

# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

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## Extraction, characterization and valorization of biomolecules issued from coffee and cacao transformation

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UNIVERSITÉ  
DE MONTPELLIER

***Science is an enterprise that can only flourish if it puts the truth ahead of  
nationality, ethnicity, class and color.***

**Jonh C. Polanyi**

***La science a-t-elle promis le bonheur? Je ne le crois pas. Elle a promis la  
vérité, la question est de savoir si l'on fera jamais du bonheur avec la  
vérité.***

**Emilie Zola**

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## Abbreviations

<b>2FMT:</b> 2-furanmethanethiol	<b>EGCG:</b> (-)-epigallocatechin gallate
<b>2MTHFT:</b> 2-methyl-3-tetrahydrofuranthiol	<b>ESI:</b> Electrospray ionization
<b>3,4-DCQA:</b> 3,4-dicaffeoylquinic acid;	<b>EtOH:</b> Ethanol
<b>3,5-DCQA:</b> 3,5-dicaffeoylquinic acid	<b>FAMES:</b> Fatty acids methyl esters
<b>3MH:</b> 3-mercaptohexanol	<b>FC:</b> Folin-Ciocalteu assay
<b>3MHA:</b> 3-mercaptohexyl acetate	<b>FDA:</b> Food and Drug Administration
<b>3MH-Cys:</b> 3-mercapto(hexan-1-ol)-L-cysteine	<b>FTIR:</b> Fourier-transform infrared spectroscopy
<b>3MH-Glu:</b> 3-mercapto(hexan-1-ol)-glutathione	<b>FRAP:</b> ferric reducing ability of plasma
<b>3MMB:</b> 3-mercapto-3-methylbutan-1-ol	<b>GAE:</b> Gallic acid equivalents
<b>3MMBF:</b> 3-mercapto-3-methylbutyl formate	<b>GC:</b> Gas chromatography
<b>4,5-DCQA:</b> 4,5-dicaffeoylquinic acid	<b>GCB:</b> Green coffee beans
<b>4MB:</b> 4-mercapto-1-butano	<b>GPC:</b> sn-Glycero-3-phosphocholine
<b>4M4M2P:</b> 4-mercapto-4-methylpentan-2-one	<b>GSH:</b> Glutathione
<b>4MMP-Cys:</b> 4-mercapto-4-methylpentan-2-one-L-cysteine	<b>HBA:</b> Hydrogen bond acceptor
<b>4MMP-Glu:</b> 4-mercapto-4-methylpentan-2-one-glutathione	<b>HBD:</b> Hydrogen bond donor
<b>5-CQA:</b> 5- <i>O</i> -caffeoyl quinic acid or chlorogenic acid	<b>HPAE:</b> High performance anion-exchange chromatography
<b>AAPH:</b> 2,2'-azobis (2-amidinopropane) dihydrochloride	<b>HPLC:</b> High-performance liquid chromatography
<b>ABTS:</b> 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)	<b>HSE:</b> Heating stirring extraction
<b>ACN:</b> Acetonitrile	<b>IL:</b> Ionic liquids
<b>ALA:</b> $\alpha$ -linolenic acid (C18:3 n-3)	<b>IS:</b> Internal standards
<b>AMVN:</b> 2,2'-azobis (2,4-dimethylvaleronitrile)	<b>IsoFs:</b> Isofurans
<b>AOAC:</b> Association of Official Analytical Chemists guidelines	<b>IsoPs:</b> Isoprostanes
<b>AUC:</b> Area under the curve	<b>LC-MS:</b> Liquid chromatography–mass spectrometry
<b>BeB:</b> Betaine:1,4-butanediol (1:2 mole ratio)	<b>LC-MS/MS:</b> Liquid chromatography tandem mass spectrometry
<b>BeGly:</b> Betaine:glycerol (1:2 mole ratio)	<b>LLE:</b> Liquid–liquid extraction
<b>BHT:</b> Butylated hydroxytoluene	<b>LOD:</b> Limit of detection
<b>CA:</b> Caffeic acid	<b>LOQ:</b> Limit of quantification
<b>CCB:</b> Choline chloride:1,4-butanediol (1:2 mole ratio)	<b>MAE:</b> Microwave assisted extraction
<b>CCGly:</b> Choline chloride:glycerol (1:2 mole ratio)	<b>MeOH:</b> Methanol
<b>CCLA:</b> Choline chloride:lactic acid (1:2 mole ratio)	<b>MRM:</b> Multiple reaction monitoring
<b>CH:</b> Cocoa husk	<b>NaDES:</b> Natural deep eutectic solvents
<b>ChCl:</b> Choline chloride	<b>ORAC:</b> oxygen radical absorbance capacity
<b>CP:</b> Coffee pulp	<b>PAD:</b> Pulsed amperometric detection
<b>CPH:</b> Cocoa pod husk	<b>PC:</b> Phenolic compounds
<b>DES:</b> deep eutectic solvents	<b>PhytoFs:</b> Phytofurans
<b>DLPC:</b> 1,2-dipalmitoyl-sn-glycero-3-phosphocholine	<b>PhytoPs:</b> Phytosterols
<b>DPPC:</b> 1,2-dipalmitoyl-sn-glycero-3-phosphocholine	<b>PUFAs:</b> Polyunsaturated fatty acids
<b>DPPH:</b> 2,2-diphenyl-1-picrylhydrazyl	<b>QTRAP:</b> Triple-quadrupole mass spectrometer combined with a linear ion trap
<b>dw:</b> dry weight	<b>SPE:</b> Solid phase extraction
<b>EC:</b> (-)-epicatechin	<b>TPC:</b> Total phenolic compounds
<b>ECG:</b> (-)-epicatechin gallate	<b>tR:</b> Retention time
<b>EGC:</b> (-)-epigallocatechin	<b>UPAE:</b> Ultrasounds probe assisted extraction.
	<b>UPLC:</b> Ultrahigh performance liquid chromatography

*Scientific  
release*



## Scientific release

### I. Scientific publications

**Application of deep eutectic solvents (DES) for phenolic compound extraction: overview, challenges and opportunities.**

*Mariana Ruesgas-Ramón, Maria Cruz Figueroa-Espinoza and Erwann Durand*

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**Toxicity of natural deep eutectic solvents (NaDES) betaine:glycerol in rats.**

*Melha Benlebna, Mariana Ruesgas-Ramón, Beatrice Bonafos, Gilles Fouret, François Casas , Charles Coudray, Erwann Durand, Maria Cruz Figueroa-Espinoza, and Christine Feillet-Coudray*

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**Identification and quantification of phytoprostanes, and phytofurans, in coffee and cocoa by- and co-products**

*Mariana Ruesgas- Ramón, María Cruz Figueroa-Espinoza, Erwann Durand, Mirna Leonor Suarez Quiroz, Oscar González Ríos, Amandine Rocher, Guillaume Reversat, Joseph Vercauteren, Camille Oger, Jean-Marie Galano, Thierry Durand and Claire Vigor*

**Submitted 2018.**

**Deep eutectic solvents: a suitable tool for adding value to coffee and cocoa by- and co-products**

*Mariana Ruesgas-Ramón, Mirna L. Suárez-Quiroz, Oscar González-Ríos, Bruno Baréa, Guillaume Cazals, Maria Cruz Figueroa-Espinoza, Erwann Durand.*

**Submitted 2018.**

**Evaluation of antioxidant activity and interaction with radical species using the Vesicle Conjugated Autoxidizable Triene (VesiCAT).**

*Erwann Durand, Yu Zhao, Mariana Ruesgas-Ramón, Maria Cruz Figueroa Espinoza, Samantha Lamy, John N. Coupland, Ryan J. Elias, Pierre Villeneuve*

**Submitted 2018**

## II. Poster communication

109<sup>th</sup> American Oil Chemists' Society (AOCS) Annual Meeting & Expo. May 2018, Minneapolis, Minnesota, USA

14<sup>th</sup> GERLI International Lipidomic meeting. St Maximin-la-Sainte Baume, France: September 30th to October 3rd, 2018

6<sup>th</sup> Euro Fed Lipid Congress and Expo. Belfast : 16-19 septembre 2018.

