos tratados a temperaturas por encima de 80°C (Ke et al., 2021). Sin embargo, este tipo de tratamientos pueden causar un efecto contrario a lo esperado ya que temperaturas superiores a 60°C pueden degradarse los compuestos fenólicos (Alañón, Castle, Siswanto, & Spencer, 2016; Alean, Chejne, & Rojano, 2016). Dentro de los tratamientos térmicos más comunes se encuentran someter el fruto durante 5 min en agua a 95°C y posteriormente realizar un choque térmi-

co con agua fría (Schinella et al. 2010).

3.1.3. Inhibidores químicos

Estos inhibidores se clasifican en cuatro grupos basados en su mecanismo de acción: agentes reductores, acidulantes, agentes acomplejantes y agentes quelantes. Los más tradicionales son el ácido cítrico, ácido ascórbico, L-cisteína y dióxido de azufre, los cuales tienen efectos inhibidores significativos sobre la actividad de la PPO (Qian-Yun, 2020).

3.2. TECNOLOGÍAS DE EXTRACCIÓN DE COMPUESTOS FENÓLICOS

Existen diferentes tecnologías de extracción que, combinadas con los tratamientos descritos anteriormente, permiten obtener extractos ricos en compuestos bioactivos con el fin de enriquecer productos alimentarios que puedan satisfacer la necesidad del consumidor actual de tener una dieta saludable. Dentro de las más comunes se encuentran la extracción por solventes, asistida por ultrasonido, fluidos supercríticos, tratamientos hidrotérmicos y el aislamiento y purificación de compuestos usando cromatografía.

3.2.1. Extracción por solventes

Su principio de extracción es sólido-líquido, el cual se da mediante la transferencia entre una fase sólida en este caso el cacao, que contiene la sustancia a extraer (compuestos fenólicos), ocurre a través del contacto entre un solvente y el sólido, las sustancias que tienen afinidad por el solvente se solubilizan y pasan de la fase sólida a la fase líquida (Ariel, 2014). Algunos de los solventes más comunes son: agua, etanol, acetona, isopropanol o mezclas de los mismos. Esta tecnología generalmente se usa a escala de laboratorio ya que industrialmente los solventes tienen un valor comercial muy alto. (Mrabet et al., 2015; Bortlik & Lainnya, 2007; Hoving et al., 2000, Steffan at al., 2012).

3.2.2. Extracción asistida por ultrasonido

La extracción asistida por ultrasonido (EAU) es actualmente una de las técnicas más importantes utilizadas para la obtención de compuestos alimentarios (Carrillo-Hormaza, Ramírez, Osorio, & Gil, 2017). Esta tecnología se aplica para intensificar los procesos de extracción, debido a que la energía inducida en el medio aplicado mejora la transferencia de masa a partir de transmisión acústica y cavitación, lo que conduce a una agitación intensa, generando efectos estructurales, físicos o químicos en las muestras tratadas. Esta técnica es comúnmente combinada con la extracción de fluidos supercríticos para mejorar los rendimientos de extracción de compuestos bioactivos de sustratos vegetales. Estas muestras generalmente requieren una disolución en disolvente líquido (Santos-Zea, Antunes-Ricardo, Gutierrez-Uribe, García-Pérez, & Benedito, 2018). Diferentes autores se han centrado en la eficacia de este tipo de extracción, para incrementar el contenido de (-)-epicatequina en diferentes matrices alimentarias, como se refleja en la Tabla 3.2, donde se presenta una revisión de las condiciones de proceso por ultrasonido para incrementar dicho compuesto.

3.2.3. Extracción por fluidos supercríticos

En la actualidad el CO2 supercrítico se ha utilizado como un solvente verde para la extracción de compuestos con propiedades activas, presentes en diferentes matrices tales como lípidos, polifenoles, aceites, pigmentos o almidones, entre otros (Santana, Santos, & Meireles, 2019). Esta técnica no exige el uso de altas temperaturas, lo que permite mantener un mejor aroma y sabor de los productos tratados. Así mismo, cabe mencionar que es un proceso más eficiente ya que los subproductos pueden ser aprovechados sucesivamente en otros procesos (Velasquez, 2008). Diferentes autores han explorado la eficacia de esta tecnología para la extracción de compuestos fenólicos, en la Tabla 3.3 se presenta una revisión del uso de esta tecnología y sus condiciones en diferentes matrices alimentarias.

Tabla 3.2 Tratamiento de muestras con ultrasonido para incrementar los valores de (-)-epicatequina.

Referencia	Matriz y compuestos	Solvente	Tiempo	Temperatura	Potencia	Frecuencia	Observaciones
(López-miranda et al., 2016)	Uva Roja (Epicatequina)	Mezcla de etanol: agua 1: 1 (v / v)	1-90 min	25°C	-	-	Los extractos con ultrasonido presentaron mejores condiciones de extracción Los valores más altos de (-)-epicatequina se obtuvieron a los 90 min de tiempo.
(Carrillo-Hormaza et al., 2017)	(1) Cacao seco y fermentado (cacao crudo); (2) granos de cacao tostados; entre otros	32.5% Agua	50 min	30°C	180W	37kHz	-
(Santos-Zea et al., 2018)	Orégano seco	Etanol	60 min	35°C	83-150 W / L)	-	83-150 kPa presión acústica Estos parámetros generaron un aumento del 9-38% en el contenido fenólico total y 10-86% de incremento en la capacidad antioxidante
(Toro-Uribe et al., 2018)	Granos de cacao sin fermentar (1g)	120 ml de etanol al 50%	30 min	-	-	20kHz	-
(Gil, 2012)	Granos de cacao fermentado	Metanol/ agua 60:40	60 min	-	-	60kHz	Tubos cónicos de 50 ml Estas condiciones fueron ideales para la extracción de epicatequina pH:9.0

Tabla 3.3. Tratamiento de muestras por fluidos supercríticos

Referencia	Matriz y compuestos	Solvente/ Co-solvente	Temperaturas	Presión	Observaciones
(Edi-soetaredjo, Ismadji, & Ju, 2013)	Epicatequina pura	CO2/Etanol	313.15 K 323.15 K 333.15 K 343.15 K	12-26 MPa.	El efecto en la solubilidad de la (-)-epicatequina fue mayor al aumentar la presión.
(Valadez-carmona, Ortiz-moreno, Ceballos-reyes, Mendiola, & Ibáñez, 2018)	Cáscara de cacao (Flavanoles totales)	CO2/ Etanol (5, 10, 15%)	40°C 50°C 60°C	100 bar 200 bar 300 bar	Las mejores condiciones de extracción fueron: 60°C, 299 bar y 13.7% co-solvente
(da Silva, Rocha-Santos, & Duarte, 2016)	*Mentha spicata* Flavonoides (Catequina, epicatequina, entre otros)	CO2/Etanol (3g/min)	60°C	200 bar	Flujo: 15g/min, Tiempo: 60min
(Sökmen, Demir, & Yousuf, 2018)	Biomasa de Té verde (Epicatequina)	CO2/Etanol (0.5ml/min)	60°C	25 MPa	Tiempo: 3h
(Santos-Zea et al., 2018)	Orégano seco Contenido total de polifenoles	2.3% de Etanol	35 ° C	35 MPa	60 min
(Santana et al., 2019)	Cáscara de cacao (polifenoles)	Etanol	70°C	10MPa	100g de material seco. Con estas condiciones obtuvieron 39-51 mgGAE/g

3.2.4. Exracción por tratamientos hidrotérmicos (PHYTOCLEAN ™)

Esta tecnología fue desarrollada por la compañía Mazza Innovation, consistiendo en un método patentado avanzado para la extracción de compuestos bioactivos que da lugar a ingredientes limpios de manera amigable con el medio ambiente. Este método aplica calor y presión al agua para disminuir su polaridad, lo que hace que se comporte como un solvente orgánico, a niveles similares al etanol. Este procedimiento consiste en cuatro etapas: extracción sin solventes, concentración de extractos, secado para la conservación de compuestos biactivos y limpieza de los extractos, como se describen en la Tabla 3.4 (Mazza Innovation, 2014).

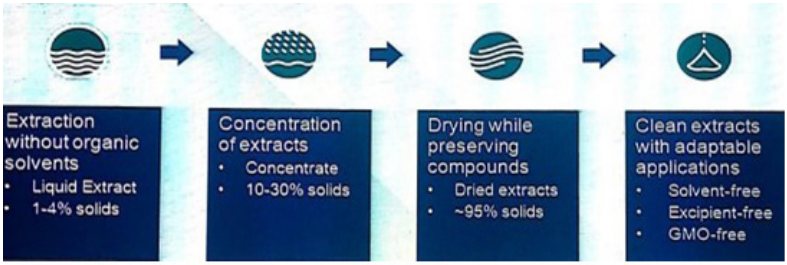


Figura 3.1. Método PHYTOCLEAN ™

3.2.5. Sistema de purificación de alto rendimiento (Reveleris®)

El sistema de purificación Reveleris® (Figura 3.2) es un sistema de alto rendimiento que combina las características de la cromatografía flash y de la HPLC preparativa en un único instrumento. El aislamiento de compuestos puros a partir de extractos de muestras naturales suele realizarse mediante cromatografía, este sistema permite una purificación altamente eficaz del compuesto de interés. Existen diferentes aplicaciones referenciadas para el aislamiento y bseparación de compuestos tales como los flavonoides de un extracto de *Ginkgo biloba*, α-Santalol y β-Snatalol de un extracto de sándalo, catequinas de un extracto de té verde, ginsenósidos de un extracto de ginseng entre otros (Buchi, 2020).

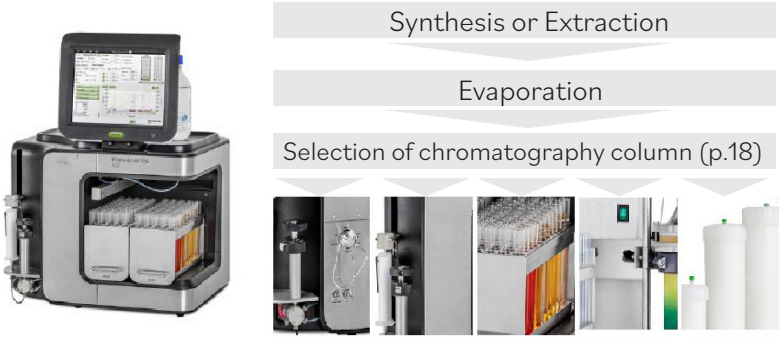


Figura 3.2. Reveleris® (Buchi, 2019)

El sistema de purificación incluye un detector UV que puede medir a tres longitudes de onda simultáneamente. Los detectores del equipo ELSD y UV ofrecen ajustes de sensibilidad para detectar y recolectar cantidades pequeñas de un analito y también compuestos con altas concentraciones. El equipo ofrece una alta modularidad en los recipientes receptores de muestra, cuenta con ocho opciones desde pequeños tubos de vidrio, pasando por recipientes de 500 ml hasta conductos especiales para llevarlos a recipientes mayores.

3.3. ANÁLISIS EXPLORATORIO DE TECNOLOGÍAS PARA LA EXTRACCIÓN DE COMPUESTOS FENÓLICOS

Para este análisis exploratorio se obtuvo un polvo de cacao sin fermentar el cual fue sometido posteriormente a diferentes tecnologías de extracción: fluidos supercríticos, extracción asistida por ultrasonido, tratamientos hidrotérmicos (*Phytoclean™*), sistema de purificación de alto rendimiento (Reveleris®), extracción por solventes, con el fin de obtener el mayor contenido posible de compuestos fenólicos de cacao, en especial la (-)-epicatequina.

3.3.1. Metodología

3.3.1.1. Obtención de polvo de cacao: Se tomó una mezcla de mazorcas de cacao de diferentes variedades, de la granja experimental de Compañía Nacional de Chocolates S.A.S. (Támesis, Antioquia). A continuación, se obtuvo un polvo de cacao sin fermentar mediante un proceso de ocho etapas (Figura 3.3) para preservar el mayor contenido posible de flavanoles.

1.Recolección de cacao

2. Las mazorcas son llevadas a la planta CNCH para ser limpiadas y desgrandas

3. Desgranado y mezclado de los granos de cacao de las variedades recolectadas

4. Eliminación de mucilago

5. Deshidratación a 45ºC hasta alcanzar una humedad entre 5-8%

6. Descacarillado

7. Desgrasado por prensado

8. Obtención de polvo de cacao sin fermentar

Figura 3.3. Proceso de obtención de polvo de cacao sin fermentar.

3.3.1.2. ***Extracción por fluidos supercríticos:*** Se tomaron 100g de muestra de cacao en polvo sin fermentar y mexclados en una malla con el solvente (cuando este se aplicó). Posteriormente se extrajo en el extractor del equipo piloto de fluidos supercríticos de la Corporación Universitaria Lasallista-CUL (Caldas, Antioquia) (Figura 3.4). Se realizaron diferentes extracciones con las condiciones reportadas en la Tabla 3.4, realizando un muestreo cada 10 minutos en los selectores 1 y 2 del equipo y se obtuvieron muestras de los selectores 1 y 2, (Figura 3.5). Estas muestras posteriormente fueron cuantificadas para establecer el contenido de (+)-catequina y (-)-epicatequina en los extractos obtenidos.

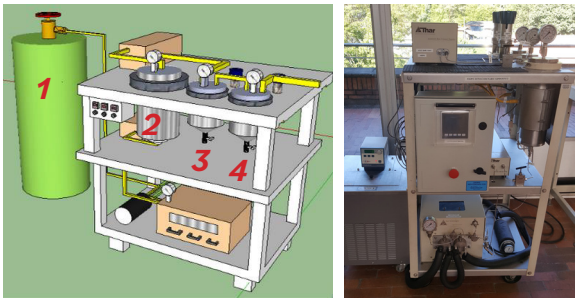


Figura 3.4 Equipo piloto para la extracción de Fluidos supercríticos.
1). Pipeta de CO2; 2) Extractor; 3). Selector 1; 4.) Selector 2.

Tabla 3.4 Condiciones de extracción fluidos supercríticos para los tratamientos evaluados

Extracción #1	Extracción #2	Extracción #3	Extracción #4
Temperatura: 50°C Presión: 350 bar Cosolvente: etanol 10% Flujo: 50g/min S1:100 bar S2: 80 bar	Temperatura: 50°C Presión: 350 bar Cosolvente: etanol 10% Flujo: 120 g/min S1:100 bar S2: 80 bar	Temperatura: 50°C Presión: 350 bar Cosolvente: etanol 10% Flujo: 180 g/min S1:100 bar S2: 80 bar	Temperatura: 60°C Presión: 250 bar Cosolvente: etanol 10% Flujo: 180 g/min S1:100 bar S2: 80 bar

Figura 3.5. Etapas de extracción por fluidos supercríticos

3.3.1.3. Extracción asistida por ultrasonido: este análisis fue llevado a cabo por la empresa Bioingred S.A. (Medellín, Colombia). Se tomaron 120g de muestra y se diluyeron en 1500g de agua. A continuación, se sometieron a tres tratamientos en las condiciones descritas anteriormente (Tabla 3.5 y Figura 3.6). Posteriormente las muestras se secaron y se cuantificó el contenido de (+)-catequina y (-)-epicatequina de las mismas.

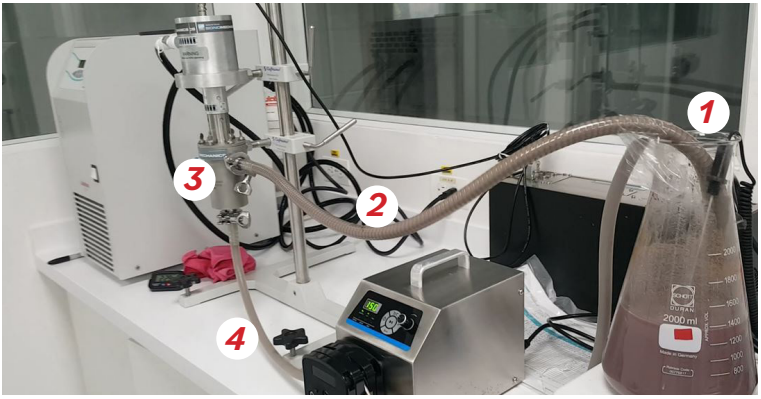


Figura 3.6 Montaje para la extracción asistida por ultrasonido. 1). Muestra diluida en agua; 2). Conducto de muestra hacia cámara de ultrasonido; 3). Cámara de ultrasonido, 4). Flujo muestra sonicada.

Tabla 3.5 Condiciones de extracción por ultrasonido para los tratamientos evaluados

Extracción #1	Extracción #2	Extracción #3
Amplitud: 70% T: 35°C t: 5min Flujo: 1L/min pH:8.0	Amplitud: 70% T: 70°C t: 5min Flujo: 1L/min pH:10.0	Amplitud: 70% T: 70°C t: 5min Flujo: 1L/min pH:2.0

3.3.1.4. Tratamientos hidrotérmicos *(Phytoclean™):* Para este tratamiento, una muestra del polvo de cacao sin fermentar se envió a la empresa Mazza Innovation (Delta, Canadá), allí se sometió al proceso de extracción descrito en la patente obtenida por Mazza Innovation, (2014). Se tomaron 39.26g de muestra y realizó una extracción en las siguientes condiciones: temperatura 130°C, volumen: 294.45mL y proporción solvente (agua)/muestra: 7.5mL/g.

3.3.1.5. Sistema de purificación de alto rendimiento *(Reveleris®)*: Para la evaluación de esta tecnología, una muestra de polvo de cacao sin fermentar se envió a la empresa Polco S.A.S. (Medellín, Colombia), donde inicialmente se extrajo con una solución de etanol y se filtó e inyectó en el *Reveleris®* a un flujo de 0.8ml/min, con una precisión de flujo menor al 2% de variación, una presión máxima de 50bares y una longitud de onda de 280nm en el detector DAD. En la muestra obtenida se cuantificó el contenido de catequinas y epicatequinas.