- **Identification and quantification of phytoprostanes and phytofurans in coffee and cocoa by- and co-products.** *Mariana Ruesgas-Ramón, Maria Cruz Figueroa-Espinoza, Erwann Durand, Mirna Leonor Suárez Quiroz, Óscar González Ríos, Amandine Rocher, Guillaume Reversat, Joseph Vercauteren, Camille Oger, Jean-Marie Galano, Thierry Durand, Claire Vigor.*

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- **Evaluation of antioxidants activity of coffee and cacao agro-residues by Vesicle Conjugated Autoxidizable Triene (VesiCAT) assay.** *Mariana Ruesgas-Ramón, Mirna Leonor Suárez Quiroz, Óscar González Ríos, Maria Cruz Figueroa-Espinoza, Erwann Durand.*

## III. Collaborations

- Fiber analysis: Denis BASTIANELLI and Elodie BABY from CIRAD-UMR SELMET (Montpellier, France).
- Monosaccharides analysis: Aurélie PUTOIS, Equipe Grains-Graines, INRA - CIRAD - Montpellier SupAgro - U. Montpellier, UMR 1208 IATE, Montpellier, France .
- Phenolic compound identification: Guillaume CAZALS, Laboratoire de Mesures Physiques, Service Commun de l'Université Montpellier 2, Plateau Technique de l'Institut des Biomolécules Max Mousseron, Université Montpellier 2, Montpellier F-34095, France.
- Murine study: Christine FEILLET-COUDRAY and Charles COUDRAY, DMEM, INRA, Univ Montpellier, Montpellier, France.

- Isoprostanoïds identification: Thierry DURAND, Claire VIGOR, and Amandine ROCHER, Institut des Biomolécules Max Mousseron, IBMM, University of Montpellier, CNRS, ENSCM, Faculty of Pharmacy, Montpellier, France.
- Thiols precursors identification: Laurent DAGAN and Aurélie ROLAND-VIALARET, Nyséos. Montpellier, France.



# *French summary*



## French summary

### **Extraction, caractérisation et valorisation de biomolécules issues de la transformation du café et du cacao**

#### **I. Contexte de la recherche**

Le défi pour alimenter environ 7,5 milliards de personnes dans le monde, mais aussi la croissance prévue de la population au cours des 40 années à venir, ont mené à l'augmentation de la production et de la transformation des aliments. Cette situation a également conduit à une importante augmentation de sous- et co-produits qui méritent d'être bien valorisés, car ils peuvent présenter des propriétés intéressantes [1].

Le café et le cacao sont consommés par des millions des personnes chaque jour dans le monde. Cependant, leurs procédés de transformation génèrent de grandes quantités de sous-et co-produits tels que la pulpe de café (CP) (représentant 45 % du poids du fruit frais), la cabosse de cacao (CPH) (représentant 74 % du poids du fruit frais [2]) et la coque de cacao (CH) (représentant 12 % du poids de la fève de cacao sec [3]). Ces résidus dérivent de la 1<sup>ère</sup> transformation du café (CP) et du cacao (CPH) et de la 2<sup>ème</sup> transformation du cacao (CH). Très souvent, ces résidus sont mal gérés et abandonnés directement dans les champs [2], provoquant ainsi de graves problèmes sociaux, économiques et environnementaux, tels que des mauvaises odeurs ou la propagation de maladies dans les plantations.

Le café est connu pour être riche en composés polyphénoliques (PC), surtout les acides chlorogéniques [4], tandis que le cacao contient des procyanidines et (-)-épicatéchines [5]. De plus, ils peuvent-être sources de lignine, protéines et minéraux, ainsi que de sucres réducteurs, mais ils ne sont pas toujours valorisés de manière intégrale en tant que déchets. Par ailleurs, leur exploitation pourrait avantager économiquement les producteurs, en plus de préserver l'environnement et de générer des technologies orientées à vers transformation des ressources naturelles. Tel serait le cas de la valorisation de ces résidus par l'extraction de molécules à l'aide des solvants eutectiques profonds (DES, deep eutectic solvents). Les DES consistent en un mélange d'un sel organique (ammonium ou phosphonium) qui fonctionne comme un accepteur de liaisons hydrogène (HBA) et d'un

donneur de liaisons hydrogène (HBD). Ils sont très facilement préparés, peuvent être liquides à température ambiante, et sont non volatils, thermiquement stables et biodégradables[6]. Les molécules extraites à l'aide des DES pourraient être utilisées comme des auxiliaires précieux en tant qu'agents de lutte contre les radicaux libres et en tant qu'agents antimicrobiens et conservateurs dans des produits alimentaires et non-alimentaires. De plus, la recherche de nouvelles molécules dans la CP, la CH et la CPH pourrait élargir leur profil moléculaire, afin de leur trouver de nouvelles applications.

Par conséquent, l'objectif de ce travail de thèse a été d'offrir des nouvelles perspectives de valorisation des principaux sous- et co-produits du café (CP, CPH) et du cacao (CH), à travers:

- (i) L'obtention d'extraits riches en biomolécules (*i.e.* composés phénoliques) à partir de la CP, la CH, la CPH et des écarts de café vert, en utilisant des solvants verts (DES).
- (ii) Compléter l'actuel portefeuille de molécules possédant des propriétés physicochimiques et/ou biologiques intéressantes issues des résidus de café et de cacao (CP, CH ou CPH) à travers la recherche d'une part, des isoprostanoïdes (phytoprostanes (PhytoPs) et des phytofuranes (PhytoFs) qui sont connus pour leurs propriétés biologiques (*i.e.* ce sont des biomarqueurs du stress oxydatif, et ils participent à la régulation de la fonction immunitaire[7][8]) et d'autre part, des précurseurs des thiols, précurseurs conjugués de la cystéine ou du glutathion, molécules inodores qui jouent un rôle clé dans la libération des arômes de divers aliments, tels que les fruits et les boissons [9][10]. De plus, à notre connaissance, ni les isoprostanoïdes ni les précurseurs des thiols n'ont jamais été décrits dans CP, CH et CPH dans la littérature.

A cela s'ajoutent deux autres études : (i) une première étude sur l'effet *in vivo* chez le rat d'un extrait riche en acides chlorogéniques (CGA), issus des grains de café vert (GCB) et obtenu à l'aide d'une extraction dans un DES, et (ii) une seconde sur l'évaluation de la capacité antioxydante à lutter contre la dégradation oxydative membranaire des extraits éthanoliques de la CP, la CPH et la CH. Cette activité a été étudiée à l'aide de la méthode VesCat (Vesicle Conjugated Autoxidable Triene) récemment développée dans notre laboratoire [11].

## II. Résultats obtenus

### a) Caractérisation chimique des échantillons

La première étape de ce travail a consisté à caractériser chimiquement chaque échantillon.

Les résultats obtenus ont montré que les CP, CPH et CH sont riches en cellulose (respectivement 17,5, 17,2 et 25,8 %), hémicellulose (respectivement 10,7, 8,3 et 9,9 %) et protéines (respectivement 10,7, 9,1 et 17,8 %). L'ose majoritaire dans tous les échantillons a été le glucose. Ces résultats sont similaires à ceux rapportés dans la littérature [2][12][13]. Les différences sont dues aux conditions de culture, aux origines et variétés du café et du cacao, ainsi qu'à la méthode d'analyse utilisée.

La caractérisation lipidique a montré que la CP est riche en acides palmitique et linoléique, qui sont également les principaux acides gras présents dans le grain de café vert [14]. Les lipides de la CPH sont riches en acides linoléique et palmitique, tandis que ceux de la CH en acides oléique et palmitique. Selon la littérature, les principaux résidus d'acides gras dans le beurre de cacao sont les acides stéarique et oléique [15], dans la CPH les acides linoléique et oléique [16] et dans la CH les acides oléique, palmitique, linoléique et stéarique[17].

### b) Extraction des biomolécules par les « Deep Eutectic Solvents (DES) »

La deuxième partie de ce travail a été dédiée à l'extraction des PC en utilisant de l'EtOH à 70 % (v/v) comme témoin et six différents DES: CCLA (chlorure de choline (ChCl):acide lactique (1 :2 ; mol/mol)), BeLa (betaine:acide lactique (1 :2 ; mol/mol)), CCGly (ChCl:glycérol (1 :2)), BeGly (bétaine:glycérol (1 :2 ; mol/mol)), CCB (ChCl:1,4-butanediol (1 :2 ; mol/mol)) et BeB (bétaine:1,4-butanediol (1 :2 ; mol/mol)). L'extraction a été réalisée soit en chauffant pendant 1 heure sous agitation (HSE) soit par des ultrasons à l'aide d'une sonde (UPAE, BRANSON SF-250: 3 min/cycle constant, 200 W). Tous les DES ont été hydratés avec 10 % d'eau (v/v) afin de diminuer leur viscosité et faciliter leur manipulation.

La teneur en composés phénoliques totaux (TPC) dans les extraits a été déterminée en utilisant la méthode Folin-Ciocalteu (FC), largement utilisée pour la quantification des PC extraits à partir de végétaux [18], ainsi que par HPLC ( $\lambda=280\text{nm}$ ). Les résultats ont montré une surestimation des teneurs des TPC en utilisant la méthode FC, quelle que soit la matrice

végétale (CP, CPH et CH) obtenue par HSE. En effet, la teneur en TPC dosée par FC a été de 5 à 85,5 fois plus élevée que celle obtenue par HPLC. Cette problématique a déjà été signalée comme principal inconvénient de cette méthode, ce phénomène étant attribué à la présence de composés tels que les sucres, les vitamines, les acides organiques et d'autres substances non phénoliques [18]. En plus, on a observé la formation d'un précipité lors de l'utilisation des DES à base de ChCl qui pourrait provenir des échanges d'ions entre ce dernier et le carbonate de potassium, conduisant ainsi à la formation de sels insolubles. C'est pourquoi, la méthode FC n'est pas recommandée dans le cas du dosage sur des extraits contenant des DES à base de ChCl, car elle ne permet qu'une approximation qualitative et non quantitative de la teneur des TPC.

A la suite de ces derniers résultats, la quantification des TPC a été faite par HPLC et la structure des molécules extraites a été confirmée par HPLC couplée à la spectrométrie de masse (MS). Les résultats ont montré que parmi le DES utilisés, le meilleur taux d'extraction a été atteint par CCLA dans le cas de la CP et la CH (respectivement  $0,96 \pm 0,13$  et  $0,92 \pm 0,08$  g GAE (équivalents d'acide gallique)/100 g base sèche (bs)). En revanche, avec les conditions testées, aucun PC n'a été détecté dans les extraits de CPH quel que soit le solvant employé (EtOH ou CCLA). Les composés suivants ont pu être identifiés par UPLC-MS ( $\lambda = 280$  et  $327$  nm)

CP : acide chlorogénique (5-CQA) et caféine

CH : caféine et théobromine

CPH : Aucun composé n'a été identifié

Lors de l'extraction avec l'EtOH 70 % (v/v) assistée par UPAE, nous avons identifié les mêmes composés cités ci-dessus. En revanche, en présence de CCLA, un nouveau composé absorbant fortement à 280 nm est apparu dans les trois résidus et a été identifié comme étant du furfural. Celui-ci se serait formé suite à la déshydratation acide des pentoses [19] libérés suite à l'hydrolyse catalysée par le DES. Des hémicelluloses présentes dans la CP, la CPH et la CH et donc favorisée par les conditions d'extraction ( $\sim 70^\circ\text{C}$ ,  $\text{pH}_{\text{CCLA}} = 0,78 \pm 0,18$ ). Ainsi, les teneurs en furfural ont été quantifiées : CPH ( $0,15 \pm 0,02$  g/100 g b.s.), CP ( $0,18 \pm 0,04$  g/100 g b.s.) et CH ( $0,06 \pm 0,03$  g/100 g b.s.).

L'extraction à l'aide du DES CCLA assistée par UPAGE a été plus efficace que celle assistée par HSE pour les rendements d'extraction de 5-CGA et caféine à partir de CP (1,09 et 1,35 fois plus élevé, respectivement), et theobromine à partir de CH (1.2 fois plus élevé). Par ailleurs, concernant les TPC, il a été possible de récupérer respectivement 1,9 ( $1,84 \pm 0,04$  g EAG/ 100 g bs) et 1,4 ( $0,84 \pm 0,12$  g EAG /100 g bs) fois plus en utilisant CCLA-UPAGE, à partir de la CP et la CH, par rapport à la méthode HSE.

D'après ces résultats, on peut conclure que les DES sont des solvants d'une grande versatilité physico-chimique capables d'extraire une large gamme de molécules, et que la composition des DES a une influence à la fois sur les rendements d'extraction mais aussi sur le type de molécules extraites. Ils peuvent donc être utilisés en tant que solvants plus respectueux de l'environnement pour la valorisation des résidus issus de la transformation du café et du cacao, à travers l'extraction de molécules bioactives.

### **c) Evaluation in vivo d'un extrait dans du DES et enrichi en composés phénoliques**

Un des principaux désavantages de l'utilisation des DES comme solvants d'extraction est que la purification des molécules cibles peut-être une étape coûteuse et compliquée. Une alternative intéressante pourrait donc être l'utilisation directe de l'extrait obtenu en tant qu'une formulation riche en PC dans du DES. Ce dernier serait donc utilisé à la fois en tant que solvant et qu'agent vecteur dans la formulation. Dans ce contexte, l'objectif de cette partie a été de tester l'effet *in vivo* chez le rat. Dans des conditions aiguës, avant de mener une étude de supplémentation nutritionnelle à long terme, d'un extrait enrichi en acides chlorogéniques (CGA) et obtenu à partir de grains de café vert (GCB, qui contiennent entre 6-10 % CGA bs [20]), par une extraction à l'aide des DES assistée par HSE (3 h, 60 °C). Six DES (CCLA, BeLA, CCGly, BeGly, CCB et BeB) ont été testés. Ce travail a été réalisé en collaboration avec l'équipe d'Endocrinologie Mitochondriale et Nutrition de l'UMR DMEM (Montpellier).

Les résultats ont montré que la capacité d'extraction des DES testés a été très similaire avec une moyenne d'extraction de 6,23 g équivalents d'acide chlorogénique CAE/100 g GCB bs. Finalement, le DES BeGly a été retenu pour l'étude, car la bétaine est un acide aminé N-triméthylé, présent chez les microorganismes, les plantes, les animaux et certains aliments

et de plus est considérée comme non-toxique [21]. Le BeGly a permis d'obtenir un extrait de GCB contenant  $9,33 \pm 1,32$  g CAE /100 g et constitué de 83,9% de mono-esters et 16,1% de di-esters de CGA. L'extrait éthanolique (témoin) a montré une composition similaire (86,1 % mono-esters et 13,9 % de di-esters de CGA). Dans les deux extraits BeGly et EtOH 70% (v/v), le composé principal a été le 5-CQA, mais la caféine a aussi été détectée à des concentrations respectives de  $1,15 \pm 0,25$  et  $0,96 \pm 0,05$  % (p/p).

Le test de toxicité a été fait sur deux groupes de 6 rats Wistar âgés de six semaines, nourris par gavage deux fois au jour pendant 14 jours, soit avec 3 mL d'eau soit avec 3 mL d'extrait BeGly (contenant 28,01 mg de PC exprimés en CAE et 5,89 mg de caféine).

L'administration orale à de fortes doses d'extrait BeGly a démontré des effets néfastes sur la santé des rats, qui se sont traduits par une faible consommation alimentaire, une consommation d'eau excessive, une perte de poids, une perte de tissu adipeux, une hépatomégalie, un stress oxydatif dans le plasma et une augmentation des taux de lipides dans le sang [22]. De plus, deux rats du groupe DES sont décédés, malgré les effets bénéfiques du 5-CQA prouvés par plusieurs études [4][5][23]. Ces effets décrits sont probablement la conséquence des conditions aiguës dans lesquelles cette étude préliminaire a été réalisée afin d'éviter la mort d'un grand nombre d'animaux lors d'une étude à long terme. Malheureusement, le comité d'éthique national français n'a pas recommandé de poursuivre l'ensemble de l'étude initialement prévue. Il est donc clair que des travaux supplémentaires sont encore nécessaires afin de déterminer et de choisir les bonnes doses, concentrations et composition de DES qui peuvent être efficaces et non-toxiques *in vivo*.

#### **d) Identification et quantification des isoprostanoïdes**

Dans le but d'élargir l'actuel portefeuille de biomolécules dérivées des sous- et co-produits issus de la transformation du café et du cacao, nous avons examiné la présence des PhytoPs et PhytoFs dans nos échantillons, ces travaux ont été réalisés en collaboration avec l'Institut des Biomolécules Max Mousseron (UMR 5247, Université de Montpellier, CNRS, ENSCM). Ces molécules sont présentes chez les plantes et dérivent de l'oxydation non enzymatique de l'acide  $\alpha$ -linoléinique (C18:3 n-3, ALA). Pour l'analyse, les lipides d'environ 0,1 g de chaque échantillon (CP, CPH ou CH) ont été extraits par la méthode Folch, puis les

phytoprostanoïdes ont été fractionnés en utilisant une cartouche Oasis MAX, et analysés par micro-HPLC couplée avec un 6460 triple quadripolaire MS/MS.