3.3.1.6. Extracción por solvente: Se realizó una extracción hidroalcohólica del polvo de cacao sin fermentar. La extracción se realizó siguiendo la metodología propuesta por Pasamar et al., (2006) se usó una solución de etanol/agua en una relación 70/30 y se mezcló dicha solución y el polvo de cacao en una relación de 1:10 (muestra:solución de etanol). La temperatura de extracción fue 60°C durante 60min. Posteriormente se eliminó el solvente usando un rotavapor® Buchi R-100 con un baño Buchi B-100, bomba de vació Buchi V-100 y Chiller Buchi F-100 (Buchi, Ciudad de México, México). Las condiciones de secado fueron: temperatura del baño 50°C, presión de 170mbar - 40mbar (fue disminuyendo a medida que se

reducía la concentración de EtOH) y temperatura del Chiller de 10°C. Para 2L de extracto líquido el tiempo de evaporación fue de 8h. Seguidamente, se realizó una liofilización para fracción acuosa en un liofilizador Christ Alpha 1-2 LD Plus (Martin Christ, Osterode am Harz,Alemania). En la fase de secado principal se tuvo una temperatura de condensación -50°C, vacío 0.040 mbar, durante 5h; y en la fase de secado final la temperatura de condensación fue -76°C, vacío 0.0010mbar y un tiempo de 3h. En la muestra obtenida se cuantificó el contenido de (+)-catequina y (-)-epicatequina.

3.3.1.7. Análisis por HPLC de (+)-catequina y epicatequinas: Para el análisis por HPLC se empleó un cromatógrafo líquido de alta resolución Agilent 1260 (Agilent Technologies, Palo Alto, CA, USA), con automuestreador automático y detectores de Fluorescencia y red de diodos (FLD y DAD) y un Computador equipado con el software Agilent OpenLAB CDS, ChemStation Edition. Las muestras se prepararon siguiendo el método propuesto por T-sanova-Savova, (2005) con algunas modificaciones. Así, 0.5g de cada una de las muestras de cada tratamiento se extrajeron con 10ml de una solución isopropanol/agua 60:40 pH 9.0, durante 60min en un baño de ultrasonidos Elma E30H (Elma Schmidbauer GmbH, Singen, Alemania) a temperatura ambiente. Posteriormente estas muestras se agitaron en vortex durante, 1min, dejando en reposo a -20°C, en un ultracongelador *(Thermo scientific, Massachusetts, USA)* durante 60min. Seguidamente se centrifugaron a 4000rpm por 8min en una centrifuga Rotofix 32 A (Andreas Hettich GmbH & Co.KG, Föhrenstraße, Almenania). Un mililitro del sobrenadante resultante se filtró por una membrana de 0.45µm (Nylon membrane filter, Millipore). Una alícuota de este filtrado se mezcló con la fase móvil (ácido acético al 0,1%), con un factor de dilución (FD) dependiendo de la coloración de la solución, (muestras oscuras: FD=5 ó 10, muestras menos oscuras: FD=4) Esto se definió para garantizar que las mediciones de las muestras no se salieran de la curva de calibración. Esta disolución se realizó en un vial de cromatografía ambar y se inyectó en el HPLC/DAD/FLD. Se usó una columna Zorbax bonus C18 (HPLC Column, 5 µm particle size, L×I.D. 25 cm×4.6 mm). La detección se realizó a 280nm y los valores procesados se analizaron mediante el uso del software OpenLab CDS Chemstation Edition. Para la cuantificación de los flavanoles y

xantinas en las muestras se usó un estándar (mezcla de (+)-catequina, (-)-epicatequina, teobromina y cafeína. Los análisis se realizaron por triplicado para cada muestra y se reportaron en mg/g.

3.3.2. Resultados

El polvo de cacao sin fermentar tuvo un contenido de 0.844mg/g de (+)-catequina y 20.00mg/g de (-)-epicatequina. Es importante indicar que era importante establecer este punto de partida para los compuestos de interés ya que se pretendía evaluar con cuál de las tecnologías se podía llegar al mayor rendimiento, para elegir aquella con la cual realizar un diseño experimental más robusto, para la obtención del extracto de cacao rico en compues tos bioactivos. El contenido de (+)-catequina y (-)-epicatequina para cada uno de los ensayos realizados se presentan en la Tabla 3.6.

Tabla 3.6 Resultados polvo de cacao sin fermentar sometido a las diferentes tecnologías de extracción.

Tecnología	(+)-catequina (mg/g)	(-)-epicatequina (mg/g)
Polvo de cacao sin fermenta	0.844	20.000
Extracción por fluidos supercríticos		
Extracción #1	0.480	12.459
Extracción #2	0.643	14.627
Extracción #3	0.588	12.394
Extracción #4	0.587	11.574
Extracción asistida por ultrasonido		
Extracción #1	0.050	0.708
Extracción #2	0.023	0.014
Extracción #3	0.196	3.371
Tratamientos hidrotérmicos (Phytoclean™)		
Extracción #1	12.364	29.432
Sistema de purificación de alto rendimiento (Reveleris®)		
Purificación de catequinas	0.756	41.5
Extracción por solventes		
Extracción Hidroalcohólica	3.3822	97.45

Tras comparar las diferentes tecnologías se observó que la extracción mediante fluidos supercríticos no es viable para la obtención de un extracto de cacao concentrado en catequinas. Esto ocurrió debido a que los granos de cacao tienen un alto contenido de grasa y no pueden llevarse al equipo desgrasados, ya que esto requiere la molienda de los mismos y llevar muestras con un tamaño de partícula reducido al equipo de fluidos supercríticos, podía ocasionar taponamientos en las líneas de flujo del mismo. Debido a esto los diferentes tratamientos evaluados solo funcionaron como un método de desgrasado, sin obtenerse en ninguno de los tiempos de muestreo ni en los dos selectores, algún lixiviado con los compuestos de interés. Sin embargo, se realizó una cuantificación a la muestra con el fin de establecer la concentración alcanzada de estos compuestos posterior a su proceso de "desgrasado" en el equipo, encontrándose valores de (-)-epicatequina entre 11-14mg/g c.a, inferiores a los del polvo de cacao inicial. Por lo tanto, al obtenerse valores inferiores de los compuestos de interés, y al ser una técnica con un costo elevado, sólo para lograr una eliminación de la grasa en el grano, se procedió a descartarla como una alternativa para la obtención del extracto de cacao.

La extracción asistida por ultrasonido fue la que presentó los menores valores de (-)-epicatequina (0.01-3.37mg/g) c.a, ya que, probablemente, las condiciones de temperatura y amplitud empleadas, pudieron causar alguna degradación de los compuestos de interés, al igual que el proceso posterior de eliminación del solvente y secado de la muestra. Por tal motivo este método tampoco fue tenido en cuenta para ser incorporado en el diseño experimental.

Las técnicas con las que se obtuvo un contenido de catequinas superior al del polvo de cacao sin fermentar, fueron Phytoclean™ (29.432mg/g), Reveleris® (41.5mg/g) y la extracción por solventes, siendo esta última la que arrojó el mayor contenido de (-)-epicatequina (97.45mg/g). Por este motivo se seleccionó esta técnica para la fase de obtención del extracto de cacao con alto contenido de compuestos bioactivos (Tabla 3.6)

La obtención de este alto contenido de (-)-epicatequina en el extracto hidroalcohólico es muy relevante, ya que incluso es superior a muchos de los valores encontrados en la literatura y algunos extractos comerciales como se presenta en la Tabla 3.7. Esto puede deberse también al contenido de estos compuestos presentes en los granos de cacao con los que se obtuvo el polvo de cacao sin fermentar.

Tabla 3.8 Contenido de flavanoles de cacao. Datos de la literatura y algunos extractos comerciales.

Referencia	Tipo de extracción	Matriz	(-)-Epicatequina (mg/g)	(+)-Catequinas (mg/g)
-	(A) Extracción hidroalcohólica con etanol al 70% (extracto liquido)	Granos de cacao secos sin fermentar, variedad CCN51	19.43	4.87
(Schinella et al., 2010a)	(B) Extracto anterior secado		62.19	25.06
-	(C) extracción con acetato de etilo		300.73	117.45
Datos no reportados	-	Granos de cacao sin fermentar	21.30	0.15
Datos no reportados	-	Granos de cacao sin fermentar	16.51	1.21
Datos no reportados	Extracción Hidroalcohólica	Granos de cacao sin fermentar	50.0	15.5
Datos no reportados	-	Biomasa de cacao obtenida por métodos biotecnológicos	5.18	1.02

CAPITULO IV:

COCOA EXTRACT WITH HIGH CONTENT OF FLAVAN 3-OLS, PROCYANIDINS AND METHYLXANTHINES

J Food Sci Technol (2021). https://doi.org/10.1007/s13197-021-05119-z

4. COCOA EXTRACT WITH HIGH CONTENT OF FLAVAN 3-OLS, PROCYANIDINS AND METHYLXANTHINES

ABSTRACT

The health benefits of cocoa depend on the flavan 3-ols, procyanidins, and methylxanthines, which decrease from the early stages of cocoa bean processing. The objective of this research was to obtain a cocoa extract high in these compounds with (-)-epicatechin as the primary reference. An evaluation of two pretreatments of cocoa beans with a control after harvesting was made: A (untreated/control), B (Frozen), and C (Polyphenol oxidase inhibition), all followed by dehydration at 45°C until obtaining a cocoa powder. In terms of (-)-epicatechin content, the best pretreatment was put on to a hydroalcoholic extraction. Flavan 3-ols, procyanidins, methylxanthines, and total polyphenols content (TPC), were quantified in the cocoa powders and the hydroalcoholic extract. The results showed that the control (A), significantly conserves the (-)-epicatechin (24.964±0.400mg/g) ca. 7 times more than conventionally sun-dried and fermented beans (3.742±1.977mg/g) ca. The hydroalcoholic extraction increased the (-)-epicatechin ca. 3 times more based on pretreatment A (84.738mg/g).

Keywords: flavan 3-ols, epicatechin, hydroalcoholic extract, cocoa.

Abbreviations: CNCH, Compañía Nacional de Chocolates S.A.S.; IMC-67 (*Iquitos Marañon Collection-67*) CCN-51 (*Colección Castro Naranjal-51*); ICS-95 (*Imperial college selection-95*); SSC-61 (*Selección Colombia Corpoica-61*); PPO, polyphenol oxidase; TPC, Total polyphenol content.

1. Introduction

Cocoa is a natural source of polyphenols, containing ca. 12-18% on a dry weight basis, which are into three groups: flavan 3-ols (catechins and epicatechins), anthocyanins and procyanidins; representing ca. 37%, 4%, and 58%, respectively, of the total antioxidants composition, reported in raw unfermented cocoa beans (Wang et al. 2019). Also, cocoa is rich in methylxanthines, containing ca. 3.2% on a dry weight basis, such as theobromine and caffeine, which represent ca. 3.7% and 0.2% (on a fat-free basis), respectively, and are considered as a central nervous system stimulant (Carrillo et al. 2014; Tan et al. 2019). It is important to consider that these contents of flavan 3-ols, methylxanthines, and procyanidins can be different between cocoa beans according to their origin, genetics, and culture conditions (Martin and Ramos 2016). Moreover, these substances are recognized for their beneficial effects and go beyond cardiovascular health, covering cognitive and other chronic diseases (Wang et al. 2019; Martin and Ramos 2021). Furthermore, different studies show that (-)-epicatechin is the first to be absorbed after oral consumption of cocoa; therefore, much of the functional and nutritional benefits are attributed to this flavanol (EFSA Panel on Dietetic Products et al. 2012; Estrada et al. 2020; Garcia et al. 2021).

In the food industry, there is an interest in searching for technologies that allow the extraction and concentration of such compounds, to incorporate them into different products to contribute to the health and well-being of consumers. In the case of the cocoa industry, this is important because the traditional cocoa processing leads to losses of these biologically active compounds (Mariatti et al., 2021). Cocoa flavanols have a reduction of 60% c.a. during fermentation and drying (Gil 2012). For this reason, strategies should be seeking to improve the nutritional quality of cocoa, which are focused on making different pretreatments to cocoa beans after harvesting, in order to preserve as much as possible these compounds (Schinella et al. 2010; Toro-Uribe et al. 2018). These kinds of treatments are combined with extraction technologies (e.g., solid-liquid) using solvents such as water, ethanol, acetone, isopropanol, or mixtures of them, the most common

solvents used (Steffan et al. 2012; Soares et al., 2020). Some regulations allow the use of these solvents. However, a minimum trace content should be in the final product. An example of this is reported by the U.S. Food and Drug Administration in the Code of Federal Regulations Title 21 about the secondary direct food additives permitted in food for human consumption (FDA 2020).

In a regulatory context, the minimum quantities required for a nutritional declaration about the antioxidant content in food are not still established in most international laws. However, in 2012, EFSA approved the request of Barry Callebaut, who, with scientific evidence, showed that consuming 200mg of cocoa flavanols per day helps cardiovascular health (EFSA Panel on Dietetic Products et al. 2012; Tanghe et al. 2021). With this in mind, this study aims to develop a solution that allows obtaining a functional cocoa extract high in bioactive compounds as (-)-epicatechin by solvent extraction for application in functional foods.

2. Materials and methods

2.1. Chemicals and reagents

All solvents (methanol, glacial acetic acid, ethanol, isopropanol, HCl, acetone) were analytical grade and supplied by Merk (Darmstadt, Germany). The water deionized in a Milli-Q water purification system (Millipore, Bedford, USA). Analytical standards of (+)-catechin, (-)-epicatechin, caffeine, theobromine, procyanidin B2, and 4-dimethylaminocinnamaldehyde (DMAC) were purchased at Sigma Aldrich (Saint Louis, USA). Gallic acid from Merk (Hohenbrunn, Germany), n-hexane, Folin-Ciocalteu were supplied by Merk (Darmstadt, Germany). Finally, Sodium Carbonate Na-2CO3 were supplied by Química Básica S.A. (Medellin, Colombia).

2.2. Pretreatments of cocoa beans

The cocoa samples were taken randomly from the experimental farm of Compañía Nacional de Chocolates S.A.S (CNCH), located in Antioquia, Co-

lombia. The samples were undried and unfermented cocoa beans mixtures, with at least 70% of the IMC-67 (*Iquitos Marañon Collection-67)* variety and 30% of a mixture of CCN-51, ICS-95 and SSC-61. These cocoas were collected and undergone three pretreatments: A (untreated/control); B (Frozen at -20°C [Gil, 2012]) using a freezer 3552A (*Thermo scientific, Massachusetts, USA*); C (Polyphenol oxidase [PPO] inhibition) this process consisted of submerging cocoa beans in water at 95°C for 5 minutes and subsequently, a thermal shock was made, with water at 25°C, as previously described (Schinella et al. 2010; Toro-Uribe et al. 2018).

After these pretreatments, cocoa beans were dehydrated under controlled conditions in an FBA_GFD1850 dehydrator (*Gourmia, Brooklyn, NY*) at 45°C+/- 1 to reach a humidity of 7-8% approximately (Tardzenyuy et al., 2020). Then, the cocoa beans were winnowing in a cocoa winnower 240-1-150 (*Capco test equipment, Ipswich, UK*), defatted in a screw press (*CNCH, Medellín, Colombia*), and milling in a processor (*Capco test equipment, Ipswich, UK*). The cocoa powders were stored at -20°C until the hydroalcoholic extraction (Figure 1).

2.3. Hydroalcoholic extraction of cocoa powder

A hydroalcoholic extraction was carried out on the cocoa powder that presented the best result in terms of (-)-epicatechin content. The cocoa powder extraction was carried out with a 70% v/v ethanol solution, in a ratio of 1:10 (cocoa: ethanol) at 60°C for 60min (Pasamar et al. 2006). Subsequently, the solvent was removed using a Buchi R-100 rotary evaporator® with a Buchi B-100 bath (50°C), Buchi V-100vacuum pump (170-40mbar which was decreasing as the concentration of EtOH was reducing). Buchi F-100 Chiller (10°C) (Buchi, Mexico City, Mexico), the rotary evaporation time for 2L of the liquid extract was 8h. After that, samples were freeze-dried in a Christ Alpha 1-2 LD Plus freeze dryer (Martin Christ, Osterode am Harz, Germany); in drying phase, the condensation temperature was -50°C with 0.040mbar vacuum for 5h, and in the final drying phase the condensation temperature was -76°C with 0.0010mbar vacuum during 3h. The cocoa extracts were stored in the dark at room temperature until

the analyses.

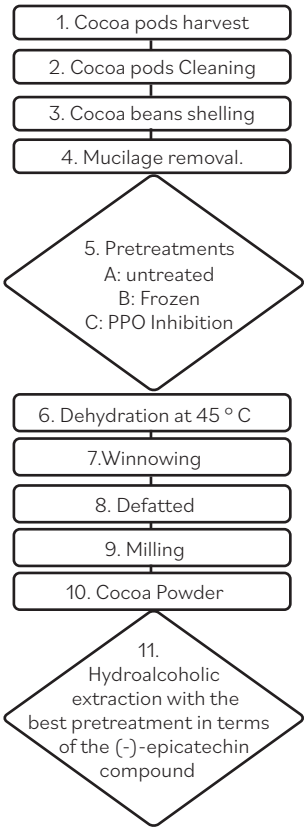


Figure 1. Pretreatment of cocoa beans and obtaining of cocoa powders with a high content of flavan 3-ols, procyanidins, and methylxanthines.