Les résultats ont montré la présence des PhytoPs et PhytoFs dans nos matières premières. En effet, nous avons pu identifier six molécules différentes de PhytoPs. Chez la CP et la CPH, le 9-*epi*-9-F<sub>11</sub>-PhytoP a été majoritaire (respectivement 171,37 et 37,12 ng/g bs,), tandis que dans la CH c'est le ent-9L1t-PhytoP (138,802 ng/g bs). La plus forte teneur en PhytoPs a été quantifiée dans la CP (654,45 ng/g b.s.). La teneur en phytoprostanoïdes chez les plantes est liée aux conditions edaphoclimatiques de culture et aux conditions de processus de transformations, tels que le séchage, le stockage et/ou la fermentation[24]. Ceci expliquerait la plus forte teneur en PhytoPs dans la CP, qui a subi un séchage. La CPH présente la teneur la plus faible en PhytoPs (155,31 ng/g b.s.), à noter qu'elle a été prélevée directement après la récolte, puis séchée pour sa conservation avant analysée

Concernant les PhytoFs, *ent*-16(*RS*)-9-*epi*-ST- $\Delta^{14}$ -10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST- $\Delta^{10}$ -13-PhytoF, and *ent*-16(*RS*)-13-*epi*-ST- $\Delta^{14}$ -9-PhytoF ont été identifiés dans les CP, les CH et les CPH. Les teneurs totales des PhytoFs quantifiées dans les échantillons ont été respectivement de 543,10 ng/g bs (CP), 393,73 ng/g bs (CPH) et 278,02 ng/g bs (CH). En plus il est possible de relier la teneur en PhytoFs avec la teneur totale en ALA (respectivement 160,73, 82,76, and 52,07 mg/g bs dans la CP, la CH et la CPH).

En conclusion, des études supplémentaires sont nécessaires, ces travaux de thèse ouvrent de nouvelles perspectives, non seulement pour la valorisation de ces déchets agricoles, mais également pour l'élargissement de cette étude aux grains de café vert et de cacao aussi qu'à leurs dérivés.

#### **e) Identification et quantification des précurseurs de thiols**

La présence de précurseurs de thiols a été explorée en collaboration avec l'entreprise Nyseos (Montpellier), avec le même but d'élargir les profils moléculaires des CP, CH et CPH.

Les thiols sont des composés organiques soufrés agissant en tant que précurseurs d'arômes de divers aliments, tels que les fruits et les boissons [9][10]. Dans de nombreux cas, ces composés ne sont pas présents dans les matières premières, mais ils sont libérés lors de la



fermentation par des précurseurs sans odeur [25], comme dans le cas de la production des vins [26].

L'identification et la quantification des précurseurs de thiols ont été faites à partir de 0.1 g d'échantillon et 2 mL d'un mélange H<sub>2</sub>O/méthanol (1 :1 v/v). Les extraits obtenus ont été caractérisés par la méthodologie proposée par Bonnaïffoux *et al.* [27], en utilisant un système de chromatographie UPLC couplé à un spectromètre de masse à triple quadripôle.

Les résultats obtenus ont montré la présence de précurseurs 3-mercaptophexanol-cystéine (3MH-Cys) et 3-mercaptophexanol-glutathione (3MH-Glu) dans la CP et la CPH. Cependant, aucune molécule de ce type n'a été identifiée dans la CH. Les précurseurs des thiols étant libérés lors du processus de fermentation [28], il est probable que dans l'hypothèse où ils étaient présents dans la CH, ils aient été libérés pendant la fermentation précédente la première transformation du cacao.

Les teneurs en 3MH-Cys et 3MH-Glu ont été respectivement de 8,7 et 0,9 µg/100 g bs pour CP et de 43 et 25,32 µg /100g bs pour CPH. Ces teneurs sont comparables à celles décrites dans la peau, les grains et le moût de différentes variétés de raisin, de 0,21 à 60,78 µg/100 g (Cabernet Sauvignon et Koshu) [26].

A notre connaissance, il s'agit de la première fois que des précurseurs de thiols soient identifiés comme sous- et co-produits issus de la transformation du café et du cacao. Ces résultats représentent une nouvelle perspective d'utilisation des extraits de la CP et de la CPH en tant que possibles suppléments dans des aliments ou boissons fermentées, où ils pourraient être utilisés pour améliorer certains arômes fruités.

#### **f) Evaluation de la capacité antioxydante en utilisant la méthode VesiCAT (Vesicle Conjugated Autoxidizable Triene)**

Dans la dernière partie de ce travail, la capacité antioxydante des extraits éthanoliques (EtOH 70% v/v) obtenus par HSE (60 °C/ 1h) à partir de la CP, la CH et la CPH a été évaluée, par un test récemment développé au sein de notre laboratoire appelé VesiCAT (Vesicle Conjugated Autoxidizable Triene) [11]. La capacité antioxydante des principales molécules

trouvées dans les extraits a été testée (5-CQA, théobromine et caféine), et le trolox a été utilisé en tant que témoin.

Le VesiCAT est une alternative efficace pour mesurer à la fois l'oxydation de la membrane des liposomes mais aussi la capacité antioxydante des molécules. Le VesiCAT utilise une nouvelle sonde phospholipidique di-éléostéarique (DEPC) synthétisée et utilisée en tant que marqueur, permettant d'évaluer l'oxydation une fois incorporée dans des systèmes membranaires modèles (liposomes). En effet, l'absorbance des triènes conjugués présents sur les résidus d'acide gras du phospholipide (acide éléostéarique) est irréversiblement perdue lors de l'oxydation, ce qui permet ainsi une évaluation directe de l'oxydation à 273 nm par microplaque. Dans cet essai, l'oxydation est induite par deux différents générateurs de radicaux (AAPH (hydrophile) et AMVN (lipophile)), avec le but de comparer l'initiation radicalaire dans la phase aqueuse et à l'interface membranaire.

Tel qu'ils étaient attendus, les résultats obtenus avec les molécules standards (5-CQA, caféine et théobromine), montrent que seul le 5-CQA possède une capacité antioxydante (respectivement de  $1,711 \pm 0,068$  et  $0,057 \pm 0,004$  TE (équivalents trolox) avec l'AAPH et l'AMVN). Étonnamment, l'extrait de la CH a montré la meilleure capacité antioxydante dans les deux essais (AAPH/AMVN), malgré l'absence de 5-CQA. En effet, la valeur TE obtenue par l'essai AAPH pour la CH a été respectivement 1,2 et 4,15 fois plus haute que celle de la CP et la CPH. Dans les tests d'oxydation induits par l'AMVN, la valeur de l'extrait de CH en TE a été également 2,6 fois supérieure à celle obtenue pour la CP et la CPH. Bien que le classement en antioxydants obtenu pour la CP, la CP et la CH dans les deux essais ait suivi la même tendance, les valeurs observées avec AMVN étaient inférieures. Les extraits de CP, de CPH et de CH semblent exprimer une meilleure activité antioxydante en phase aqueuse. Il est probable que des PC non détectés dans les conditions d'analyse de cette étude, contribuent à l'activité antioxydante de nos extraits.

L'évaluation de la capacité antioxydante de CP, de CH et de CPH à l'aide de VesiCAT a montré que cette méthode constitue un bon outil pour examiner la performance antioxydante des extraits naturels.

### III. Conclusions et perspectives générales

Ces travaux de thèse ont montré l'énorme richesse en biomolécules des sous- et co-produits issus de la transformation du café et du cacao à travers la caractérisation chimique de la pulpe de café (CP), de la coque de cacao (CH) et de la cabosse de cacao (CPH).

L'utilisation de différentes combinaisons de DES (Deep eutectic solvents), en tant que solvants d'extraction a montré des résultats intéressants. En effet, les DES possèdent une capacité d'extraction efficace pour le 5-CQA (CP), la caféine (CP et CPH) et la théobromine (CH), présents dans nos échantillons. En plus, il a été possible de mettre en évidence la formation de furfural à partir de la CP, la CH et la CPH lors de l'extraction par un DES acide assistée par UPAE. Ce résultat est encourageant, car il ouvre une nouvelle voie de valorisation de ces résidus par la synthèse de cette molécule directement à partir du traitement de la biomasse par des DES acides, dès lors que le furfural a un large éventail d'applications industrielles. Ces solvants verts, simples, économiques et efficaces peuvent donc être utilisés pour l'extraction de biomolécules à partir de la biomasse végétale. Par ailleurs, une autre alternative attractive d'emploi des DES est de se servir de l'ensemble des molécules cibles extraites-DES pour des applications alimentaires, pharmaceutiques et/ou cosmétiques. Dans ce contexte, nous avons montré la possibilité d'utiliser une formulation DES riche en acides chlorogéniques, obtenue à partir des grains de café vert, afin de tester sa toxicité *in vivo* chez le rat. Bien que les résultats obtenus soient préliminaires et qu'il y a encore un long chemin à parcourir en ce qui concerne la toxicité des DES, cette recherche est pionnière, et les futurs travaux contribueront à comprendre les facteurs et les risques liés à l'application des DES *in vivo*.