2.4. HPLC analysis of flavan 3-ols and methylxanthines.

An Agilent 1260 high-resolution liquid chromatography (*Agilent Technologies, CA, USA*) with automatic autosampler, fluorescence, and diode array detectors (FLD and DAD) was used to HPLC analyzes. The signals were processed using the Agilent OpenLAB CDS software, ChemStation Edition. Samples were prepared following the methodology proposed by T-sanova-Savova. (2005). The extraction of the cocoa powders samples

and hydroalcoholic extract (0.5g) was done with 10ml of a 60:40 pH 9.0 isopropanol/water solution during 60min in an Elma E30H ultrasonic bath (*Elma Schmidbauer GmbH, Singen, Germany*), at room temperature. Subsequently, these samples were stirred in a vortex for 1min and stored at -20°C in a freezer for 60min. They were centrifuged at 4000rpm for 8min, in a Rotofix 32A centrifuge *(Andreas Hettich GmbH & Co.KG, Schwerin, Germany*). The supernatant (1ml) was filtered by a 0.45µm Nylon membrane filter Millipore (*Membrane Solutions, Auburn, EEUU*), an aliquot of this filtrate was mixed with the mobile phase (0.1% acetic acid), with a dilution factor (DF) between 4 to 10 depending on the color solution. This solution was performed in a chromatography vial; next it was injected into the HPLC/DAD/FLD. A Zorbax bonus C18 HPLC Column was used (5µm particle size, L×ID 25cm×4.6mm), the detection was at 280nm. The intensity of the acquired signals processed through the OpenLab CDS Chemstation Edition software. An external standard was applied for flavanols and methylxanthines quantification (a mixture of the four compounds (+)-catechin, (-)-epicatechin, caffeine, and theobromine). The analyses were performed in triplicate for each sample and reported based on dry cocoa solids in mg/g.

2.5. Procyanidin analysis

In the samples of cocoa powders and hydroalcoholic extract were quantified the procyanidin content, following the methodology proposed by Payne et al. (2010). All samples were defatted with hexane to achieve a fat percent less than 5%. The DMAC solution was prepared with 0.030g of this reagent in 3ml HCl prepared in 27mL of methanol. 0.500g of each sample was taken and added in an AWAA solution (Acetone, water, and acetic acid) (70+29.5+0.5, v/v), with vortex stirring. A Centrifugation was done at 3000rpm for 5min, the solution was filtered using a 0.45µm acrodisk. In the microwells of the reading plate were put 240µl of DMAC solution and 40µl of the sample. The used standard was the procyanidin B2, corresponding to the main dimers of flavanols in cocoa (Kothe et al. 2013). Finally, the measurement was with an absorbance of 650nm in an ELx800 reader and the Ridasoft® software (*Biotek, Winooski, USA*).

2.6. Total polyphenol analysis (TPC)

The total polyphenol content was determined by UV-VIS spectroscopy using the micro-scale method of Folin-Ciocalteu (*Merk, Darmstadt, Germany*), described by Rover and Brown (2013). 300µl of extract diluted (1/100), 150µL Folin-Ciocalteu reagent, 2250µL of distilled water and 300µL of Na2CO3 at 20% were mixed and, after 60min incubation at room temperature, the absorbance of the color was at 760nm against a blank sample on a GENESYS 10S UV-Vis spectrophotometer (*Thermo Scientific, Waltham, Massachusetts*). The TPC was expressed as equivalent milligrams of gallic acid per gram of dry sample material (mgGAE/g). All measurements were performed in triplicate and reported based on dry cocoa solids.

2.7. Statistical analysis

A completely randomized block model was performed, where the main factor was the pretreatment, which had three levels: A (untreated/control), B (Frozen at -20°C), and C (PPO inhibition). The time was a block with two levels (t1 and t2), which correspond to the cocoa harvesting period (the difference between the two blocks was eight months) to eliminate the experimental noise associated with seasonal differences in the harvest. The assignment of the treatments to the experimental units brought in each event was random. An ANOVA analysis of variance and a Tukey test was done to establish significant differences between treatments. The statistical treatment of the data was using the R statistical language (R Development Core Team, 2019).

3. RESULTS

3.1. Flavan 3-ols

The average values of (-)-epicatechin were approximately 24.0mg/g for A and B treatments without statistically significant differences ($p<0.05$)

between them. In contrast, an average value of 17.40±0.903mg/g was determined for pretreatment C, lower than the treatments mentioned above, and with a statistical difference ($p>0.05$) (Figure 2a-b, Table 1). These results have a similar behavior from the reported data by Kim and Kenney (1984), which found higher values of (-)-epicatechin in frozen beans (35-43mg/g) and lower values in the treatment in which PPO was inhibited (21mg/g). The differences between the (-)-epicatechin values reported by these authors and the values reported in this research, could be because the cocoa varieties evaluated in both studies were different (Trinidad-Jamaican hybrids, Catongo, Forastero, and Colombian Trinitario cocoa beans, respectively). Additionally, the process of PPO inhibition, because in their study, cocoa beans were heating at 105°C into a microwave. This work used a thermal shock with water at 95°C and 25°C.

Different studies have reported the PPO inhibition as one of the best treatments for the conservation of cocoa flavanols (Schinella et al. 2010), however, in this research, C was not the best pretreatment to the conservation of flavan 3-ols, especially the (-)-epicatechin. The lower concentration of (-)-epicatechin by enzymatical inhibition may occur by the exposure conditions of cocoa beans at temperatures above 60°C, which can deteriorate it (Alañón et al. 2016). Hurst et al. (2011) presented comparative values of (-)-epicatechin at different stages of the industrialization process of cocoa beans. The highest contents were in unfermented raw ripe beans (13.35mg/g) and the lowest in beans subjected to roasting (0.66mg/g). Their results showed that the highest total polyphenol content was without high temperatures. A hypothesis about the results of A and B (higher (-)-epicatechin content than C), could be associate with dehydration at 45°C. It is necessary to highlight that the PPO was not inhibited in these pretreatments, but this behavior is possible because the PPO is sensitive to drying; this does not mean that the enzyme loses its reaction power but this process is slower. Wollgast and Anklam (2000) mentioned that the PPO activity in dried beans is only 2% (the case of pretreatment A). For B, where the cocoa beans were frozen, the low temperatures reduce or inactivate the enzymatic activity, which delays the effect of degradation of bioactive compounds. This result allows mentioning that the traditional PPO inhibition (using temperatures of 95°C) is not the best way to preser-

ve the (-)-epicatechin content. On the other hand, this is possible using dehydration under controlled conditions or freezing of cocoa beans after harvesting.

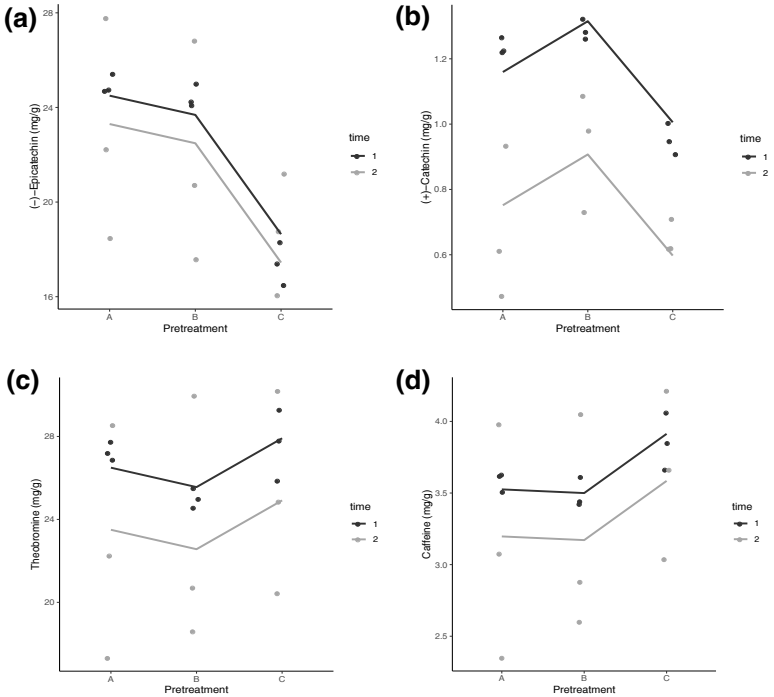


Figure 2. Flavan 3-ols (a. epicatechins and b. catechins) and methylxanthines (c. theobromine and d. caffeine) in cocoa powers (1 and 2 correspond to cocoa harvesting time).

Table 1. Content of flavan 3-ols and methylxanthines in cocoa powders in t1 and t2.

	Flavan 3-ols				Methylxanthines			
Time	t1*		t2*		t1*		t2*	
Pretreatment	(-) -epicatechin (mg/g)	(+)-catechin (mg/g)	(-)-epicatechin (mg/g)	(+)-catechin (mg/g)	Theobromine (mg/g)	Caffeine(mg/g)	Theobromine (mg/g)	Caffeine(mg/g)
A	24.965±0.400 [a]	1.238±0.025 [ac]	22.832±4.678 [a]	0.674±0.236 [ac]	27.279±0.436 [abc]	3.586±0.067 [abc]	22.712±5.625 [abc]	3.1361±0.816 [abc]
B	24.456±0.489 [ab]	1.289±0.031 [ab]	21.715±4.701 [ab]	0.933±0.183 [ab]	25.023±0.471 [abc]	3.493±0.104 [abc]	23.097±6.048 [abc]	3.1778±0.769 [abc]
C	17.403±0.904 [c]	0.953±0.048 [c]	18.681±2.575 [c]	0.649±0.052 [c]	27.658±1.714 [abc]	3.859±0.199 [abc]	23.168±4.886 [abc]	3.6388±0.588 [abc]

*The values correspond to the mean and the standard deviation of the three analyses. Mean values with different letters in the same column show statistically significant differences ($p < 0.05$). *t1 and t2 correspond to cocoa harvesting time.*

Table 2. Content of procyanidins and total polyphenol content (TPC) cocoa powders in t1 and t2.

	Procyanidins		TPC	
Time / Pretreatment	t1*	t2*	t1*	t2*
	Procyanidin B2 (mg/g)	Procyanidin B2 (mg/g)	TPC (mgGAE/g)	TPC (mgGAE/g)
A	167.429 ± 31.080[abc]	137.583 ± 9.703 [abc]	153.0678 ± 2.034 [abc]	113.6442 ± 27.883[abc]
B	109.750 ± 16.181[abc]	141.870 ± 25.472[abc]	149.1456 ± 47.499[abc]	104.1996 ± 28.945[abc]
C	157.929 ± 54.522[abc]	158.745 ± 11.169[abc]	202.1441 ± 6.491[abc]	103.6454 ± 10.105[abc]

*The values correspond to the mean and the standard deviation of the three analyses. Mean values with different letters in the same column show statistically significant differences ($p < 0.05$). *t1 and t2 correspond to cocoa harvesting time*

There were no differences between t1 and t2 for (-)-epicatechin ($p>0.05$), contrary to (+)-catechins content in which statistically significant differences were found ($p<0.05$), this may occur due to the variations that exist in cocoa beans baches between the analyzed times (eight months) since the climatic and stationary harvest conditions occurred at different times. However, these variabilities are acceptable in experiments of this type because during cocoa cultivation and harvest, many environmental variables are difficult to control (Carrillo et al. 2014). In that way, Quelal-Vásconez et al. (2020) quantified the flavanols and methylxanthines content of cocoa powders, highlighting the natural variability of this type of unprocessed samples due to their origins, which influences the dispersion of the values of these compounds.

In previous studies, Compañía Nacional de Chocolates S.A.S. performed an analysis of (-)-epicatechin content in fermented and dried Colombian cocoa; the results showed that the samples evaluated did not exceed 8mg/g of (-)-epicatechin (unpublished results). Nazaruddin et al. (2006) reported values of (-)-epicatechin in fermented and dried Malaysian cocoa beans of 4.61mg/g. Samaniego et al. (2020) showed values of (-)-epicatechin content in fermented and dried Ecuadorian cacao beans, between 3.45-13.16mg/g. This behavior occurs because of about 60% of the polyphenol content decrease in cocoa pre-industrialization processes (Gil 2012). Montoya et al. (2021) reported values of (-)-epicatechin of different unfermented and dried cocoa beans around 10.26-16.71mg/g. The comparison of the average values presented in these studies and the results of A and B (24.0mg/g) and C (17mg/g), allows mentioning that these processes (untreated and freezing respectively, with dehydration at 45°C in both), could be a more appropriate way to preserve (-)-epicatechin content. Also, it is worth mentioning that it is necessary to control the drying process because that affects the content of these compounds. Additionally, it is relevant to highlight that Colombian cocoa compared to other cocoas, seems to have great potential to obtain cocoa extracts with a high content of flavan 3-ols.

According to the results obtained, it is possible to conclude that the better pretreatments to preserve the (-)-epicatechin content in cocoa beans are A and B. However, freezing can become a treatment that generates higher processing costs. Therefore, there were no statistical differences between both pretreatments; thus, it is possible to select treatment A as better scalability. Subsequently, the hydroalcoholic extract was obtained, applying this extraction to the cocoa powder of pretreatment A, as mentioned before. For the cocoa extract, the average values of (+)-catechins and (-)-epicatechin were 3.699±1.297mg/g and 84.738±4.805mg/g, respectively. These results represent an increase of approximately 30% of (-)-epicatechin compared to the obtained in the cocoa powder A, which is relevant to future applications in the production of functional foods. This concentration occurs due to the high polarity of the flavanols; therefore, they are more soluble in solvents such as ethanol, methanol, and water (Okiyama et al. 2018).

Other emerging technologies were previously explored by CNCH for the extraction and concentration of these compounds, as presented in Table 3. However, the best (-)-epicatechin content was achieved from hydroalcoholic extraction, as discussed previously. Technologies such as supercritical fluids in cocoa are an alternative for defatting the bean, which is expensive, but the (-)-epicatechin obtained was 14.620mg/g. Moreover, technologies such as extraction by hydrothermal treatments also were tested (Mazza Innovations LTD 2014), but the (-)-epicatechin content was 29,430mg/g. On the other hand, the (-)-epicatechin value reported in this study in the hydroalcoholic extract (84,738±4.805mg/g) is higher than the reported in a commercial cocoa extract (50mg/g), this may be due to the contribution of the pretreatments A and B to preserve the content of flavan 3-ols. Additionally, as was stated above, Colombian cocoa has a high content of these compounds compared to other cocoa beans from other regions (Urbanska and Kowalska 2019).

Table 3. Obtaining cocoa extracts from unfermented beans using different technologies (unpublished Results).

Technology/Commercial Extract	(-)-epicatechin(mg/g)	TPC(mgGAE/g)
Unfermented cocoa powder	24.960	153.067
Extraction by supercritical fluids	14.620	-
Chromatography extraction for compound purification	41.500	-
Extraction by hydrothermal treatments	29.430	188.400
Extract obtained by biotechnological methods.	5.187	15.172
Ultrasound-Assisted Extraction	3.371	-
Commercial extract	50.000	197.220

3.2. Methylxanthines

The theobromine values in t1 were between 25-27mg/g (B, A, and C), and in the caffeine was between 3.5-3.8mg/g (A, B, and C). In t2 were between 22-23mg/g (A, B, and C) and 3.1-3.6mg/g (A and B, C), theobromine and caffeine, respectively (Figure 2c-d, Table 1). There were no statistically significant differences among pretreatments and between harvest time ($p>0.05$). The theobromine was higher than the catechines; this is common in cocoas and does not correspond to the effect of any treatments performed in this study. Carrillo et al. (2014) also reported that in different Colombian cocoas, finding that methylxanthines are present in a higher proportion than catechines, being the theobromine the predominant compound. Brunetto et al. (2007) described a similar result. In the case of the hydroalcoholic extract, theobromine and caffeine values were 83.418±5.354mg/g and 12.681±0.437mg/g, respectively, which represented an increase of 25% approximately.

On the other hand, cocoa has classification into three genetic varieties: Forastero, Trinitario, and Criollo, and it is possible to validate this classification through the theobromine/caffeine ratio (Carrillo et al. 2014). The theobromine/caffeine ratio of the cocoa mixtures used in these experiments showed average values between 7.22±0.123; it is in the range of Tri-

nitario cocoa variety (5-10) with average amounts of xanthines.

3.3. Procyanidins content

According to the statistical analysis, it was possible to establish that there are no differences between A, B, and C pretreatments or t1 and t2 ($p>0.05$), indicating that the procyanidin content is not affected by any of the pretreatments made to cocoa beans. Table 2 and Figure 3a present the results obtained for the content of procyanidins of the pretreated cocoa beans. The results had a high variation, Payne et al. (2010) mentioned that this variability might occur due to the colorimetric method used for the measurement of this variable.

Gültekin-Ozgüven et al. (2016) mention that cocoa and its derivatives contain a variable content of procyanidin B2 between 0.144mg/g to 0.457mg/g. CNCH sampled different post-harvested cocoa varieties in a conventional way (fermented and dried), where procyanidin values were between 10.9-43.1mg/g (unpublished results). Kramer et al. (2019) also reported procyanidin B2 value for a cocoa extract of 21.39mg/g. That allows mentioning that the pretreatments evaluated in this study contribute to the conservation of the phenolic compounds present in the cocoa bean. In the case of the hydroalcoholic extract, the procyanidin value was of 823.6±26.715mg/g. Compared to pretreatment A, it was possible an increase in the procyanidin content of 20%. That is relevant to applying this extract in the development of functional food; since consuming cocoa-based products with high procyanidin content can contribute to health benefits.

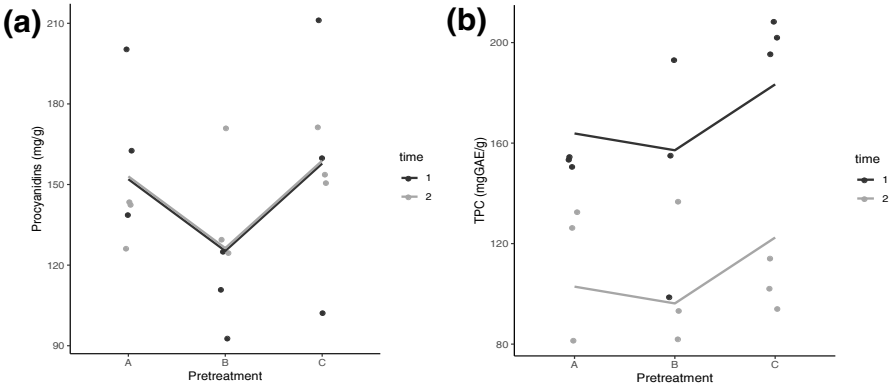


Figure 3. a. Procyanidins and b. TPC in cocoa powders (1 and 2 correspond to cocoa harvesting time).