L'identification et la quantification pour la première fois des PhytoPs, PhytoFs et précurseurs de thiols ont permis d'élargir la gamme actuelle de biomolécules reportées dans la CP, CH et CPH, et aussi d'ouvrir la voie à des nouvelles perspectives de valorisation. Cependant, des études complémentaires sont nécessaires afin d'établir, par exemple, une relation entre les conditions agronomiques de cultures et industrielles de transformation sur la concentration des PhytoPs, PhytoFs et précurseurs de thiols dans les sous- et co-produits issus de la transformation du café et cacao.

Dans le cas des précurseurs de thiols, d'autres études sont nécessaires afin (i) de mieux les caractériser dans la CP, la CPH et la CH et d'associer leur teneur à l'influence des conditions de culture et des procédés post-récolte, (ii) de déterminer les meilleures conditions nécessaires pour leur formation dans ces résidus et enfin, (iii) d'examiner leur application possible dans des aliments fermentés ou des boissons.

Finalement, l'utilisation de la méthode VesiCAT pour évaluer la capacité antioxydante des extraits de CP, CPH et CH a montré que les molécules présentes dans ces matières premières, sont plus efficaces dans un milieu aqueux.

Dans certains rapports scientifiques, on considère que les CP, CH, et CPH, sont encore sous-exploités, ce travail de recherche a donc démontré que ces résidus sont des sources intéressantes de biomolécules qui méritent d'être d'avantage étudiées. Ces travaux de thèse ont donc contribué à enrichir la connaissance des biomolécules à haute valeur ajoutée présentes dans les résidus issus de la transformation du café et du cacao, tout en utilisant la chimie verte à l'aide des solvants respectueux de l'environnement pour leur extraction.

**Ces travaux de thèse ont été soutenus pour la bourse doctorale numéro 405131 accordée par le Conseil National de la Science et de la Technologie (CONACyT) Mexique. L'étude *in vivo* chez les rats a été soutenue pour le Council Scientifique de Montpellier SupAgro.**

# *General introduction*



## General introduction

The food and drink industries are an important sector of the world's economy. Nowadays, food production is facing the challenge to feed about 7.5 billion people in the world. Even worst, it has been estimated that the global demand for food will continue to increase at least over the next 40 years [29]. Indeed, the earth's population is expected to grow to nearly 10 billion in 2050, with a stronger increase in developing countries where already important issues exist regarding food security, natural resource management and poverty alleviation. Additionally, these countries are more vulnerable to climate-change conditions, due to their low capacity of adaptation [30]. This situation will increase the inequality between rich and poor countries. Thus, the challenge in the next years is not only to maximize the food production, but also looking for solutions to face food safety and security, as well as social equality to build sustainable farming systems capable of feeding human beings while preserving the environment.

One of the immediate consequences of population increases has been the increment of agricultural lands to produce crops predestined to human and animal consumption. As consequences, the increase of waste from food industrialization has been also intensified, either derivate from (i) the agro-residues which are left in the field directly after crops harvest or (ii) those separated from the product while processing [31].

Coffee and cocoa are important crops consumed by millions of people every day. They have gained popularity throughout the history due to their flavor. Additionally, both products have a medicinal, recreational, and social role around of the world [32][33].

The importance of coffee and cacao is such that in 2016 the worldwide production of green coffee and cocoa beans reached 9.22 and 4.47 million tons, respectively [34]. However, this production has led to important social, economic, and environmental impacts. The industrial transformation of coffee and cocoa generates large quantities of by- and co-products, which become unused waste when they are simply discarded. By-products are defined as the plant products that are left-over after separation of the main value product, *e.g.* cocoa pod husk (CPH) derived from the primary processing from cocoa; and coffee pulp (CP), parchment skin, mucilage, and low-grade green coffee derived from the primary wet coffee processing.

Co-products are defined as inedible ingredients derived from the food secondary processing, *e.g.* cocoa husk (CH) separated from cocoa bean after roasting. These residues may have valuable properties (i) to be used for other goods, (ii) to be transformed once into another useable product, (iii) as alternative renewable energy sources, or (iv) to be used as a source of bioactive compounds possessing interesting physicochemical and/or biological properties, which might be helpful for several food and non-food purposes (*e.g.* food, nutraceutical or drug ingredients, additives, enzymes, bulk chemicals, biologically active secondary metabolites, etc.) [35].

Considering the annual production of coffee beans (2016), it is estimated that 9.72 million tons of solid wastes (including hulls, skin mucilage, and pulp) were generated in that year [36]. In the case of cocoa, only 10 % of the total fresh fruit weight corresponds to the beans [37][38]. Moreover, 40.19 million tons of CH and CPH were produced in 2016.

Since the 70's, several studies have been carried out in order to add-value to coffee and cocoa by- and co-products. Despite the fact that they could be an inexpensive and rich source of valuable molecules, their management, reduction, and re-utilization is still a challenge. Several solutions and alternative uses of CP, CPH, and CH that have been attempted include their use as fertilizers, livestock feed, compost, vermicompost, for the cultivation of moulds and yeasts, or to produce mushrooms, enzymes, alcohol, organic acids, aroma compounds, gas, or to obtain enriched extracts in pectins, alkaloids or phenolic compounds, etc. [39]. In addition, since these organic residues have no clear use, sometimes they are burned or spilled in landfill site, leading to landscape deterioration, generating CO<sub>2</sub>, pollution of water resources, annoyances by off-odors, mold botanical diseases, and insects proliferation [40].

The management of coffee and cocoa agro-residues is also affected by the economic and social situation in the producing countries. Therefore, in this field, studies are still necessary in order to improve environmental, economic, and social aspects around the coffee and cocoa industry. Reducing the amount of wastes and increasing the reuse of by- and co-products derived from the coffee and cocoa processing, may contribute (i) to decrease the negative environmental impact of the agricultural waste derived from coffee and cocoa processing, and to respond to the “zero-waste” society requirements, by improving

sustainable practices and integrated coffee and cocoa production systems, in collaboration with actors of the coffee and cocoa transformation chain, (ii) to improve rural and local industrial development by diversifying uses of these agro-wastes, (iii) to complete the actual portfolio of interesting bio-molecules obtained from the coffee and cocoa by- and co-products, and (iv) to contribute to the development and economical situation in the producing countries by creating an additional income for coffee and cocoa producers, as the benefits from the expansion of the coffee industry do not always trickled down to them.

CP, CH, and CPH are abundant and concentrated in the production areas, which is an advantage to facilitate their transformation, on condition that a proper collection and rapid transformation are made, as these residues deteriorate very fast.

CP, CH, and CPH are thus ultimate substrates for the recapture of functional compounds and the development of new products with a market value. They could represent an inexpensive source of high-added value biomolecules possessing interesting physicochemical and biological properties that can be used for the production of value-added products. For example, phenolic compounds could be applied as natural antioxidants in food or beverage; pectin could be used as gelling agent in confectionary or as fat replacement; water insoluble fibers (e.g. hemicelluloses) can be used in food products to improve water retention and texture or in the formulation of functional foods to improve intestinal regulation; alkaloids can be used in soft and energy drinks, etc. [36][37].

The recovery of valuable compounds from natural resources is conducted using the so called “5-stage universal recovery processing” that includes: (i) macroscopic pretreatment, (ii) separation of high-molecular from low-molecular compounds, (iii) extraction, (iv) purification/isolation, and (v) encapsulation of product formation [41]. For each step, different conventional processing techniques have been proposed in the literature to recover biomolecules from CP, CPH, and CH, such as *e.g.* drying or freeze drying, reduction of particle size, followed by alcohol precipitation (when necessary) and water or solvent extraction. Other more efficient and environmentally friendly methods like enzyme-, ultrasound-, and microwave-assisted extraction, and supercritical fluid extraction have been reported.



In this context, the main interest of this thesis research was to offer new perspectives to the valorization of the main co- (CPH) and by-products (CP, CH) derived from processing of coffee and cocoa, by:

- An innovative environmental friendly approach using green solvents (Deep Eutectic Solvents called DES), in order to obtain from CP, CPH, CH, and green coffee, enriched extracts in bioactive molecules such as *e.g.* phenolic compounds, which can be used in food industry and for health or non-food applications. DES were considered for their green chemistry standpoint. They are non-volatile, easy to prepare, and inexpensive, and may be biodegradable and non-toxic. Additionally, DES offer the possibility to improve extraction yields, compared to other conventional solvents. In addition, depending on the nature of starting molecules involved in the DES formulation, tunable DES may be prepared to design task-specific medium for targeting molecule extraction.
- A broaden investigation to discover new interesting bio-molecules (namely isoprostanooids and thiols precursors) in order to expand the actual portfolio of value-added molecules from the coffee and cocoa residues. Isoprostanooids and thiols precursors possess interesting properties and, as far as we know, they have never been reported on coffee and cocoa agro-residues.