3.4. Total polyphenol content (TPC)

The average values of total phenols were between 103-202mgGAE/g, and there were no statistically significant differences ($p>0.05$) between the treatments evaluated (Figure 3b, Table 2). On the other hand, significant differences occurred when the two evaluated times were analyzed ($p<0.05$) since t2 had lower values of TPC (t1: 153-202mg/g; t2:103-113mg/g), which is due to the variability of cocoa. Additionally, it is possible to observe that the TPC is slightly higher in treatment C in t1 (202.144± 6.49mg/g). Gil (2012) reported that the PPO has a direct relationship with the reduction of the (+)-catechin and (-)-epicatechin content. However, not all the antioxidants present in cocoa are a substrate of the PPO, that is the reason because the TPC is not significantly affected. Statistical analysis shows that there are no differences between the three treatments. The Colombian cocoa has a TPC average of 37.81mgGAE/g (fermented and dried cocoa beans), reported by Urbanska and Kowalska (2019). That allows concluding that it is possible to conserve approximately 90% of the TPC present in unprocessed cocoa beans through the pretreatments evaluated in this research. In the cocoa extract, the average TPC was 348,967±6,653mgGAE/g, which doubles the content of compounds present in pre-treated

cocoa beans (A: 153.067± 2.034 mg/g) and exceeding those reported by some commercial extracts and obtained by other extraction technologies (Table 3).

Todorovic et al. (2015) conducted a study on the content of methylxanthines and polyphenols in cocoa products. The authors found that the average TPC was 35.35mgGAE/g for powdered chocolates and dark chocolates between 7.21–12.65mgGAE/g; these contents are below the regulatory limits to make a nutritional claim. Different clinical studies have reported that daily consumption above 100mg/day of these compounds is required to obtain such benefits (EFSA Panel on Dietetic Products et al. 2012). That demonstrates the need for enriching a cocoa-based product since the TPCs at the end of the transformation processes are below the recommended daily doses required.

CONCLUSIONS

Antioxidant-rich cocoa powder was obtained using unfermented beans and immediately dehydrated at 45°C contributing to cocoa polyphenols preservation.

The extraction of cocoa antioxidants using hydroalcoholic treatments significantly concentrates them, even obtaining better yields comparing to some emerging technologies such as supercritical fluids, hydrothermal treatments, and ultrasound.

The polyphenol oxidase inhibition at 95°C significantly affects the content of epicatechin.

In future works are recommended, scaling up and optimization of control (A) and hydroalcoholic extraction. Additionally, it is relevant to evaluate some aspects, such as the antioxidant capacity and functionality of the cocoa extract obtained and its application in a cocoa-based product, to establish its applicability in functional products.

CAPITULO V:

BIOAVAILABILITY AND RADICAL SCAVENGING POWER OF PHENOLIC COMPOUNDS OF COCOA AND COFFEE MIXTURES

Food Sci Technol Int (Accepted)
https://doi.org/10.1177/10820132211023258

5. BIOAVAILABILITY AND RADICAL SCAVENGING POWER OF PHENOLIC COMPOUNDS OF COCOA AND COFFEE MIXTURES

ABSTRAC

Cocoa and coffee are natural sources of phenolic compounds, which are degraded during beans processing of both. For this reason, there is an interest in obtaining extracts of these bio compounds. The aim of this study was uncovering the radical scavenging activity (AC) of mixtures of cocoa and coffee extracts, and the bioavailability of their phenolic compounds, resorting to in vitro models: DPPH and ORAC antioxidant methods, and the characterization of the trans-epithelial transport of cocoa and coffee phenolics through Caco-2 cells monolayer model of the intestinal barrier. The cocoa displayed a higher AC than the coffee regarding both DPPH and ORAC assays, while the mixtures increased in parallel with the percentage of cocoa in the blends. The combination index was calculated to set up the type of interaction in the cocoa-coffee mixtures, obtaining that the mixture 25:75 was moderately antagonistic, 50:50 nearly additive, and 75:25 slightly synergistic. The absorption efficiency of the cocoa phenolic compounds was between 87.9-97.4%, in the coffee compounds was 100%. The mixtures varied according to the proportion of cocoa and coffee. The results obtained allowed concluding that the phenolic compounds present in cocoa and coffee, respectively, are featured by high bioavailability and a valuable antioxidant capacity, while no pattern was found in the mixtures concerning the real benefit of using them combined.

Keywords: Flavanols; Catechins; Chlorogenic acids; Biological activity; Radical scavenging capacity.

Abbreviations: AC, antioxidant capacity; APPH, 2,2´-Azobis(2-methyl-propionamidine) dihydrochloride, CI, combination index; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; IC50, half maximal inhibitory concentration; LDL, low-density lipoproteins; NEAAs, non-essential amino acids; Trolox, (±)-6-Hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid; TEER, trans-epithelial electrical resistance.

1. Introduction

Cocoa is a natural source of polyphenols, containing 12-18% on a dry weight basis, which are classified into three groups: flavan 3-ols (catechins and epicatechins), anthocyanins, and procyanidins; representing 37%, 4%, and 58% respectively, of the total antioxidant composition reported in raw unfermented cocoa beans (Y. Hu et al., 2016; Wang, Feltham, Suh, & Jones, 2019). Alternatively, chlorogenic acids are the most abundant phenolic compounds present in coffee, including several quinic acid esters, representing from 7 to 10% of its dry matter. The most relevant chlorogenic acid is 5-O-caffeoylquinic, an ester of caffeic acid and quinic acid (Gil, 2012; Budryn et al., 2016). In the food industry, there is an interest in searching for technologies that allow the extraction and concentration of such compounds, to incorporate them into different products to contribute to the health of consumers. In the case of the cocoa and coffee industry, this is important because the traditional processing of them leads to losses of these biologically active compounds (Todorovic et al. 2015).

The term "bioactive compound" is referred to nutrients and non-nutrient present in foods and responsible, after intestinal absorption, for an array of biological activities that could contribute to maintaining human health and well-being (Fernandes, Nave, Gonçalves, Freitas, & Mateus, 2012). In this respect, to the present date, several studies have allowed identifying the beneficial effects of bioactive compounds supplied by cocoa- and coffee-based products on health, being the bioactivities identified so far, mainly associated with their radical scavenging capacity. Hence, nowadays, it has been reported the benefits of cocoa on cardiovascular health, diabetes, and blood pressure, as well as on cognitive and brain functions (Balzer et al., 2008; Pokorney & Piccini, 2017; Sokolov et al., 2013). On the other hand, the health benefits of the bioactive compounds present in coffee have been associated with their preventive role against inflammatory and mutagenic events, as well as cardiovascular and rheumatic diseases (Budryn et al., 2016), while other studies have shown that antioxidant compounds present in coffee are competent to inhibit lipid peroxidation on low-density lipoproteins (LDL)-cholesterol, thus playing a role regar-

ding the modulation of oxidative stress and reducing the risk of atherosclerosis (Agudelo-ochoa et al., 2016; Lara-Guzmán et al., 2016).

Despite the currently generated knowledge on these plant-based food matrices, there are not many studies focused on combinations of cocoa and coffee as sources of bioactive compounds, including the exploration of interactions between bioactive compounds supplied by each separate matrix in terms of bioavailability and bioactivity. In this aspect, Çelik and Gökmen, (2018) studied possible molecular interactions and their effect on the antioxidant capacity of flavanols present in cocoa infusions: (+)-catechin and (-)-epicatechin. This research allowed describing, in some specific mixtures, synergistic, additive, and antagonistic behaviors. Other authors (Ribeiro et al., 2014) have evaluated cocoa, coffee silverskin, and green coffee minimally processed mixtures, describing that the obtained blend (94% roasted coffee powder *(Coffea canephora cv. Robusta and Coffea arabica, 70/30, w/w*), 3% cocoa powder, 2% coffee silverskin, and 1% golden coffee (green coffee minimally processed), was featured by the highest content of bioactive compounds and radical scavenging power.

Based on these premises, the present research aims to understand the interaction of phenolic compounds in cocoa (flavanols) and coffee extracts (chlorogenic acid) and diverse mixtures (0-100, 25:75, 50:50, 75:25, 100:0 cocoa:coffee) to establish synergies or antagonism and understand their bioavailability through the transepithelial transport by Caco-2 cells.

2. Materials and methods

2.1. Chemicals and reagents

All solvents (methanol, glacial acetic acid, ethanol, isopropanol, HCl, acetone) were analytical grade and supplied by Merk (*Merk, Darmstadt, Germany*). The water deionized in a Milli-Q water purification system (*Millipore, Bedford, USA*). The standards of (+)-catechin, (-)-epicatechin, chlorogenic acid, (±)-6-Hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-Azobis(2-me-

thyl-propionamidine) dihydrochloride (APPH), non-essential amino acids (NEAAs), fetal bovine serum (FBS), Eagle's minimum essential medium (EMEM), and L-glutamine were purchased to Sigma Aldrich (*Steinheim, Germany*). Methanol was from Carlo Erba, France and ethanol, sodium di-Hydrogen phosphate anhydrous (Na_2HPO_4), and di-Sodium Hydrogen phosphate anhydrous (NaH_2PO_4) from Panreac ApplichemGmbH (*Darmstadt, Germany)*. Fluorescein was purchased to Fluka analytical (*England, United Kingdom*).

2.2. Plant material and preparation of coffee-cocoa mixtures

The cocoa powder extract was supplied by the Compañía Nacional de Chocolates S.A.S.-CNCH (Rionegro, Colombia) and the coffee powder extract was from Industria Colombiana de Café S.A.S (Medellín, Colombia). The blends of coffee and cocoa were prepared to mix them at the proportions referred to in Table 1. Mixed samples (10mg) were diluted in 10mL of deionized water, stirred by vortex, and centrifuged at 10.500rpm for 5min (*Sigma 1-13 centrifuge, B. Braun Biotech International, Osterode, Germany*), at room temperature. The supernatants were filtered through a 0.45mm PVDF membrane Millex HV13 (*Millipore Sigma, Bedford, U.S.A.*) and appropriately diluted according to each further determination's sensitivity.

Table 1. Cocoa and coffee mixtures.

Mixture	Cocoa (%)	Coffee (%)
M1	0	100
M2	25	75
M3	50	50
M4	75	25
M5	100	0

2.3. HPLC analysis of flavanols of cocoa

The chromatographic analysis was developed on an Agilent 1260 high-re-

solution liquid chromatography (*Agilent Technologies, CA, USA*) with automatic autosampler, fluorescence, and diode array detectors (FLD and DAD). The signals were processed using the Agilent OpenLAB CDS software, ChemStation Edition. The samples were prepared following the methodology proposed by Tsanova-Savova et al., (2005). The extraction of the cocoa samples (0.5g) was done by adding 10 mL of isopropanol/deionized water (60:40, v/v), pH 9.0 for 60min in an Elma E30H ultrasonic bath *(Elma Schmidbauer GmbH, Singen, Germany*), at room temperature. Afterward, these samples were stirred in a vortex for 1 min and stored at -20°C for 60min. The samples were centrifuged at 4000 rpm for 8min in al Rotofix 32A centrifuge (*Andreas Hettich GmbH & Co.KG, Schwerin, Germany*). The supernatant (1mL) was collected and filtered through a 0.45µm Nylon membrane filter (Millipore, Membrane Solutions, Auburn, USA) and performed in a chromatography vial to be analyzed by HPLC/DAD/FLD. The chromatographic separation was in a Zorbax bonus C18 HPLC Column (250 x 4.6 mm, 5µm particle size). The chromatograms were recorded at 280nm and analyzed using the OpenLab CDS Chemstation Edition software. A pool of authentic external standards of (+)-catechin, (-)-epicatechin, caffeine, and theobromine was applied to quantify the compounds. The analysis of the diverse mixtures was performed on three replicates and reported as micrograms per milliliter (µg/mL).

2.4. HPLC analysis of chlorogenic acids of coffee

The evaluation of chlorogenic acids content in the coffee extract was carried out by reverse-phase high-resolution liquid chromatography (RP-HPLC), coupled to a UV-Vis or DAD detector, following the methodology described by the Deutsche Institut für Normung in the DIN 10767 (1992). Accordingly, for the analysis, it was required a methanolic extraction to guarantee the precipitation of proteins. The samples were then centrifuged at 4000rpm for 10min in a Rotofix 32A centrifuge (*Hettich, Tuttlingen, Germany*) and filtered through a 0.45µm Nylon membrane filter (*Millipore, Membrane Solutions, Auburn, USA*). Afterward, the samples were injected into the HPLC system, including a G1311C quaternary pump and a G1315D diode array detector. The chromatographic separation was on a

ZORBAX Eclipse XDB C18 column (150 x 4.6mm, 3.5µm particle size) (*Agilent, Santa Clara, USA*). The detection was at 325 nm. In this chromatographic system, nine chlorogenic acids were separated: 5-caffeoylquinic acid (5-CQA), 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), caffeic acid, ferulic acid, isopherulic acid, 3,4-dicaffeoylquinic acid (3,4 di-CQA), 4,5-dicaffeoylquinic acid (4,5 di-CQA) and 3,5-dicaffeoylquinic acid (3,5 di-CQA). The total content of chlorogenics was reported as the sum of each concentration quantified to authentic external standards and expressed as ug/ml of chlorogenic acids.

2.5. DPPH and ORAC-based antioxidant capacity

2.5.1. DPPH assay

For the assessment of the samples on their radical scavenging capacity, DPPH assays were developed using 96-well microplates (*Nunc, Roskilde, Denmark*), following the methodology previously described by Migues et al., (2018). The antioxidant activity was evaluated measuring changes of absorbance at 515nm, after 35min of reaction with the radical DPPH$^{\cdot}$ (2µL of the sample diluted in H2O (1:2, v/v) + 250 µL of DPPH$^{\cdot}$ solution up to absorbance ≈1) using and an Infinite® M200 microplate reader (*Tecan, Grödig, Austria*). The standard curve exhibited linearity between 10 and 200 mM Trolox. The results were expressed as millimolar of Trolox equivalents per gram of dry weight (mM TE/g dw) by applying the indicators stated in Table 2.

Table 2. Equations for the calculation of the DPPH-based antioxidant capacity.

Indicator[Z]	Equation		Ref.
%I	'%I = (ABSmixture / ABSDPPH) x 100'	(1)	(Jiménez, Sánchez, Martínez, 2012)
IC_{50}	'IC_{50} = ((DPPH (Mm TE/g dw) x 50) / %I)'	(2)	(Sabahannur & Alimuddin, 2018)
CI	'CI = ((IC_{50} of the mixture x ration cocoa in the mixture) / IC_{50}-cocoa) + ((IC_{50} of the mixture x ration coffee in the mixture) / IC_{50}-coffee) '	(3)	(Rahadian, Muhammad, Prasetiangga, Walle, & Dewettinck, 2017)

[Z] *%I, percentage of inhibition of the DPPH radical; IC50: half maximal inhibitory concentration that indicates the amount of the bioactive compound that can inhibit 50% of the DPPH radical; CI: the combination index to establish the type of interaction between the mixtures (CI=1, additive interaction; CI>1, antagonism; CI<1, synergism, according to Enko & Gliszczyńska-Świgło, 2015.*

2.5.2. ORAC assay

The assessment of samples on the ORAC-based antioxidant activity was carried out according to the methodology described by Paulsen et al., (2018), using 96-well microplates. Briefly, peroxyl radical was prepared using 2,2′-azobis (2-amidino-propane) dihydrochloride for each run, and fluorescein was used as a substrate of the reaction. For this method, the samples were diluted in water (1:50, v/v). Fluorescence conditions were as follows: excitation at 485 nm and emission at 520 nm, both read using an Infinite®M200 microplate reader (*Tecan, Grödig, Austria*). The standard curve was linear between 10 and 200 mM Trolox. The results obtained were expressed as mM TE/g dw.

2.6. Assessment of main phenolics transport with Caco-2 model

The human colorectal adenocarcinoma cell line (Caco-2, ATCC®HTB37) was obtained from the American Cell Culture Collection (*ATCC, Rockville, MD, USA*). These cells were grown at 37 °C in a humidified atmosphere containing 5% CO2, in Eagle's Essential Minimum Medium (EMEM), supplemented with 10% fetal bovine serum, 1% (v/v) NEAAs, and 2 Mm L-glutamine. The cells used in this study corresponded to the pass number 15.

To assess phenolic compounds' bioavailability, present in the cocoa, coffee, and cocoa-coffee mixtures, an in vitro model of intestinal barrier developed using Caco-2 cells was applied. For this purpose, Caco-2 cells were seeded at a density of 3x105 cells/well in 300µL of culture medium on advanced inspection systems (HTS)-Transwell®-12 well, semi-permeable (0.4µm pore diameter polyester membranes) (apical chamber) (*Costar, Corning, Birmingham, UK*). In the lower compartment (basolateral compartment), 600µL of culture medium was added.

Once confluent, seeded Caco-2 cells were allowed to differentiate for 21 days before the experiments, replacing the culture medium every 48-72 hours. The cell monolayer's integrity was evaluated by determining the

transepithelial electrical resistance (TEER), using a commercial measurement system Millicell ERS (*Millipore Co., Bedford, MA*) that includes Ag-AgCl electrodes, according to the manufacturer's instructions. The final values expressed in Ω/cm2 were calculated according to the following equation: 'TEER = (R - Rb) x A', where 'R' is the electrical resistance of the filter insert with the cell layer, 'Rb' is the resistance of the filter alone, and 'A' is the growth area of the filter in cm2. Only those inserts that presented a TEER value higher than 600 Ω/cm2 were used for the bioavailability experiments.

For the development of the bioavailability experiment, 20µL of the analytical extracts were added to 300 µL of the fresh medium in the apical chamber. The transport from the apical to the basolateral chamber was evaluated after 120min of incubation at 37°C in a humidified atmosphere containing 5% CO2. That time was defined because it simulates the regular period in which food remains in the small intestine (Bonetti et al., 2016). Afterward, the apical and basolateral chambers' culture media were collected and dried using a SpeedVac concentrator Savant SPD121P (*Thermo Scientific, MA, USA*). The dry residue was suspended in methanol, filtered through 0.45 µm microfilters, and stored at -20 ºC until chromatographic analysis.