The present manuscript is organized into four main chapters. The first one corresponds to the bibliographic review. It introduces the thesis research areas, provides information about coffee and cocoa crops, but also a description of the Deep Eutectic Solvents and the studied biomolecules. The second chapter aimed at listing the materials and detailing the methods used for experiments. The third chapter (Results and discussions) covered the extraction and identification of biomolecules. Moreover, the *in vivo* effect of DES formulation in murine model and the evaluation of antioxidant capacity (using VesiCAT: vesicle conjugated autoxidizable triene assay [11]) were performed and discussed. Finally, the last chapter was devoted to the general conclusions and perspectives of this work.

# *Literature review*



# Literature review

## I. Coffee

### 1.1 Description, consumption and production

Coffee beans are important agricultural and economical commodities around the world [42] [1], which have been consumed for over 1,000 years [43].

*Coffea arabica* L. knew as Arabica coffee, is originally from the province of Kaffa in Ethiopia [44]; however Arabia was responsible for the coffee culture propagation. Then the habit of coffee drinking was spread all over the Moslem Middle East and reached Cairo in the period 1500-1510, Constantinople (now Istanbul) and Turkey in 1554, then Venice in 1560. It was the popularity of the coffee houses for all classes that promoted the drinking of coffee among the people of the Middle East.

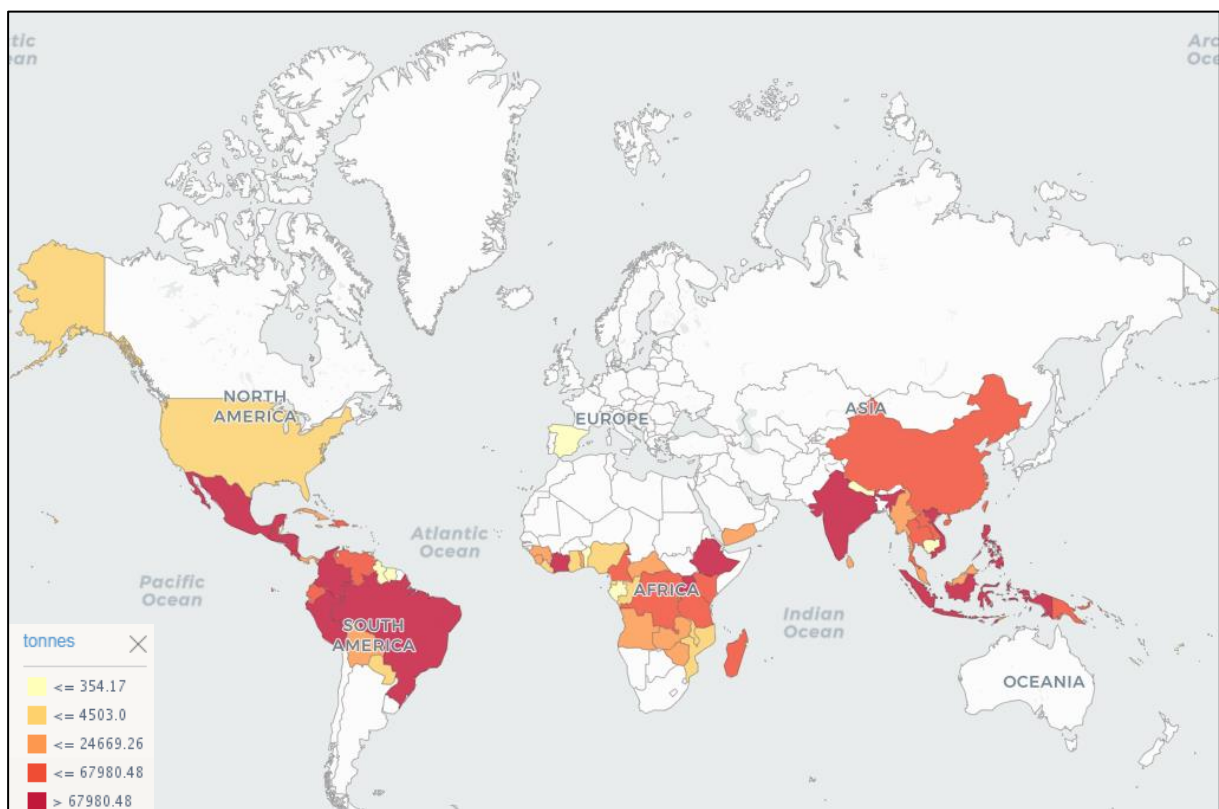
Robusta coffee (*Coffea robusta* L.) occurs wild in the equatorial forest from West Africa to Lake Victoria, mainly between 10° north and south of the equator, from sea level to 1500 m. Its origin is unknowing, but exist a debate between Uganda and elsewhere in Equatorial Africa, where it was planted around 1900, before the arrival of Europeans [45]. On the other side, Arabica coffee develops well in high altitudes (600–2,000 m), while Robusta coffee plantations adapt well in altitudes below 600 m [43].



**Figure 1. Ripe coffee beans.**  
From: [46]

The coffee tree or shrub is a perennial evergreen dicotyledon that belongs to the *Rubiaceae* family of the genus *Coffea*. Both the Robusta and Arabica coffee plant can grow to heights between 10-12 m if not pruned, but producing countries will maintain the coffee plant around 2-4 m for easy harvesting. They have a main vertical trunk (orthotropic) and primary, secondary, and tertiary horizontal branches (plagiotropic). In most species, the flowers are born with leaves, but in some, the flowers are born on leafless branches (Figure 1).

The coffee bean comes from the cherry or berry of the coffee plant which is actually the plant's fruit. It is produced after the withering of a white flower. Each cherry consists of two seeds (beans) covered by a thin parchment like hull and further surrounded by pulp. The cherries are green at first and turn to red when they are ripened, this takes between 6 to 14 months, depending on the species of tree [45] [43].



**Figure 2. Green coffee production in the world.**

From: [47]

Nowadays, although 70 different species of coffee are referenced, the coffee beverage is made with roasted coffee beans from Arabica and Robusta coffee [1]. Arabica coffee, which

has higher quality and aroma, provides between the 75-80 % of the world's production. While, Robusta coffee, which is more acid and more resistant to plagues, with higher caffeine content, provides the rest of the 20 % worldwide production. Coffee beverage is consumed around the world, but the main consumer countries are Finland, Norway, and Iceland, with 12, 9.9, and 9 kg/person/year, respectively [48].

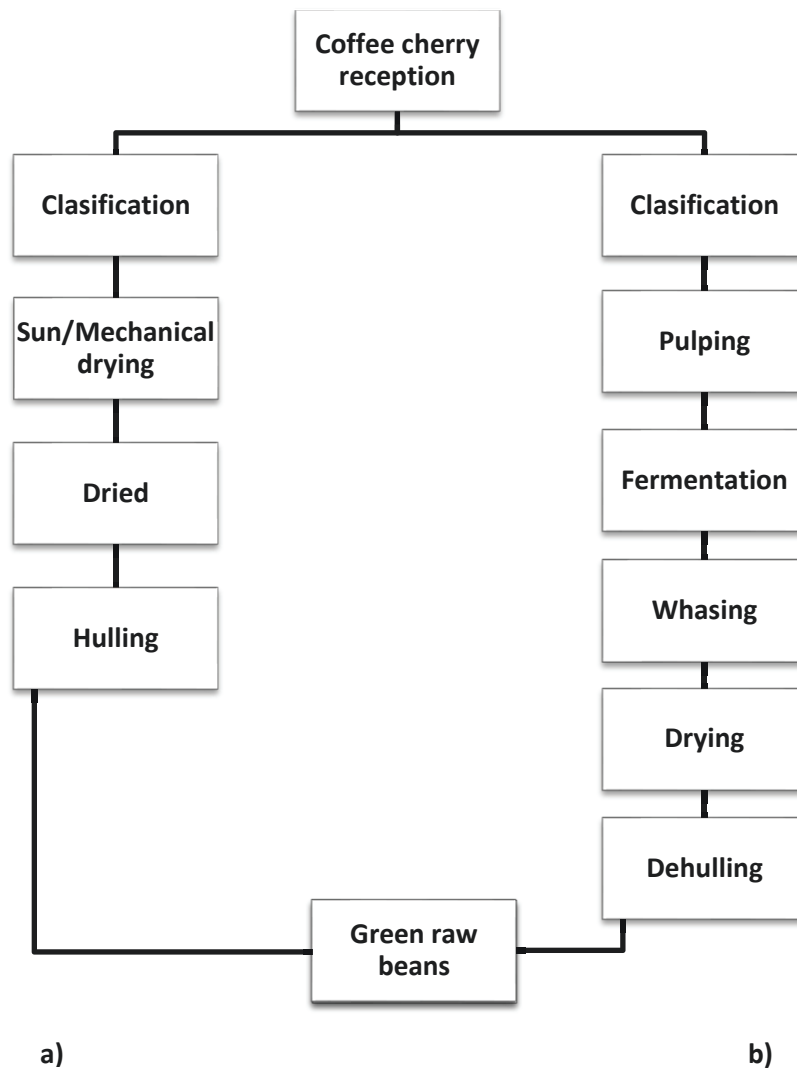
On the other hand, the main producer country in 2016 was Brazil, with around 3 million tons of green coffee, following by Vietnam, Colombia, Indonesia and Ethiopia [34] (Figure 2). In the same year, Mexico produced 151,714 tons, ranking in the eleventh position in the worldwide production. The main producers are in the South and Center of the country, specifically Chiapas, Veracruz, and Puebla with 339, 194 and 129 thousand tons, respectively [49].

## **1.2 Coffee processing**

The first goal of the coffee processing is to obtain the green coffee beans from the cherry coffee. This process is carried out in the producing countries, and starts with the removal of both the pulp and hull. To achieve this goal, three different process are used: (I) dry processing of natural coffee, (II) wet processing of washed coffee (Figure 3), and (III) intermediate semidry (pulped natural) process [1].

- (I) Dry process is the oldest and the simplest. It consists in three steps: classification, drying, and dehulling [1], [50]. The classification consists in the removal of unripe and damaged cherries; other materials and dirt. Then the whole cherries (including exocarp, mesocarp, and endosperm, i.e. pulp, parchment, and bean) are dried, with either a natural (sun-dried) or an artificial (in furnaces) process [44] to obtain the parchment coffee. The drying rate of parchment is dependent on the initial moisture of the parchment, ambient air temperature, humidity, thickness of the spread, and periodicity of stirring [1]. Finally, the dried coffee husk are removed by a dehulling machine, to release the coffee beans [50]. The dry process is employed by more than 80 % of Brazilian, Ethiopian, and Yemen Arabica coffees and for almost all Robusta coffees in the world.

- (II) The coffee wet process is the only method used in Mexico, and the principal method that is used worldwide for Arabica coffee. This process leads to better quality of coffee, resulting in a higher price. It is based on the following steps: classification, pulping, fermentation, washing, drying and dehulling. Classification is carried out in a large tank filled with flowing water. Usually the first step is carried out in two or more stages, first to remove stones and dirt, and second to separate the damaged and unripe berries by flotation [50]. Then the outer skin and most pulp of the sunken fruits are mechanically removed by pressing the fruit in water through a pulper machine. The obtained beans are fermented to remove a layer of pulp remnants and the mucilage layer. The time of fermentation varies according to the location, with temperature being an important factor, from about 6 to as long as 80 h. But in general, the degradation of mucilage takes approximately 24-36 h for Arabica and 72 h for Robusta [44]. After fermentation, beans are washed and dried in drying barbecues and spread evenly to a thickness of 5 cm average. Here, the coffee is stirred with wooden rakes to ensure uniform drying and reduce parchment splits [44]. Coffee beans are dried until the moisture content reaches around 12 %, and then de-hulled to remove the parchment.
- (III) In the semidry process, often referred to as “the pulped natural process”, the mucilage is not removed after pulping, and parchment and mucilage are dried together. Green coffee is obtained by hulling dry parchment with the dried mucilage adhering to it. The semidry process is used in Brazil and Burundi [1].



**Figure 3. Coffee dry (a) and wet (b) processing.**

Adapted from: [51]

The resulting solid wastes (parchment and silverskin) are collectively named “parchment husks”. Overall, for every one ton of clean coffee produced, 0.28 ton of parchment husks and 0.5 ton of pulp are generated [52]. Thus, coffee pulp (CP) is the main and first by-product obtained during the wet processing of coffee. CP represents 40 % of the fresh weight of coffee cherries [53].

Green coffee non suitable for consumption is called “low-grade green coffee” and is widely known to adversely affect the beverage quality. These defective coffee beans (immature, broken, bleached, insect damaged beans, black beans...) are rejected after separating the graded ones according to the size, color and aspect. They represent about 15–20 % dw of

coffee production and attempts are being explored for their utilization, as they are a problem for disposal. According to Ramalaskshmi *et al.* [54], there are marginal differences in the physical and chemical parameters among the graded and defective coffee beans, in particular CGA, making them suitable as a source of natural antioxidant compounds and other interesting biomolecules (Table 1). Considering the nine main isomers of CGA: 5-CQA, 4-CQA, and 3-CQA; 3,5-diCQA, 4,5-diCQA, and 3,4-diCQA; 5-FQA, 4-FQA, and 3-FQA, in order of abundance in green coffee beans. 5-CQA alone is responsible for about 56-62 % of total CGA. DiCQA isomers account for about 15-20 % of total CGA in green coffee beans and FQA isomers, for 5-13 % of total CGA. *p*-CoQA isomers, CFQA isomers and the newly identified diferuloylquinic acids and dimetoxycinnamoylquinic acid derivatives account together for the remaining percentage [55].

**Table 1. Chemical composition of Arabica green coffee beans**

Component	Content (% w/w dw)
Cellulose	42.0 ± 1.0
Hemicellulose	7.5 ± 2.5
Lignin	2.0 ± 1.0
Total sugars	10.8 ± 1.8
Protein	10.25 ± 1.75
Lipids	16.5 ± 1.5
Total polyphenols	7.9 ± 1.3
Caffeine	1.1 ± 0.3
Ash	4.2 ± 1.2

From: [43]

### 1.3 Chemical composition of coffee pulp

CP is an organic material with a rich composition (Table 2). This by-product is especially rich in carbohydrates and proteins. It is also a source of interesting biomolecules such as tannins and the caffeine. This latter is a powerful and addictive stimulant, considered as the principal substance causing the mild stimulation effect of coffee. In CP, the caffeine content represents about 1.5 % of the dry matter. On the other hand, tannins and the caffeine are generally considered antinutritionals, so their use in animal feed cannot be greater than >10 % [51].



**Table 2. Concentration of major molecules found in coffee pulp (CP).**

Components	Content (% dw) *
	[36] [43] [56] [36]
Proteins	11.5 ± 2.0
Cellulose	17.7
Hemicellulose	2.3 ± 1.0
Lignin	17.5 ± 2.2
Lipids	2.0 ± 2.6
Pectin	6.5 ± 0.9
Ash	0.4
Total sugars	14.4 ± 0.9
Tannins	3.0 ± 5.0
Caffeine	1.5 ± 1.0
Chlorogenic acid	2.4 ± 1.0
Total phenolic compounds (GAE**)	2.62

\*dw= dry weight. \*\* GAE: gallic acid equivalents (Folin-Ciocalteu assay)

CP is also source of other phytochemicals for the food and pharmaceutical industries [57], such as phenolic compounds (PC). In Arabica coffee pulp, the four major classes of PC are flavanols, hydroxycinnamic acids (*e.g.* caffeic, ferulic, and *p*-coumaric acids [58]), flavonols and anthocyanidins, Ramírez-Martínez and co-workers [58] identified by HPLC in fresh coffee pulp the following PC: chlorogenic acid (5-*O*-caffeoylquinic acid, IUPAC numbering [59]; 42.2 % of the total of identified phenolic compounds), epicatechin (21.6 %), 3,4-dicaffeoylquinic acid, (5.7 %), 3,5-dicaffeoylquinic acid (19.3 %), 4,5-dicaffeoylquinic acid (4.4 %), catechin (2.2 %), rutin (2.1 %), protocatechuic acid (1.6 %), and ferulic acid (1.0 %).

Despite its composition in valuable molecules, the CP does not have a defined use in the industry, and its management still remains a challenge. Indeed, in the producing countries coffee wastes and by-products constitute a source of contamination. Among them, CP represents the most serious environmental problem, due to its high moisture content (76.7 %), which contributes to its rapid putrefaction [36]. However, due to its versatile composition in different classes of molecule, with relatively high content, CP can be used for different applications.

## 1.4 Coffee pulp applications

The large production of coffee around the world produce as residue the CP, which represent a pollution problem. In order to manage this challenge, CP has been utilized as fertilizers, livestock feed, and compost. Nevertheless, these applications consume only a fraction of available CP and the methods are not technically efficient [42]. For this reason, research has increased its interest in exploring new perspectives for its use. Here is a review of the different coffee pulp applications.