The phenolic transport was evaluated, collecting culture media from the apical and basolateral chambers. Subsequently quantified regarding their content of (+)-catechin, (-)-epicatechin, and chlorogenic acid by HPLC-DAD-ESI-MS/MS analysis. Results on absorption efficiency were expressed as a percentage of the compounds available for absorption in the apical chamber, according to the following equation: '*Absorption efficiency* (%) = (*Concentration in the basolateral chamber culture media at min 120 / Concentration in the apical chamber culture media at min 0) x 100*' (4).

2.7. HPLC-DAD-ESI-MS/MS analysis

As referred above, the samples were analyzed by reverse-phase chromatography HPLC-DAD-ESI-MS/MS, applying the method proposed by Kern

et al., (2003). The phenolic compounds detected in the extracts (catechins, epicatechins, and chlorogenic acids) were separated using a C18 Luna column (250 × 4.6mm, 5μm particle size) (*Phenomenex, Macclesfield, UK*). For the chromatographic separation, the solvents A (acetonitrile/formic acid (99:1, v/v)) and B (methanol), were used upon the following linear gradient scheme (t in minutes; %B): (0; 5%), (15; 15%), (30; 30%), (40; 50%), (45, 95%), and (50; 5%). The flow rate and volume of injection were 1 mL/min and 10μL, respectively. The chromatographic analysis was done on an Agilent HPLC 1100 series chromatograph equipped with a binary pump model G1312A, autosampler model G1313A, in-line degasser model G1322A, diode detector model G1315B, and an in-line mass spectrometer (*Agilent Technologies, Waldbronn, Germany*), controlled by the ChemStation software (Agilent, version 08.03). The phenolic acids were quantified based on standard curves developed using authentic standards at the following wavelengths: chlorogenic acids 320 nm, (+)-catechin, and (-)-epicatechin 280nm.

2.8. Statistical analysis

The results were presented as the mean ± SD (n=3). A one-way analysis of variance (ANOVA) was performed to establish significant differences between mixtures. The statistical treatment of the data was done using the R-statistical language (*R Development Core Team, 2019*). Statistically significant differences were set-up at $p<0.05$.

3. Results and discussion

3.1. Quantitative profile of (+)-catechin, (-)-epicatechin, and chlorogenic acid of cocoa, coffee, and cocoa-coffee mixtures

For the analysis of the bioavailability of flavanols and phenolic acids present in the cocoa-coffee mixtures the concentration of (+)-catechin, (-)-epicatechin was measured, catechin dimer, and chlorogenic acids. As mentioned before, the compounds were identified according to their retention time, DAD-spectra, and fragmentation pattern in comparison with

authentic standards. Thus, catechin dimer, (+)-catechin, and (-)-epicatechin corresponded to peaks recorded at retention times 9.2, 10.7, and 13.9 min., respectively, with the maximum absorption at 280 nm wavelength. The fragmentation patterns (arbitrary mass units (amu) of the parent and specific productions) for these flavanols were m/z 289 > 109, 123 ((-)-epicatechin), m/z 289 > 123, 145 amu ((+)-catechin), and m/z 576.8 > 289.1, 124.7 amu (catechin dimer). On the other hand, the peak corresponding to chlorogenic acid was found at 12.5 min and presented the following fragmentation pattern m/z 353.0 > 191.0 amu, recorded its highest absorption ratio at 320 nm.

The concentration of these compounds was closely dependent on the mixture (Table 3). In each compound, the concentrations between M1 and M5 (0:100; 100:0 cocoa: coffee, respectively) were as follows: in the case of catechin dimmer, the values were between 8.06-2641.48 µg/mL. In the (+)-catechin 15.61- 51.51 µg/mL, (-)-epicatechin 0.00-568.92 µg/mL and chlorogenic acid 0.43-0.00 µg/mL. Regarding flavanols, the highest concentration of the catechin dimer was from 25:75 to 100:0 in cocoa-coffee mixtures (2744.93 µg/mL, on average). The highest concentration obtained was in 25:75 (2848.38µg/mL). A similar trend was observed to (+)-catechin, and (-)-epicatechin; the highest concentrations were from 50:50 to 100:0 in cocoa-coffee mixtures ((+)-catechin=44.08µg/mL and (-)-epicatechin=492.03µg/mL, on average). The behaviour of the chlorogenic acids was inverse to that of the flavanols, corresponding the highest concentration to the 0:100 (cocoa-coffee) mixture (0.43µg/mL), which decreased in parallel with the augment of the cocoa percentage up to the absence of this phenolic acid in the 100:0 (cocoa-coffee) mixture.

Table 3. Concentration in the mixtures (µg/mL) of the flavanols and phenolic acids considered for the bioavailability analysis

Mixture (cocoa%-coffee%)	Flavanols			Phenolic acids
	Catechin dimmer	(+)-catechin	(-)-epicatechin	Chlorogenic acids
M1 (0-100)	8.06	15.61	0.00	0.43
M2 (25-75)	1219.71	17.55	194.32	0.26
M3 (50-50)	2087.90	36.90	370.53	0.15
M4 (75-25)	2848.38	43.84	536.66	0.04
M5 (100-0)	2641.48	51.51	568.92	0.00

3.2. Radical scavenging activity of coffee-cocoa mixtures

3.2.1. DPPH-based antioxidant capacity

Parallel to the cocoa proportion in the mixtures, the AC increases, which would indicate a more prominent antioxidant activity of the cocoa phenolics. Hence, the average DPPH• scavenging activity for the M1 (0:100 cocoa-coffee) mixture was 1.109 mM TE/g dw, which is in good agreement with the results reported by Çelik and Gökmen, (2018) for different coffee infusions (1.095-1.184 mM TE/g dw). However, this was the lowest AC relative to the other mixtures assessed in the present work (Table 4). However, the highest average DPPH• scavenging activity corresponded to the 100% cocoa extract (M5), with almost 2.5 mM TE/g dw. In addition, significant differences were found between the later matrix and the additional mixtures characterized ($p<0.05$) that exhibited values in the following decreasing order: 75:25 (cocoa-coffee) (1.939 mM TE/g dw) > 50:50 (cocoa-coffee) (1.743 mM TE/g dw) > 25:75 (cocoa-coffee) (1.513 mM TE/g dw) (Figure 1 and Table 4).

Concerning the DPPH• scavenging capacity of the cocoa-coffee mixtures, significant differences were founded between 25:75 and 75:25 that in turn exhibited significantly higher and lower AC relative to 100% cocoa and coffee extracts, respectively (Table 4). These findings allow assuming the high radical scavenging potential of the cocoa extract used in low concentrations to inhibit a significant number of free radicals. This attribute is desirable in ingredients intended to be used as sources of bioactive compounds of interest in functional foods design, mainly because the high AC of cocoa could achieve similar functional effects with low doses.

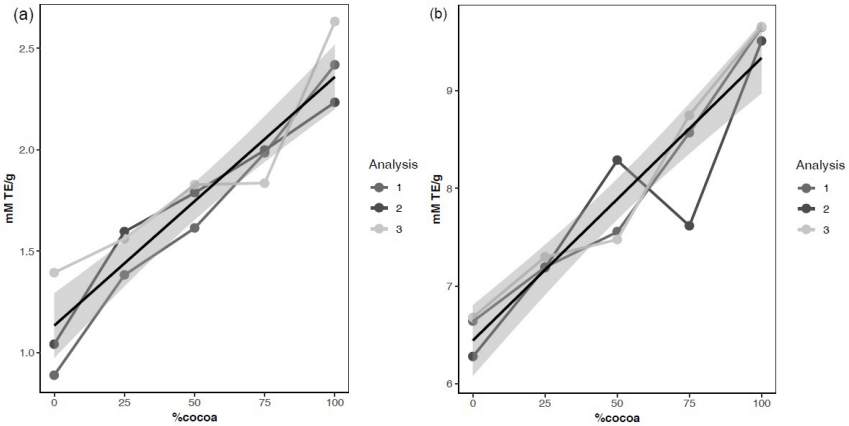


Figure 1. Antioxidant capacity of cocoa-coffee mixtures determined by the DPPH (a) and ORAC (b) methodology (The values 1, 2, 3 correspond to the means of three analyses).

Table 4. DPPH and ORAC-based antioxidant capacity (mM TE/g dw) and interaction of the mixtures.

Mixture (Cocoa%-coffee%)	DPPH	% of inhibition[Z]	IC50[Y]	CI[X]	Type of interaction	ORAC
M1 (0-100)	1.109 ± 0.243[a]	4.114 ± 1.776 [a]	15.030 ± 4.469[a]	1.699 ± 0.505[a]	----	6.532 ± 0.309[a]
M2 (25-75)	1.513 ± 0.173[b]	6.477 ± 1.533[b]	12.018 ± 1.739[b]	1.237 ± 0.179[b]	Moderate antagonistic	7.225 ± 0.663[ab]
M3 (50-50)	1.743 ± 0.194[bc]	7.944 ± 1.747[bc]	11.235 ± 1.477[bc]	1.043 ± 0.137[bc]	Nearly additive	7.771 ± 0.644[bc]
M4 (75-25)	1.939 ± 0.135[dc]	9.059 ± 1.109[dc]	10.802 ± 1.108[bcd]	0.894 ± 0.092[cd]	Slightly synergistic	8.312 ± 0.574[cd]
M5 (100-0)	2.428 ± 0.358[e]	12.461 ± 3.203[e]	9.976 ± 1.341[cde]	0.725 ± 0.097[de]	----	9.603 ± 0.542[e]

The values correspond to the mean and the standard deviation of the three analyses. Mean values with different letters in the same column show statistically significant differences ($p < 0.05$). [Z]%I, percentage of inhibition of the DPPH radical; [Y]IC50: half maximal inhibitory concentration that indicates the amount of the bioactive compound that can inhibit 50% of the DPPH radical; [X]CI: the combination index to establish the type of interaction between the mixtures

These results agree with some authors, whose have reported AC values in unfermented cocoa beans based on the DPPH-assay, such as 0.367 mM TE/g dw (Ramirez et al., 2013; Zapata et al., 2013). Thus, the AC described currently in the literature is lower than the presented in this work. This result could be because the unfermented cocoa beans used in this study have undergone a process to concentrate phenolic compounds through a hydroalcoholic treatment which usual exhibit higher concentration.

The IC50 (half maximal inhibitory concentration) corresponds to the quantity of compound required to inhibit 50% of the DPPH radical (Sabahannur & Alimuddin, 2018). This indicator is used in the pharmaceutical field to establish a drug's concentration to inhibit 50% of a particular biological process (Navre, 2019). The IC50 of the cocoa-coffee mixtures with high antioxidants content was calculated to identify their radical scavenging potential. In this aspect, 100% coffee and cocoa extracts (samples M1 and M5, respectively) presented differences among the mixtures. M1 (0:100) showed the highest value of IC50 (15.030 mM TE/g dw) and M5 (100:0) the lowest one (9.976 mM TE/g dw), indicating that a lower amount of cocoa in the mixtures would allow achieving 50% inhibition of the radical, while it is required up to 5-times higher coffee extract than cocoa to attain similar effects.

Moreover, regarding IC50, no statistically significant differences ($p>0.05$) were found between mixtures, which presented a significantly lower inhibition capacity than 100% coffee extract. Indeed, the cocoa-coffee mixtures' IC50 values decreased in parallel with the cocoa percentage increase in the mix. So, this result noticed a close relation between both extracts, demonstrating a dependency on the concentration of cocoa to reach an optimal effect with a low dose.

The interaction between the mixes was defined by the value obtained in the combination index (CI). In this aspect, some authors have described that a CI=1 between two antioxidants can be considered additive, a CI>1 antagonistic, a CI<1 synergistic (Enko and Gliszczyńska-Świgło, 2015). However, Rahadian et al. (2017), referring to the work done by Chou

(2006), provided additional details essential for a more accurate establishment of the interaction between two given antioxidants by presenting intermediate ranges of CI as follows: 0.3-0.7 (synergetic), 0.7-0.85 (moderate synergetic), 0.85-0.90 (slight synergetic), 0.90-1.10 (nearly additive), 1.10-1.20 (slight antagonistic), 1.20-1.45 (moderate antagonistic), and 1.45-3.3 (antagonistic) (Chou et al., 2006). Based on this information and the results obtained for the CI values presented in Table 4 is possible to say that as increased, the percentage of cocoa in the mixtures increased the synergistic interactions between antioxidant extracts, although this synergism was slight. In this regard, the cocoa-coffee mixtures 25:75, 50:50, and 75:25 exhibited moderate antagonism, nearly additive, and slightly synergistic behaviors, respectively. This classification is relevant because it allows establishing the combined effects of two compounds supplied together, which may have more significant benefits (Lane et al., 1999). Based on these premises and according to the results from this study, the proportion of cocoa plays a central role in the interaction type definition.

Currently, there is a gap of information about the interaction between phenolic compounds of cocoa and coffee; few studies to date have described this. In respect to this interaction, Ribeiro et al., (2014) showed how the mixture of 94% roasted coffee powder (*Coffea canephora cv. Robusta and Coffea arabica, 70:30, w/w*), 3% cocoa powder, 2% coffee silverskin, and 1% golden coffee, is featured by a high content of phenolic compounds relative to other types of commercial coffee. However, the synergistic/antagonistic interaction between antioxidants present in these products was not analyzed. On the other hand, Çelik and Gökmen (2018) provided valuable information on the interactions between the insoluble fractions of different coffee infusions and main cocoa flavanols (catechin and epicatechin), just as the interactions between other coffee infusions with dark chocolate. According to this study, for the insoluble fractions with cocoa flavanols, the AC values were between 0.953-1.84 mM TE/g dw. For the coffee infusions and dark chocolate, the AC was between 0.045-0.105 mM TE/g dw. In the first case, the CI was higher than the AC of the mixtures assessed in the present study. However, it is important to clarify that the

products referenced in this studies corresponded to the evaluation between coffee products enriched with pure compounds as catechins, while in the present research, were evaluated concentrated extracts.

Additionally, the interactions between the insoluble fractions and the pure catechins presented additive (CI=1) and antagonist (CI>1) synergies, respectively. Interactions between coffee infusions and dark chocolate were synergistic (CI<1). These results highlight that the interactions between cocoa and coffee don't have a pattern of interaction, which seems to be influenced by the purity of the compounds or their concentration.

3.2.2. ORAC-based antioxidant capacity

The average ORAC value for 100% coffee extracts was 6.532 mM TE/g dw. In this matrix, Narita & Inouye (2012) reported ORAC-based antioxidant values for Arabica green coffee extracts using water as a solvent (similar process to obtaining the coffee extract characterized in the present work), ranging between 0.354-2.629 mM TW/g dw. Besides, Getachew and Chun (2017) evaluated the AC of green coffee (*Coffee arabica*) extracts from different origins, using subcritical water extraction method to modify the native structure of the polysaccharides present in the coffee matrix. The values reported in this study were 0.06-0.08 mM TE/g dw. Hence, the cocoa extract's AC evaluated in this study presents a superior performance compared to some values reported in the literature (Narita and Inouye, 2012). The 100% cocoa extracts exhibited an average ORAC-based AC of 9.603 mM TE/g dw.

On the other hand, Cádiz-Gurrea et al. (2014) reported the ORAC-based AC of different fractions of cocoa phenolic compounds that ranged between 1.4 and 9.6 mM TE/g dw, corresponding 100% cocoa to the upper limit of this range. This fact suggests a high AC for this type of extract, even higher than the 100% coffee extract achieved. Concerning the other mixtures characterized in the present work, no statistical differences were found between 25:75 nor between 75:25 cocoa-coffee mixtures (Figure 1b and Table 4).

According to these results, this study evidences that cocoa extracts have a higher antioxidant capacity than coffee extracts. We could emphasize that the cocoa extracts are a valuable source of polyphenols for enriching food products with antioxidant phenolic compounds. Additionally, the use of these natural compounds in the elaboration of functional products could protect the cells from oxidative stress, which entails health benefits associated with reducing the risk of cardiovascular diseases and atherosclerosis (Martinez et al., 2020).

3.3. Bioavailability analysis.

In the last years, several studies have demonstrated the bioavailability of phenolic compounds, describing that most of them are easily detected in plasma after consumption of plant-based foods (Parada and Aguilera, 2007). Generally, the potential of a bioactive compound depends on its bioavailability. It has been demonstrated using a Caco-2 cells monolayer model, an in vitro experimental approach that simulates the so-called "transepithelial transport" occurring in the small intestine (Herrera et al., 2012). Thus, this cellular model has been internationally used to study drug absorption (Fernandes et al., 2012). In food research, this model has allowed observing some light on the benefits to human health of plant-based foods since once demonstrated their content of beneficial bioactive compounds, establishing the extent to which such compounds are absorbed at the gastrointestinal level (Colorado, 2017).

Based on these, the absorption of polyphenols present in cocoa, coffee, and different cocoa-coffee mixtures can be studied using this in vitro model. The compounds detected in higher concentrations were a catechin dimer, (+)-catechin, (-)-epicatechin, and chlorogenic acids. They were the most predominant in the pure extracts evaluated and were assessed on their capacity to cross the intestinal barrier. On this fact, other authors have reported that the transport of cocoa flavanol through Caco-2 cells monolayer is associated primarily with catechin monomers and dimers (Kosińska

& Andlauer, 2012). The absorption of these compounds was calculated as the percentage of the target phenolic in the basolateral chamber relative to the concentration of compounds added in the apical chamber medium after 120min. Thus, Figure 2 shows the percentage of transport (absorption efficiency) of the phenolic compounds.

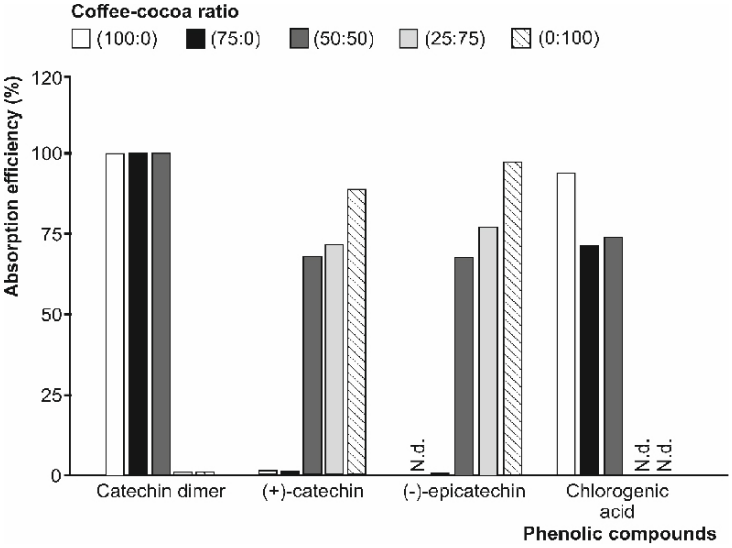


Figure 2. Absorption efficiency (%) in Caco-2 cells after 120 min. N.d. Not detected. Results representative of two independent experiments.

In the case of catechin dimer found in the cocoa-coffee mixtures assessed, this presented an absorption efficiency ranging between 59 and 93%. In this regard, the 100%-coffee extract showed the highest percentage of this compound (93.6%). Although this compound was not identified, it is important to mention that cocoa and coffee can contain this type of phenolic structures. Cocoa flavanols can be monomers (catechin and epicatechin) and dimers or oligomers (procyanidins) (Quiñones and Aleixandre, 2012). Regarding the bioavailability of these compounds, some authors have reported that transepithelial transport of procyanidins does not occur or has a low absorption rate (<5%). Mnatsakanyan et al. (2010) also reported the presence of some dimers present in the coffee (carboxylic acids)

To the (+)-catechin was observed to increase transport efficiency in parallel with the cocoa percentage rise in the mixture. This behaviour has a direct relation with the concentration of this compound in the mixes. This compound was not detected in the basolateral compartment during the bioavailability assay of the extracts that exhibited the lowest cocoa ratio in the mixture (100% coffee and 25:75 cocoa-coffee mixtures). This result could be a consequence of its degradation under the culture conditions (37 ºC and 70% humidity) during the assay (2h). In contrast, in extracts featured by higher ratios of cocoa in the mixtures (50%-100%), which displayed a higher (+)-catechin concentration between 36.90 and 51.51µg/mL, the bioavailability verified was up to ca. 88% (Figure 2), without statistically significant differences 50:50 and 75:25 cocoa-coffee mixtures, as well as 100% cocoa.

The presence of (-)-epicatechin was found in the basolateral compartment (bioavailable fraction) in the 50:50 and 75:25 mixtures (67% and 77%, respectively); as well as when applying the 100% cocoa extract (97.0% of bioavailability), this is similar with the bioavailability described in the literature (Kieu et al., 2020). However, no statistically significant differences between the diverse extracts analyzed were observed (Figure 2). Finally, the identification of bioavailable chlorogenic acid was in all extracts with the highest coffee extract values and decreased in 25:25 and 50:50 cocoa-coffee mixtures. In the 75:25 mix and 100% cocoa, these compounds were not detectable (Figure 2).

Not many studies on the bioavailability of cocoa polyphenols have been reported using the Caco-2 cells monolayer model of the intestinal barrier. Gómez-Juaristi et al (2020) and Liu et al. (2016) described the bioavailability of flavanols and hydroxycinnamic acids (5-caffeoylquinic and 3,5-dicaffeoylquinic acids), demonstrating that chlorogenic acids are featured by a higher intestinal absorption than the flavanols.

Conclusions

The cocoa extract evaluated showed superior performance than the coffee extract in terms of antioxidant capacity. With a lower dose of cocoa extract, it is possible to reduce more radicals, demonstrating its potential effect on health significantly. Besides, this could be a cost advantage for the development of functional products. The antioxidant capacity of the mixtures was directly proportional to cocoa extract present in them (higher % cocoa, higher AC).

In terms of bioavailability, both phenolic compounds present in the coffee and cocoa have high absorption efficiency, which is interest for the biological properties attributed to these compounds. The mixtures were not identified with a strong interaction between both extracts, which does not allow us to conclude about the real benefit of using them in combination. Instead, it is possible to mention that the cocoa and coffee extracts isolated have better potential functionality.

CAPITULO VI:

STABILITY AND QUALITY
OF A COCOA EXTRACT AND
A COCOA-COFFEE MIXTURE.

6. STABILITY AND QUALITY OF A COCOA EXTRACT AND A COCOA-COFFEE MIXTURE

ABSTRACT

Cocoa and coffee are two sources of antioxidants, and several studies have reported their health benefits. This study aimed to evaluate the stability and quality of a cocoa extract and a cocoa: coffee blend. The stability analysis was carried out for twelve weeks under storage at 25°C and 40°C, each week, the content of flavan 3-ols ((+)-catechin and (-)-epicatechin), procyanidin B2, methylxanthines (theobromine and caffeine), and chlorogenic acids were quantified to establish their behavior over time. The quality of both ingredients and their potential for food fortification were evaluated through sensory analysis and composition analysis while their safety through an MTT cytotoxicity test in CHO-K1 cells. The results obtained showed that the most stable compounds in cocoa extract were (+)-catechin and caffeine. While, in the mix were (+)-catechin, and in the case of chlorogenic acids, 5-CQA and caffeic acid. The rest of compounds, (-)-epicatechin, theobromine, procyanidin B2, 3-CQA, and the total content of chlorogenic acids, although they presented a high variability, no statistically significant differences were found between weeks 0 and 12, which demonstrates the stability over time of the phenolic compounds present in both extracts. Respect the sensory profile, the most predominant flavor notes were astringent, bitter, and green taste typical of phenolic compounds and unfermented cocoa and coffee beans. Additionally, the ingredients have a high potential for food fortification about minerals such as B, Cu, Fe, Mn, Rb, Si, and Zn. Regarding cytotoxicity, the cocoa extract was not cytotoxic, and the coffee extract used in the mixture, before this study, was recognized as GRAS (Generally Recognized as Safe).

Key words: cocoa, coffee, phenolic compounds, stability, taste, minerals, safe

1. Introduction

The food industry has been developing different strategies to offer consumers products that contribute to the prevention of some diseases, working on researches for healthy ingredients with potential for incorporation in the development of functional foods (Ribeiro et al. 2014). Many of these ingredients have bioactive compounds, which are substances present in plants that have health benefits. Cocoa and coffee are two food matrices with a high content of antioxidants. Some studies report cocoa benefits on cardiovascular health, diabetes, blood pressure, cognitive and brain health (Balzer et al., 2008; Pokorney & Piccini, 2017; Sokolov, Pavlova, Klosterhalfen, & Enck, 2013, Bravo, 1998). While, coffee has benefits on anti-inflammatory, antimutagenic, cardiovascular, and rheumatological diseases (Budryn et al. 2016).

Both cocoa and coffee are considered sources of phenolic compounds. Cocoa contains mainly flavanols, which are into three groups: catechins or flavan-3-ols, anthocyanins, and proanthocyanidins (Schinella et al., 2010). The main cocoa flavanol monomers are (-)-epicatechin and (+)-catechin. Procyanidin is composed of oligomers and polymers of catechin and epicatechin (Hu, Kim, & Baik, 2016; Rabadán-chávez et al., 2016). Chlorogenic acids are the most abundant phenolic compounds present in coffee. Green coffee beans have the highest content of these compounds, approximately 7-10% of its dry matter. Some of the most abundant caffeoylquinic acids in coffee are 5-Caffeoylquinic acid, 3-Caffeoylquinic acid and Caffeic acid (Budryn, Zaczynska, & Oracz, 2016; Gil, 2010; Gemechu, 2020).

The development of extracts woth high content in bioactive compounds has different technological challenges. Ingredients stable whit cost-competitive, safe for consumption and with scientifically proven functional benefits are desirable. However, these kinds of compounds are sensitive to different deterioration factors such as temperature, light, and oxygen. These could affect their bioactive content over time, for this reason, is relevant to guarantee the highest content of these compounds over time

through adequate storage and packaging conditions (Alean, Chejne, & Rojano, 2016; Alañón, Castle, Siswanto, & Spencer, 2016). Additionally, high concentrations of phenolic compounds are responsible for the astringent and bitter taste, which limits their use in high concentrations (Belscak et al., 2009; Brunetto et al., 2007). Another factor is to guarantee that these types of products are safe for consumers, and from a legal point of view, it is also necessary to prove this aspect.

The objective of this project was to evaluate the performance of a cocoa extract and a mixture of cocoa and coffee to development of functional products, through its stability in different storage conditions, the sensory performance, its potential for food fortification, and its safety through a toxicity test.

2. Materials and methods

2.1. Chemicals and reagents

All solvents (methanol, glacial acetic acid, ethanol, isopropanol, HCl, acetone) were analytical grade and supplied by Merk (*Merk, Darmstadt, Germany*). The water deionized in a Milli-Q water purification system (*Millipore, Bedford, USA*). The standards of (+)-catechin, (-)-epicatechin, and chlorogenic acid. Methanol was from Carlo Erba, France and ethanol, sodium di-Hydrogen phosphate anhydrous (Na_2HPO_4), and di-Sodium Hydrogen phosphate anhydrous (NaH_2PO_4) from Panreac ApplichemGmbH (*Darmstadt, Germany*). The Methyl Methane Sulfonate from Sigma-Aldrich (*Saint Louis, USA*). Dulbecco's Modified Eagle's Medium (DMEM) from Caisson (*Virginia, USA*), fetal bovine serum (FBS) from Eurobio *(Les Ulis, France*), trypsin and the sodium phosphate (Na_2HPO_4) were from Sigma-Aldrich (*Saint Louis, USA*), Ethylenediaminetetraacetic acid (EDTA) from Amresco (*Ohio, USA*), potassium chloride (KCL) and sodium chloride (NaCl) were supplied by Merk (*Merk, Darmstadt, Germany*). The dipotassium phosphate (KH_2PO_4) was from Panreac ApplichemGmbH (*Darmstadt, Germany*).

2.2. Plant material and preparation of coffee-cocoa mixture

The cocoa powder extract was supplied by the Compañía Nacional de Chocolates S.A.S.-CNCH (Rionegro, Colombia) and the coffee powder extract was from Industria Colombiana de Café Colcafé (Medellín, Colombia). A blend of cocoa and coffee were prepared to mix them in a proportion of 75:25 cocoa-coffee.

2.3. Stability analysis

In order to measure the stability of cocoa extract and mix, samples of both were packed hermetically in bags of a three-layer PET/BOPPmet/PE material with high barrier to water vapor and oxygen. Additionally, it was stored at two temperatures: ambient (25°C) and accelerated temperature (40°C) and relative humidity (75%) conditions in a CTWIN 480 climatic chamber (Comtitronic, Medellín, Colombia) for 12 weeks. A weekly comparison test was carried out between the product stored under accelerated conditions (climatic chamber) and the product stored under ambient conditions. The phenolic compounds and methylxanthines present in the samples: (+)-catechin, (-)-epicatechin, procyanidin B2, theobromine, caffeine and chlorogenic acids were quantified as described below.

2.3.1 HPLC analysis of flavanols of cocoa extract

The chromatographic analysis was developed on an Agilent 1260 high-resolution liquid chromatography (*Agilent Technologies, CA, USA*) with automatic autosampler, fluorescence, and diode array detectors (FLD and DAD). The signals were processed using the Agilent OpenLAB CDS software, ChemStation Edition. The samples were prepared following the methodology proposed by T-sanova-Savova, (2005). The extraction of the cocoa samples (0.5g) was done by adding 10 mL of isopropanol/deionized water (60:40, v/v), pH 9.0 for 60 min in an Elma E30H ultrasonic bath (*Elma Schmidbauer GmbH, Singen, Germany*), at room temperature. Afterward,

these samples were stirred in a vortex for 1 min and stored at -20 °C for 60 min. The samples were centrifuged at 4000 rpm for 8 min in al Rotofix 32A centrifuge (*Andreas Hettich GmbH & Co.KG, Schwerin, Germany*). The supernatant (1 mL) was collected and filtered through a 0.45µm Nylon membrane filter (*Millipore, Membrane Solutions, Auburn, USA*) and performed in a chromatography vial to be analyzed by HPLC/DAD/FLD. The chromatographic separation was in a Zorbax bonus C18 HPLC Column (250 x 4.6 mm, 5 µm particle size). The chromatograms were recorded at 280 nm and analyzed using the OpenLab CDS Chemstation Edition software. A pool of authentic external standards of (+)-catechin, (-)-epicatechin, caffeine, and theobromine were applied to quantify the compounds. The analysis of the diverse mixtures was performed on three replicates and reported as mg/g.

2.3.2. HPLC analysis of chlorogenic acids of mix

The evaluation of chlorogenic acids content in the mix of cocoa-coffee was carried out by reverse-phase high-resolution liquid chromatography (RP-HPLC), coupled to a UV-Vis or DAD detector, following the methodology described by DIN 10767 (1992). Accordingly, for the analysis, it was required a methanolic extraction to guarantee the precipitation of proteins. The samples were then centrifuged at 4000 rpm for 10 minutes in a Rotofix 32A centrifuge (*Hettich, Tuttlingen, Germany*) and filtered through a 0.45µm Nylon membrane filter (*Millipore, Membrane Solutions, Auburn, USA*). Afterward, the samples were injected into the HPLC system, including a G1311C quaternary pump and a G1315D diode array detector. The chromatographic separation was on a ZORBAX Eclipse XDB C18 column (150 x 4.6 mm, 3.5 µm particle size) (*Agilent, Santa Clara, USA*). The detection was at 325 nm. In this chromatographic system, nine chlorogenic acids were separated: 5-caffeoylquinic acid (5-CQA), 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), caffeic acid, ferulic acid, isopherulic acid, 3,4-dicaffeoylquinic acid (3,4 di-CQA), 4,5-dicaffeoylquinic acid (4,5 di-CQA) and 3,5-dicaffeoylquinic acid (3,5 di-CQA). The total content of chlorogenics was reported as the sum of each concentration quantified to authentic external standards and expressed as mg/g of chlorogenic acids.

2.4. Quality Analysis

The quality of the cocoa extract and mix were evaluated in terms of its antioxidant potential and bioavailability as reported in previous work (Chapter V). In this occasion, attributes such as sensory profile, its potential for food fortification and its safety for consumption (cytotoxicity) were evaluated.

2.4.1. Sensory analysis

For the flavor attributes evaluated in the cocoa extract and the mix, a flavor profile test was conducted by consensus, based on NTC 3929 of 1996. The tests were carried out in the sensory panel of the Centro de Investigation Desarrollo y Calidad-CIDCA of Compañía Nacional de Chocolates in Rionegro, Colombia, in the tasting cabins, using the specialized software in sensory analysis FIZZ Acquisition V 2.47. (Biosystems, France). The panel consists of five panelists previously trained under GTC 245 standards (Icontec, 2014).

2.4.2. Potential of cocoa extract and mix in food fortification-mineral-assay

The analysis of minerals was carried out following the methodology proposed in the UNE-EN ISO 11885 (ISO 11885:1996) (UNE, 2010) standards by means of an atomic emission spectrometer with inductively coupled plasma (ICP) Thermo ICAP 6500DUO (*Thermo Scientific, Madrid, España*). 0.20g of cocoa extract and mix were taken a 25ml digestion tube where 4 ml of concentrated nitric acid and 1 ml of 33% hydrogen peroxide were added. They were then taken to a microwave for digestion. Then 300 ml of ultrapure water, 30 ml of 33% oxygenated water and 2 ml of concentrated sulfuric acid were added. The tubes were subjected to the following temperature and pressure ramp: T inicial=40bar while increasing the pressure to 10bar/minute during 30 minutes and reaching a T final=220ºC, the

samples were kept at that temperature during 20 minutes. They are then cooled and made level in a 25mL double gauging tube with ultrapure water, homogenized and left to stand for analysis together with a blank (ultrapure water) and a control sample (this control is a solution composed of the following elements: Na, K, Ca, Mg, B, Cr, Fe, Ni, Cu, Zn, Cd, Pb, Al, Co, Tl, S, P, As, Mo, Se, Sb, Ti, Be, Bi, V, Sr, Li, Rb, Mn, La and Si, at different concentrations. It is called QC-1.0, and was the pattern standard for the construction of the calibration curve). Both the samples and the calibration curve are evaluated at the corresponding wavelengths for each element. The concentrations of the compounds are read in mg/l and the data are reported as presented in equation (1) '*mg Compound/Kg= (Concentration x Dilution Factor)/ sample weight'* (1).

2.4.3 Cytotoxicity assay MTT

This test was performed following the methodology proposed by Mosmann et al. (1983) and the guidelines to evaluate cytotoxicity ISO 10993-5:2009. A CHO-K1 (Chinese Hamster Ovary) cell line was obtained from cell banks recognized as ATCC and was used to measure cytotoxicity using MTT methodology.

Cell culture: CHO-K1 cells were cultured in DMEM medium with 10% fetal bovine serum (SBF). The cultures were maintained at 37°C and 5% CO2 and 90% relative humidity (RH). Confluent cells will be shed with trypsin at 0.25% and EDTA at 0.05% for five minutes. Cells were sub-cultured every three days at a concentration of $1x10^4$ cells/ml. The cell line was maintained in the in vitro toxicity unit's cell bank and stored in liquid nitrogen fumes, which allows maintaining the same batch of cells evaluated for the absence of mycoplasma.

Only was evaluated the cytotoxic effect of the cocoa extract, because previously the green coffee extract of Colcafé S.A.S. received GRAS denomination (Generally Recognized as Safe) by FDA (U.S. Food and Drug Administration). The maximum concentrations were defined based on the

solubility and potential changes in pH and osmolarity of the sample. The evaluated concentrations of cocoa extract were: 2 g/ml, 0.2 g/ml, 0.02 g/ml, 0.002 g/ml and 0.0002 g/ml, a negative control (PBS 10%) and a positive control (MMS 200 µg/ml), for a 24h exposure period. Six repetitions of each treatment were carried out including the controls.

Cell suspension from 100µl at a concentration of $1x10^4$ cells/ml were seeded in culture boxes from 96 wells, for 24 hours. After this time, a change of culture medium was made. The negative control was made with 10% PBS, the positive control with methyl methanesulphonate (MMS) to 200µg/ml, and the different concentrations of the test substance for 24 hours. Then, a fresh culture medium, supplemented with 100µl of MTT (5 mg/ml in PBS) was added to the culture. The cells were incubated in the dark at 37°C and 5% CO_2 for 3 hours. Subsequently, the culture medium was removed and 100µl of DMSO was added to each well to dissolve the purple formazan crystals. The absorbance was measured in a spectrophotometer (*Thermo Scientific, Massachusetts, USA*) at a wavelength of 570nm, three times. Cytotoxicity was determined as a measure of relative viability (RV) (equation 2), which is the product of the absorbance of the well containing the cells exposed to a specific concentration of the test substance, over the average absorbance of the negative control per 100. Potential changes in cell morphology and/or occurrence of vacuolization and/or granulation were also reported. 'RV= (*absorbance of the evaluated concentration) / (average absorbance of the negative control (C-)) x100%'* (2).

2.5. Statistical analysis

In the stability analysis, the results were presented as the mean ± SD (n=3). A one-way analysis of variance (ANOVA) was performed to establish significant differences between samples. The temperature was a block with two levels (25 and 40°C), which correspond to storage conditions of samples. Data analysis for the cytotoxicity test was performed using a Kruskal Wallis rank-sum test and a comparison of medians using a Wilcoxon rank-sum test, with the p-value adjusted by Holm's methods. The statistical treatment of the data was done using the R-statistical language (R

Development Core Team, 2019). Statistically significant differences were set-up at $p<0.05$.

3. Results and discussion

3.1. Stability analysis

According to the design parameters of cocoa extract and cocoa-coffee mix, the compounds such as flavanols, methylxanthines, and chlorogenic acids had a stable behavior during the storage. Tables 1 and 2, and Figure 1 and 2 show the evolution over time of the concentrations of these compounds, finding variations, which may be associated with the measurements or the samples in each time. These slight variations are a good result because phenolic compounds are sensitive and easily degraded. This result allows us to say that the designed ingredients are stable and the packaging used for storage is adequate. In the case of phenolic compounds from cocoa extract (catechin, epicatechin, and procyanidin B2) and methylxanthines (Theobromine and caffeine) no statistically significant differences ($p>0.05$) were found between samples over time or between storage temperatures (25 and 40°C).

Additionally, in Figure 1 is possible to identify that the most stable compounds with little variability were catechin and caffeine in the case of the cocoa extract in both storage conditions (Figure 1a and b). In the cocoa-coffee mix, the compound with the highest stability was catechin too, at both temperatures (25-40°C) (Figure 1 c and d). The compounds with the biggest fluctuations over time were epicatechin and theobromine in cocoa extract and cocoa-coffee mix under storage conditions. Gil, (2012) reported that epicatechin is one of the most sensitive compound to deterioration during primary processing of cocoa beans. However, the variations found were not considered significant, which allows mentioning that the designed extract has high stability. Besides, the packaging used with the light barrier is adequate to preserve the phenolic compounds. Hernández-Hernández et al., (2019) Hernández-Hernández et al., (2019) reported a study where was evaluated the stability during 28 days of olive

oil added with a cocoa shell extract containing antioxidants. In this study, the concentrations of theobromine and epicatechin were quantified over time, finding a slight decrease in their contents with insignificant variations. A similar result was found in this work.

Another similar result was described by Ribas-agustí et al. (2014), who analyzed the shelf life of fermented dry sausages added with an extract of phenolic compounds from cocoa and grape seeds. These authors found at the end of the time of the products, the content of catechin and epicatechin was 54-61% respectively. The results obtained in this research, comparing the weeks 0 and 12 in each storage condition were, epicatechin for cocoa extract stored at 25°C was 82.4% and at 40°C was 64.9%. This allows concluding that temperature plays an important role in the deterioration of these compounds. The best behavior of cocoa extract was in the storage at 25°C with the packaging defined for this study.

Table 1. Analysis of phenolic compounds of cocoa extract during storage.

Sample	Time (weeks)	Theobromine (mg/g)	Caffeine (mg/g)	(+)-Catechin (mg/g)	(-)-Epicatechin (mg/g)	Procyanidin B2 (mg/g)
Cocoa extract	0	55.125 ± 0.246 [a]	16.059 ± 0.063 [a]	3.771 ± 0.022 [a]	91.428 ± 0.459 [a]	45.926 ± 0.134 [a]
Cocoa extract storage at 25°C	1	78.595 ± 3.936 [a]	12.976 ± 0.472 [a]	5.278 ± 0.119 [a]	77.379 ± 3.734 [a]	39.751 ± 2.183 [a]
	6	65.468 ± 5.385 [a]	10.212 ± 0.885 [a]	4.268 ± 0.365 [a]	64.417 ± 1.985 [a]	32.568 ± 2.588 [a]
	12	85.431 ± 6.144 [a]	10.212 ± 0.949 [a]	5.547 ± 0.346 [a]	75.380 ± 0.573 [a]	44.489 ± 3.367 [a]
Cocoa extract storage at 40°C	1	78.904 ± 5.880 [a]	13.246 ± 1.212 [a]	5.074 ± 0.610 [a]	77.491 ± 6.129 [a]	38.760 ± 2.743 [a]
	6	63.620 ± 3.923 [a]	9.862 ± 0.541 [a]	3.799 ± 0.304 [a]	57.933 ± 3.788 [a]	29.930 ± 1.977 [a]
	12	76.944 ± 5.401 [a]	10.273 ± 0.664 [a]	4.794 ± 0.316 [a]	70.934 ± 4.312 [a]	39.984 ± 2.896 [a]

Different letters in a column indicates statistically significant differences ($p \leq 0.05$) between samples according to the Tukey test

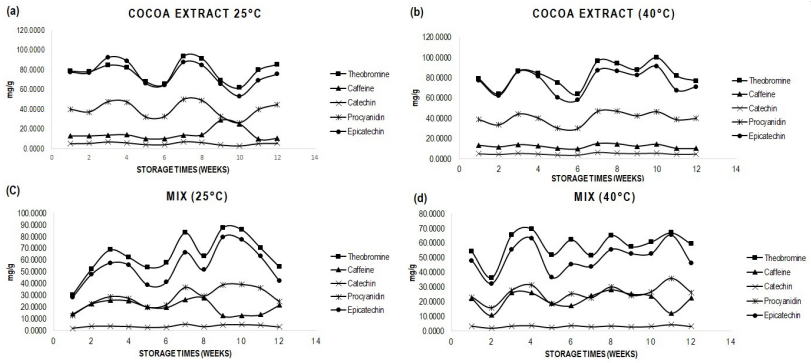


Figure 1. Stability over time of phenolic compounds present in the cocoa extract and cocoa-coffee mix during 12 weeks of storage at 24 and 40°C.

In the analysis of the chlorogenic acids (CGAs) present in the cocoa: coffee mix, fluctuations in the behavior of these compounds were found (Figure 2). As mentioned above, this may occur because the samples measured were different each time. As for the cocoa flavanols, the CGAs were stability and the differences between each observation time were not significant ($p<0.05$) between samples (Table 2) and between storage blocks (25 and 40°C). This behavior is desirable in this kind of ingredient because the concentration of the bioactive compounds influences the costs and possible claims associated with their functional benefits in a food product depend on it.

Table 2. Analysis of phenolic compounds of mix during storage

Sample	Time (weeks)	5-CQA (mg/g)	3-CQA (mg/g)	Caffeic Acid (mg/g)	Total CGAs (mg/g)
Mix	0	5.085 ±2.436 ^{a}	29.900 ± 9.601^{a}	0.030 ± 0.000^{a}	54.734 ± 3.802^{a}
	1	4.018 ± 0.087^{a}	31.874 ± 0.862^{a}	0.031 ± 0.001^{a}	36.433 ± 1.134^{a}
Mix storage at 25°C	6	5.659 ±0.207 ^{a}	45.802 ± 1.551^{a}	0.029 ± 0.001^{a}	54.997 ± 2.169^{a}
	12	6.445 ± 0.349 ^{a}	51.139 ± 2.227^{a}	0.018 ± 0.001^{a}	63.564 ± 3.319^{a}
Mix storage at 40°C	1	5.684 ± 0.534^{a}	46.073 ± 4.136^{a}	0.025 ± 0.004^{a}	55.861 ± 5.841^{a}
	6	4.604 ± 0.304^{a}	36.940 ± 2.663^{a}	0.019 ± 0.002^{a}	43.433 ± 3.635^{a}
	12	6.364 ± 0.000^{a}	42.405 ± 0.150^{a}	0.019 ± 0.000^{a}	44.191 ± 1.192^{a}

Different letters in a column indicates statistically significant differences ($p \leq 0.05$) between samples according to the Tukey test

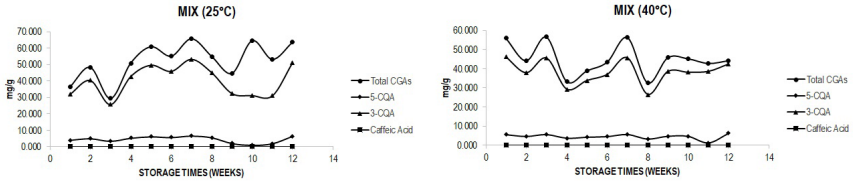


Figure 2. Stability over time of chlorogenic acids present in the cocoa-coffee mix during 12 weeks of storage at 24 and 40°C.

Although the CGAs were stable over time, the mix samples stored at 40°C presented a slight decrease. Vargas-Muñoz et al. (2020) presented a study where they evaluated the stability of 5-caffeoylquinic acid of a cocona powder at 25 and 35°C, they found higher stability of this compound at 25°C in comparison with the values obtained at 35°C. Them conclude that chlorogenic acids are very susceptible to thermal degradation, which could influence this reduction during the analysis of this condition. For this reason, store the mix at 25°C is adequate to preserve the content of these compounds.

The most abundant caffeoylquinic acids in the mix were 5-CQA, 3-CQA, and caffeic acid; Table 2 shows the average values of each of them at times 0, 1, 6, and 12, at 25 and 40°C. There were no statistically significant between samples at any of the times analyzed. In the blocks, differences ($p<0.05$) were detected for caffeic acid because a slight decrease in its content was observed at 40°C. Additionally, the most stable compounds at both conditions were 5-CQA and caffeic acid, a result similar to that reported by Sopelana et al. (2013), while the total content of CGAs and 3-CQA fluctuated (Figure 2). In figure 2 is possible to see a similar pattern of behavior between the values of 3-CQA and the total CGAs, which may be due to the high value of 3-CQA. This caffeoylquinic acid is predominant in green coffee, which was used in the preparation of the cocoa: coffee mix (Farah et al., 2008).

3.2. Quality Analysis

3.2.1. Sensory analysis

The results obtained in the sensory analysis of the cocoa extract and the mix, show that the attributes with the lowest values in both extracts were fermented (cocoa extract=0.3±0.70; mix=0.17±0. 35), roasted (cocoa extract=0.0±0.00; mix=0.17±0.35), and sweet (cocoa extract=0.2±0.35; mix=0.17±0.73) (Figure 2). This result is consistent because both extracts were made from unfermented cocoa and coffee beans without the roasting process. The highest ratings were associated with the attributes astringent (cocoa extract=8.2±1.59; mix= 6.17±1.60), bitter (cocoa extract=7.7±1.69; mix= 6.17±1.60) and green notes (cocoa extract=5.5 ±1.59; mix=4.67±1.16) (Figure 3). This result is also consistent since phenolic compounds are responsible for bitter and astringent flavors in many foods. Astringency is defined as a sensation of dryness in the mouth. This sensation may occur to a complexation reaction between polyphenols and proteins in the mouth and saliva (Drewnowski and Gomez-Carneros, 2000). Soares et al. (2013) also mentioned that bitter attributes are typical of products rich in phenolic compounds even if present in low concentrations. Additionally, these authors found that compounds such as epicatechin activate the human bitter taste receptors.

Other attributes with intermediate values such as acidity (cocoa extract=1.0±1.125; mix=1.2±1.49), fruity notes (cocoa extract=1.8±2.13; mix=1.17±1.60) and total impression (cocoa extract=1.8±1.06; mix=3.20±3.75) were also evaluated. Similar values were found between cocoa extract and mix in the acidity and fruity notes, however, in the total impression, the mix had a higher rating probably associated with the coffee flavor (cocoa extract= 0.0±0.00; mix=0.33±0.70). Also, the values obtained for the astringent, bitter, and green notes were slightly lower in the mix compared to the cocoa extract, which could improve its impression rating. This could be associated with the cocoa solids present in the cocoa beans. Mazo et.al, (2018) reported in this study a relationship between this variable and the bitter taste in chocolate beverages.

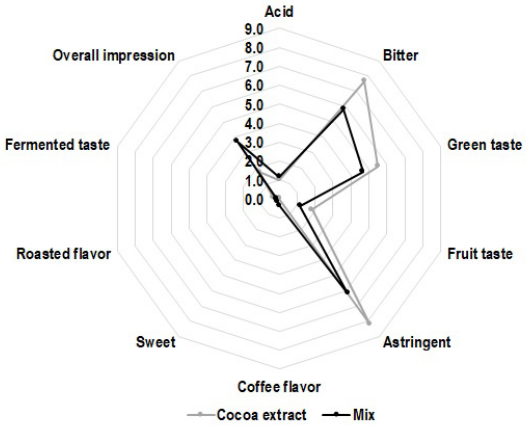


Figure 3. Flavor profile of cocoa extract and mix

One of the biggest technological challenges in the development of functional foods with high content of phenolic compounds is to obtain a sensory profile with low affectation due to the astringent and bitter flavors provided by these compounds (Belscak et al., 2009; Brunetto et al., 2007). However, different technologies have been reported and recommend the encapsulation of this type of ingredient improving the stability of phenolic compounds and the sensory perception of the products obtained (Budryn et al., 2016; Estrada et al., 2020).

3.2.2. Potential of cocoa extract and mix in food fortification

Minerals are compounds that provide different functionalities to the body, a deficiency in them could cause pathologies. These compounds have a fundamental role in biological functions (Wang et al., 2021). The content of essential and toxic minerals in the cocoa extract and the mix was evaluated as shown in Table 3. Additionally, the legal limits or daily reference values (DRV) reported in Colombian and the Food and Drug Administration (FDA) were reviewed to establish the potential of both ingredients for fortification.

Some minerals, such as boron (B), were detected in high concentrations (cocoa extract= 22.35 ± 3.57mg/Kg; mix= 25.77 ± 4.95mg/Kg). This mi-

cro mineral together with other minerals (e.g., Mg and P) may improve the estrogen concentrations in postmenopausal women and healthy men; embryonic development; cellular and organ membrane functions (Mohammad et al., 2017). No reference values for this mineral were found in the standards consulted. Charlene et al. 1999 reported that some foods with the highest amount of boron are raisins 22mg/kg and peanuts with 17mg/kg. The values obtained in cocoa extract and the mix, exceed the values mentioned.

Cadmium is a non-essential element considered one of the most toxic heavy metals as even in low doses could be toxic to any living organism (Gilbert et al., 2011). Regulation (EU) No 488/2014 of the European Commission on May 12, 2014, amending Regulation (EC) No 1881/2006 concerning the maximum content of cadmium in foodstuffs, specific in cocoa powder-based products, should not exceed 0.60mg/kg. The value found in the cocoa extract and mix was 20 times below this limit.

Another mineral with a high proportion was copper (Cu) (cocoa extract= 20.08±3.40mg/Kg; mix= 19.16±4.68mg/Kg). This compound is essential for iron metabolism, the proper functioning of different metabolic organs, stimulates the immune system, favors tissue regeneration, and helps neutralize free radicals (Mohammad et al., 2017). Some foods with high iron content are meat (1.21 mg/kg) and wheat bran (9.65mg/kg). The data obtained in the ingredients evaluated in this study exceeded these referenced values, this result may occur because the cocoa has a high content of this mineral (Cu in cocoa powder=50mg/kg) (Lurie et al., 1990).

The iron (Fe) content in both ingredients was also significant (cocoa extract= 4.29±0.59mg/kg; mix= 9.32±1.09mg/kg). A higher iron value was found in the mix because the coffee extract evaluated in isolation presented an iron content of 22.65 mg/kg, exceeding 5.3 times the cocoa extract (4.29 mg/kg). Fe is required for hemoglobin formation in red blood cells, energy metabolism and has an important role in enzyme systems of various tissues (Mohammad et al., 2017). Gómez-Álvarez, (2004) presented the contents of some iron-rich foods, such as meat (20mg/kg) and vege-

tables (10mg/kg), although these values are higher than those found in the extracts, it is possible to mention that a slightly higher dose of these ingredients in a functional product could approach the daily reference value reported in the normative.

Manganese (Mn) was also present in a high proportion, especially in the mixture (cocoa extract= 9.13±1.43/Kg; mix=22.43±0.12mg/Kg) as the coffee extract has an Mn content of 57.90mg/Kg. This mineral is essential for health as it contributes to wound healing, digestion, reproduction, and cellular energy regulation (Freeland-graves et al., 2016). Some foods rich in Mn are whole wheat bread (25mg/Kg), legumes (13.6mg/Kg) and, spinach (5mg/Kg), values close to those found in this study. Also, the results obtained can contribute significantly to supplement the daily reference values reported for this mineral

Rubidium (Rb) is another mineral found in the highest proportions in both the cocoa extract and the mix (cocoa extract= 69.26±12.54mg/Kg; mix=80.67±15.15mg/Kg). Both cocoa extract and coffee extract presented high values of this compound (77.68 and 69.26mg/Kg respectively). Rb is an almost essential element for living organisms. The human body treats Rb ions similarly to potassium ions. Some studies have shown that rubidium has antidepressant properties. The daily human intake of Rb is approximately 3 mg through foods such as beef and soybeans (Dixit et al., 2017).

Silicon (Si) was found in a high proportion in both ingredients, being higher in the cocoa extract (cocoa extract= 47.37 ± 4.76mg/kg; mix=13.44 ± 2.24mg/kg). This mineral serves to reduce the incidence of moniliasis and increase yield in cocoa crops, which could be a reason for the increase of this compound in cocoa beans (Miraval, 2019). Additionally, silicon has beneficial effects on health, participates in the formation and maintenance of connective tissue, and prevents cardiovascular and neurodegenerative diseases. Natural sources of silicon are fruits (e.g. mango 20mg/kg), vegetables (e.g. green beans (cooked) 24mg/kg) and cereals (e.g. rice 12.4mg/kg) (Sadowska, 2020). The results obtained in this research are similar or

have higher levels than reported; therefore, the cocoa extract and mix could be sources of this compound for the fortification of food products.

Zinc (Zn) is an essential mineral, a deficiency of which can affect the growth of a fetus and good cognitive development. Consuming Zinc contributes to tissue repair and strengthening the immune system (Ohanenye et al., 2021). The values found in the cocoa extract and the mix were 8.42 ± 2.07 and 9.25 ± 1.65 respectively. These values are close to the DRV reported in the regulations.

Table 3. *Contents of essential and toxic minerals of cocoa extract and mix.*

Mineral element	Cocoa extract (mg/Kg)	Mix (mg/Kg)	Legal limit or DRV
Al	1.51 ± 0.27	3.24 ± 1.48	-
B	22.35 ± 3.57	25.77 ± 4.95	-
Ca (mg/100g)	0.005 ± 0.00	0.04 ± 0.00	1300mg* 1000mg**
Cd	0.03 ± 0.01	0.03 ± 0.01	0.60 (mg/kg)***
Co	4.08 ± 0.61	3.74 ± 0.93	-
Cr	0.23 ± 0.03	0.15 ± 0.02	35 mcg* 120ug**
Cu	20.08 ± 3.40	19.16 ± 4.68	0.9mg* 2mg**
Fe	4.29 ± 0.59	9.32 ± 1.09	18 mg*, **
K (g/100g)	1.26 ± 0.08	2.35 ± 0.04	4700 mg * 3500 mg**
Li	0.00 ± 0.00	0.00 ± 0.00	-
Mg (g/100)	0.09 ± 0.01	0.17 ± 1.78	420mg* 400 mg**
Mn	9.13 ± 1.43	22.43 ± 0.12	2.3 mg* 2 mg**
Mo	0.00 ± 0.00	0.12 ± 0.03	45mcg* 75ug**
Na (g/100)	0.004 ± 0.00	0.01 ± 0.00	2300mg* 2400mg **
Ni	6.99 ± 1.05	6.38 ± 1.58	-
Pb	0.00 ± 0.00	0.15 ± 0.01	-
P	0.15 ± 0.02	0.19 ± 0.03	4700mg* 1000mg**
Rb	69.26 ± 12.54	80.67 ± 15.15	-
Sb	0.30 ± 0.02	0.00 ± 0.00	-
Si	47.37 ± 4.76	13.44 ± 2.24	-
S (g/100)	0.13 ± 0.02	0.13 ± 0.03	-
Sr	0.24 ± 0.10	2.33 ± 0.08	-
Ti	0.21 ± 0.01	0.29 ± 0.02	-
Zn	8.42 ± 2.07	9.25 ± 1.65	11 mg* 15 mg**

Data are presented as median ± SD and legal limits established for foods in different regulations:
*FDA, **Resolución 333 in Colombia, ***Reglamento UE de la Comisión No. 488 / 2014

3.2.3. Cytotoxicity assay MTT

As mentioned in the materials and methods section, this test was only performed on the cocoa extract since the safety of coffee extract was proven previously, which received the GRAS (Generally Recognized as Safe) designation by FDA (U.S. Food and Drug Administration). Additionally, Caro-Gómez et al. (2019) found that animals treated with Colcafé S.A.S. green coffee did not show any clinical alterations (appearance and behavior) or biochemical alterations, as indicated by the evaluated markers of liver function.

In the analysis of cocoa extract were no observed changes in cell morphology (granulations or vacuolization) in CHO-K1 cells after exposure to the extract for 24 hours in the test. The test substance is considered non-cytotoxic if it shows significant statistical differences with the positive control and/or does not show a trend of reduced Relative Viability (RV) with increasing test substance concentration. Significant differences ($p<0.05$) were found between the concentrations of cocoa extract and positive control. No statistically significant difference ($p<0.05$) was found between the concentrations of 2 mg/ml, 0.2 mg/ml, 0.02 mg/ml, 0.002 mg/ml, 0.0002 mg/ml and the negative control. Figure 4 shows the average relative viability of each concentration of cocoa extract, the standard deviation, and the error bars for each treatment, including the negative and positive controls.

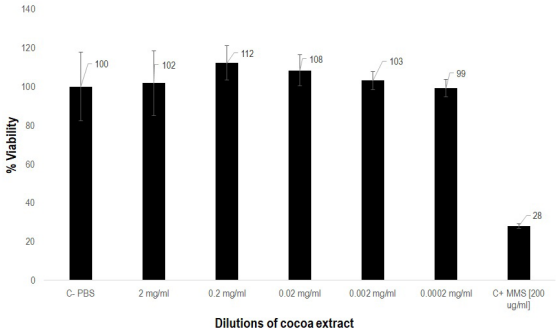


Figure 4. Mean and standard deviation of the relative viability (RV) of the different cocoa extract treatments including the negative control (C-, PBS 10%) and the positive control (C+, MMS 200g/ml).

Tabla 4. The relative viability of CHO-K1 cells exposed to the different concentrations of cocoa extract, including positive (MMS 200 µg/ml) and negative (PBS 10%) controls.

Dilution	Average relative viability
C- PBS (10%)	100,00 ± 4,31 [a]
2 mg/ml	101,8 ± 4,31[a]
0.2 mg/ml	112,2 ± 4,31[a]
0.02 mg/ml	108,3 ± 4,31[a]
0.002 mg/ml	103,0 ± 4,31[a]
0.0002 mg/ml	99,1 ± 4,31[a]
C+ MMS [200 µg/ml]	28,1 ± 4,31[b]

Different letters in a column indicate statistically significant difference ($p < 0.05$) between samples, according to the Kruskal Wallis rank test and the Wilcoxon rank sum comparison test, with a p-value adjusted by the "holm" method.

C-: Negative control
PBS: Phosphate buffered saline
C+: Positive Control
MMS: Methyl methane sulfonate

The concentrations of 2 mg/ml, 0.2 mg/ml, 0.02 mg/ml, 0.002 mg/ml, and 0.0002 mg/ml of the test substance have no significant statistical difference ($p>0.05$) of the RV with the negative control. Significant differences ($p<0.005$) were found between the different concentrations evaluated and the positive control (Table 4). Under the experimental conditions in the present study, the cocoa extract is considered non-cytotoxic. Baranowska et al., (2020) evaluated the cytotoxicity of a cocoa extract obtained under similar conditions (alcoholic extraction), through the MMT test with a human HT29 Cell Line, they found a similar result to the presented in this study, the short exposure (6h) of the substances did not inhibit cell growth in any of the concentrations evaluated at prolonged time conditions (24 and 72h) if inhibition of cell growth was detected but it did not exceed 20%.

CONCLUSIONS

The phenolic compounds present in the cocoa extract and the cocoa: coffee mix (75:25) have adequate stability under storage conditions at room temperature (25°C) and under accelerated conditions (40°C) since no significant changes in the concentrations of flavan 3-ols, procyanidins, methylxanthines, and chlorogenic acids were evidenced. However, it is better to maintain at 25°C the extracts in a light barrier packaging such as the one used in this study. Lower reductions were found in the content of phenolic compounds using these parameters.

The predominant flavor notes in both ingredients are astringent and bitter due to the high content of phenolic compounds. For this reason, we recommended their encapsulation or use of a flavor masker to prevent deterioration of the sensory profile of a product.

Both cocoa extract and mix have a high potential for food fortification in terms of some essential minerals as B, Cu, Fe, Mn, Rb, Si and Zn. They are also two safe ingredients for consumption as no evidence of toxicity was found.

CAPITULO VII:
DISCUSIÓN GENERAL

El cacao y el café son dos matrices con muchas bondades funcionales, actualmente Colombia es un gran productor y exportador de ambos ya que sus cultivos cuentan con unos altos estándares de calidad en términos de sabor y aroma. Sin embargo, esas bondades funcionales no se explotan comercialmente de manera frecuente en el mercado colombiano, ya que los compuestos bioactivos responsables de los beneficios en salud, se degradan en los procesos de industrialización tanto de los granos de cacao como de café, lo que impide realizar declaraciones en salud en un producto final debido a que no se alcanzan las concentraciones necesarias para comunicar al consumidor dicho beneficio, además no se cuenta con muchos estudios científicos que puedan respaldarlo. Este proyecto permitió proponer una alternativa de ingredientes con un alto potencial en términos del contenido de los compuestos bioactivos presentes en el cacao (flavan 3-oles y metilxantinas) y el café (ácidos clorogénicos), además de una primera aproximación a su potencial funcional para el desarrollo de productos alimentarios enriquecidos con estos. Para conseguir este resultado se realizaron diferentes ensayos con el fin de determinar el mejor sistema de extracción de los compuestos bioactivos, su conservación, el comportamiento tras incluirlos en una mezcla con café, así como su estabilidad.

El primer objetivo planteado para el presente trabajo era conservar la mayor concentración posible de la composición bioactiva de granos cacaos, concretamente de (-)-epicatequina, ya que este compuesto es considerado con un alto potencial funcional. Para ello, partiendo de estudios previos de técnicas descritas para la conservación de compuestos fenólicos en el grano de cacao, tras ser cosechado (congelación, inhibición de la enzima polifenol oxidasa y la deshidratación controlada de los granos), se aplicaron dichas técnicas obteniendo que on la congelación (24.96mg/g) y la deshidratación controlada (24.47mg/g) de granos de cacao sin fermentar, es posible aumentar la concentración de este compuesto siete veces más que el contenido presente en granos de cacao secos y fermentados (3.74mg/g). Por otro lado, con el procedimiento para inhibir la PPO se obtuvo el menor valor de (-)-epicatequina (17.40mg/g). Aunque no se encontraron diferencias estadísticamente significativas entre el proceso de congelación y de deshidratación. la congelación puede convertirse en un

tratamiento que encarece el procesado, por lo tanto, se seleccionó el tratamiento de deshidratación inmediata de los granos sin fermentar ya que este proceso resulta más práctico y con mayor rentabilidad para la industrialización.

Con el fin de optimizar la técnica de extracción de compuestos bioactivos, del extracto de cacao, se evaluaron diferentes métodos con el fin de establecer con cuál se podía obtener el mayor contenido de flavan 3-oles, en especial (-)-epicatequina como se mencionó anteriormente. Entre los métodos ensayados la extracción hidroalcohólica (97.45mg/g), resultó ser la más idónea, seguida del sistema de purificación de alto rendimiento (Reveleris®) (41.50mg/g), tratamientos hidrotérmicos (Phytoclean™) (29.43mg/g), la extracción por fluidos supercríticos (12.76mg/g) y la extracción asistida por ultrasonido (1.36mg/g). Por tanto, la extracción hidroalcohólica es la mejor a nivel técnico y económicamente viable para un proceso a escala industrial, comparada con las demás metodologías evaluadas.

A partir de la combinación del pretratamiento de granos de cacao sin fermentar (deshidratación controlada) y la extracción hidroalcohólica se obtuvo un extracto de cacao con alto contenido de (+)-catequina (3.7mg/g), (-)-epicatequina (84.7mg/g), teobromina (83.4mg/g), cafeína (12.7mg/g) y procianidinas (823.6 mg/g). Cabe destacar que dichos valores superaron los reportados en algunos extractos comerciales o referenciados en la literatura.

El fin último de este trabajo era la obtención de una mezcla de cacao y café con alto contenido de compuestos bioactivos. Para conseguir este objetivo, a partir del extracto de cacao obtenido en la primera fase de este este estudio y un extracto de café verde obtenido por Industria Colombiana de Café con alto contenido de ácidos clorogénicos, se realizó una evaluación de diferentes mezclas de cacao: café variando la composición porcentual en las proporciones de cada uno de estos extractos (0:100; 25:75; 50:50; 75:25; 100:0). En esta parte del estudio se evaluó el poder de captación de radicales (capacidad antioxidante) de las mezclas y la biodisponibilidad

de sus compuestos fenólicos, a través de modelos in vitro como el DPPH y ORAC, y la caracterización del transporte transepitelial o biodisponibilidad de los compuestos fenólicos del cacao y del café a través del modelo de células Caco-2 de la barrera intestinal. Se encontró que tanto el extracto de cacao como el de café tienen una alta capacidad antioxidante (DPPHcacao=2.428 mM TE/g vs. DPPHcafé=1.109 mM TE/g; ORACcacao=9.603 mM TE/g vs. ORACcafé=6.532 mM TE/g) y una alta biodisponibilidad (90-100% respectivamente), lo que hace que cada uno de ellos tenga un gran potencial funcional. Sin embargo, no se encontró que el uso de ambos extractos de forma combinada tuviera un beneficio diferenciado ya que no se obtuvo una relación sinérgica fuerte sino débil en la mezcla 75:25 cacao:café, mientras que en las demás mezclas evaluadas se encontró una relación moderadamente antagónica (25:75) o simplemente aditiva (50:50). Estos resultados no permitieron establecer una conclusión clara en relación al uso combinado de ambos extractos. Además, se encontró que tanto la capacidad antioxidante como el tipo de interacción es dependiente de la concentración de extracto de cacao presente en las mezclas.

Sin embargo, la calidad del extracto de cacao y la mezcla 75:25 cacao: café se evaluó en términos de su perfil sensorial, su potencial para la fortificación de alimentos de acuerdo a su contenido de minerales y su seguridad a través de un análisis de citotoxicidad, además de la estabilidad en el tiempo de los compuestos fenólicos presentes en ambos extractos en condiciones ambientales (25°C) y extremas de almacenamiento (40°C). Los resultados obtenidos demostraron los flavan 3-oles y metilxantinas más estables en el extracto de cacao fueron la (+)-catequina y la cafeína, en el mix fue la (+)-catequina. Para el caso de los ácidos fenólicos los compuestos más estables fueron el 5-CQA y el ácido cafeico. El resto de compuestos (-)-epicatequina, teobromina, procianidina B2, 3-CQA y el contenido total de ácidos clorogénicos, aunque presentaron una alta variabilidad no se encontraron diferencias estadísticamente significativas entre la semana 0 y 12 lo que demuestra la estabilidad en el tiempo de los compuestos fenólicos presentes en ambos extractos. Este comportamiento estable es deseable ya que uno de los atributos más importantes en el desarrollo de este tipo de ingredientes, es la concentración de los compuestos bioacti-

vos debido a que de ello dependen los costos y las posibles declaraciones asociadas a sus beneficios funcionales en sus aplicaciones en productos alimentarios. Además, se puso en evidencia que ambos ingredientes tienen un alto potencial para la fortificación de alimentos en relación a minerales como B, Cu, Fe, Mn, Rb, Si and Zn. En cuanto al perfil sensorial, las notas de sabor más predominantes fueron las astringentes, amargas y el verdosas típicas de los compuestos fenólicos y los granos de cacao y café sin fermentar. En relación a la citotoxicidad se encontró que el extracto de cacao no era citotóxico y el extracto de café utilizado en la mezcla, previamente a este estudio fue reconocido como GRAS (Generally Recognized as Safe), por lo tanto, el consumo de estos ingredientes no presenta perjuicio para la salud.

CAPITULO VIII:
CONCLUSIONES Y RECOMENDACIONES

1. El mejor sistema de conservación de los compuestos fenólicos del cacao, es la deshidratación controlada inmediatamente el grano es cosechado, por encima de mecanismos como la inhibición tradicional de la enzima polifenol oxidasa. Además, que representa la opción más viable en términos económicos para una producción a escala industrial.

2. De todas las tecnologías de extracción analizadas (fluidos supercríticos, tratamientos hidrotérmicos, ultrasonido y extracción hidroalcohólica), la extracción hidroalcohólica al 70% es la que presenta mejores rendimientos en cuanto a las concentraciones de compuestos fenólicos. A su vez es económicamente la más rentable para la industria.

3. El extracto de cacao evaluado mostró un desempeño superior al extracto de café en términos de la capacidad antioxidante. Esto es relevante porque con una menor dosis de este ingrediente, es posible reducir una mayor proporción de radicales libres, lo que indica que puede tener un mayor potencial funcional.

4. En las mezclas de cacao y café no se identificó una interacción sinérgica fuerte entre ambos extractos, lo que no permite concluir sobre el beneficio real de utilizarlos de forma combinada. Ambos extractos de forma aislada tienen una mejor funcionalidad. Sin embargo, de considerarse su mezcla, la mejor alternativa sería en una proporción cacao:café 75:25

5. Los compuestos bioactivos presentes en el extracto de cacao y la mezcla de cacao y café obtenidos en este estudio, tienen una adecuada estabilidad en el tiempo, lo que indica que sus concentraciones se mantienen en un rango aceptable.

6. Ambos ingredientes tienen un alto potencial para la fortificación de alimentos por su alto contenido de algunos minerales esenciales. En relación a la seguridad de consumo, puede recomendarse su aplicación en productos de consumo humano ya que se tuvo evidencia de que no generan algún efecto citotóxico.

7. Este proyecto permitió proponer una alternativa de ingredientes con un alto potencial en términos del contenido de los compuestos bioactivos presentes en el cacao (flavan 3-oles y metilxantinas) y el café (ácidos clorogénicos), además de una primera aproximación a su potencial funcional para el desarrollo de productos alimentarios enriquecidos con estos.

8. En futuros trabajos se recomienda realizar pruebas industriales del proceso para la obtención del extracto de cacao. Además de la evaluación de la funcionalidad con voluntarios tanto del extracto de cacao como de la mezcla de cacao: café, con el fin de establecer sus beneficios potenciales sobre la salud del consumidor y el soporte científico para la obtención de un aval legal para una declaración nutricional en el marco del desarrollo de un alimento funcional.

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