### 1.4.1 Composting and vermicomposting

The composting and vermicomposting are a traditional application for CP. Composting can be defined as a solid-waste management system that accelerates the process of decomposition of biomass [51]. When this process is not controlled, it may result in several problems due to the grow-up of macro- and micro-fauna (*Acarida*, *Coleoptera*, *Collembola*, *Diptera*, *Eisenia*, *Oligochaeta*, *Perionyx*, *Thysanoptera*, etc.) [39]. On the other hand, vermicomposting takes advantage of this process; resulting in a very effective biological process to stabilize organic wastes by transforming them into a source of nutrients and soil conditioner [1].

Several species of earthworm found in CP such as *Amyntas gracilis*, *Dichogaster sp.*, *Eisenia andrei*, *Eisenia fetida*, *Perionyx excavates*, have been exploited for vermicomposting with good results. Indeed, the vermicomposting carried out by the earthworm *Eisenia fetida* has showed to increase the P, Ca, and Mg available, but decrease the K content. Other study showed the efficiency of exotic earthworm (*Eudrilus eugeniae*) to degrade the CP. These studies indicated that the CP vermicomposting enhanced the nutrient value, the microbiological quality of compost, and became a source of plants nutrition [60].

### 1.4.2 Livestock feed

Traditionally, agricultural residues have been used for livestock feed. In the case of CP, several studies have been carried out to evaluate its nutritional characteristics and potential, as well as its limitations, in feeding systems. The evidence tends to indicate that the

antinutritional factors, protein digestibility, and nitrogen retention are major factors limiting the use of CP as animal food [1]. Nevertheless, the CP effects in the animal feed have been investigated in the diets of different farm animals. The results showed that it can be used until 20 % in cattle diet, 5 % in poultry feed, 3 % in bird and 16 % in pigs [61]. Indeed the possible negative effects due to the presence of caffeine, tannins and other polyphenols in CP, can be reduced or neutralized using mixtures with poultry litter. Studies have also been carried out to use the CP as a feed ingredient for fishes. Results showed that neither fresh nor ensiled CP, was considered as suitable foodstuff for Nile tilapia. However, CP showed potential as a feed ingredient in fish diets, if fish are reared in earthen ponds or pens instead of concrete tanks or raceways [51]. On the other hand, the fermentation could be an option to detoxify the CP and to facility its use [39].

### 1.4.3 Silage

Silage is a very common process usually used to treat the agrowastes. In general, it involves simple technology, starting to decrease the water content in CP from 80-85 % to 65-70%; commonly through sun dehydration. Once the desired moisture content is reached, CP can be ensiled without additional processing, with or without additives [36].

In recent studies, silage has been tested to reduce the anti-physiological compounds in CP in order to be used as organic fertilizers without any treatment, or for animal feed. For example, the CP silage, and its mixtures with sugar cane molasses, or with molasses and ammonia, showed important reductions in caffeine and total polyphenols [62]. Others compounds such as sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ ), urea ( $\text{CH}_4\text{N}_2\text{O}$ ), calcium carbonate ( $\text{CaCO}_3$ ), and dicalcium phosphate ( $\text{CaHPO}_4$ ) have been tested in ensiling CP, but none of these additives improved the quality of the silage [36].

### 1.4.4 Detoxification

The presence of compounds such as alkaloids, tannins, and PC, gives the anti-physiological and anti-nutritional character of CP. Consequently, CP is not a suitable substrate for bioconversion processes. Thus, detoxification of CP has been studied through physical, chemical, and microbial approaches. The use of fungi strains such as *Aspergillus*, *Rhizopus*,

*Phanerochaete*, and *Penicillium* have shown the highest ability to degrade caffeine from CP [63]. On the other hand, other studies showed that anaerobic fermentation of CP resulted in about 13 to 63 % reduction of caffeine in 100 days. In contrast, aerobic fermentation resulted in 100 % degradation of caffeine in 14 days [64].

#### 1.4.5 Enzymes production

The CP lignocellulosic fraction has been studied in order to produce enzymes such as pectinase, caffeinase, xylanase, etc. For example, the pectinase production increased four-fold using CP in solid state fermentation (SSF) by *Aspergillus niger* V22B35 [65]. Other study explored the use of coffee by-products for the production of xylanase under SSF. The maximum yields were reached by *Penicillium sp* using CP as support [1]. Additionally, the production of  $\alpha$ -amylase using different coffee industry substrates was carried out using fungal organisms such as *Neurospora crassa*, *A.oryzae*, and *A. niger* under SSF. The results showed that CP produces the highest activity of  $\alpha$ -amylase [66]. Other enzymes such as cellulases, proteases,  $\beta$ -amylase,  $\beta$ -glucosidase, and lipases were produced using anaerobic fermentation of CP [39].

#### 1.4.6 Ethanol and biogas production

Bioethanol is a biofuel that is considered as a promising substitute for fossil fuels. However, it is necessary to reduce production costs by using alternative biomass feedstock. Recently, agricultural residues have been investigated to produce biofuels, as renewable and alternative sources for energy production.

The potential production of bioethanol from dry and wet (80-85 % moisture) CP was studied for Shenoy *et al.* [67] using *Saccharomyces cerevisiae*. They found theoretical ethanol yields up to 9.35 and 40 % for dry CP and wet CP, respectively, compared to those of several cereal straws and hays (52.00-65.82 %) reported by Chet *et al.* [68].

In another study five different fermentation media were tested for the ethanol production. The substrates were sugarcane juice or molasses diluted with water or with CP extract, and a medium with only CP extract [69]. The addition of CP extract to sugarcane juice or molasses did not influence the fermentation or yeast viability. Adding coffee pulp extract has the

advantages of utilizing the sugars present in it and using less water during the fermentation process, with a theoretical ethanol yield of 51 %.

Further, the CP has the potential for direct conversion into biogas through anaerobic digestion. Biogas is the name of a mixture of CO<sub>2</sub> and methane (CH<sub>4</sub>) produced by bacterial conversion of organic matter [51]. According to the estimation, about 131 m<sup>3</sup> of biogas could be produced by anaerobic digestion from 1 ton of CP, which would be equivalent to 100 L of petrol in fuel value [70]. The biogas production by the co-digestion of CP and cow-dung mixture showed that after the first month, CH<sub>4</sub> content in the biogas reaches 50 % of the yield. This content increased up to 60 %, and remained almost constant for at least 8 months after further digestion [71]. Robusta and Arabica coffee solid residues were found to generate about 650 and 730 m<sup>3</sup> of CH<sub>4</sub> per ton of volatile solids, respectively. These yields were highest in comparison with any other agro- industrial residues (230, 400, and 450 m<sup>3</sup> for sugar filter, sisal, and maize bran, respectively) [72].

The CP pressing juice has been also studied for the biogas production by one- and two-phase anaerobic systems. This last one system carried out by acidogenic and methanogenic phases produces the best conditions to reach 75 % of CH<sub>4</sub> in the gas [73].

#### **1.4.7 Food products and aroma compounds**

The agro-residues have been studied for their applications in the fabrication of food commodities. For example, the fiber content in CP (cellulose, hemicelluloses, lignin, and others polysaccharides) could confer a wide range of health benefits including prevention of gastrointestinal diseases, cardiovascular diseases, and obesity. In addition, the possible synergistic effect between antioxidants and fibers could justify the use of CP as nutraceutical and preservative in food formulations. Furthermore, fresh CP can be easily processed into various food commodities like jam, juice, concentrate, jelly, and flavoring [42]. More recently, CP from Nicaragua has been commercialized by a Spanish company (Casa Eguía) to make tea. Additionally, the lignocellulosic biomass in CP can be converted into a number of added products by chemical and enzymatic treatments, such as fermentable sugars [1].

On the other hand, the production of aroma compounds from CP for their application in food industry has been also studied. For example, Pedroni Medeiros *et al.* [74] tested the ability of two different strains of *Ceratocystis fimbriata* for fruity aroma production by SSF on CP and coffee husk complemented with glucose. The main produced volatiles compounds were ethyl acetate, ethanol and acetaldehyde. This result shows the great potential for the use of CP as substrate to microbial aroma production by SSF. The production of fruity flavors with the same microorganism has been also investigated by Soares *et al.* [75], but the SSF was carried out using the coffee husk obtained during the processing of coffee cherries by dry process. The production of pineapple and banana aroma was achieved with different concentrations of glucose (20–46 %), and under optimized conditions, a total of 13 compounds were produced.

#### 1.4.8 Source of biomolecules

CP is a rich source of different biomolecules, among them, PC are the most important. The four major classes of polyphenols found in CP are hydroxycinnamic acids, flavanols, flavonols, and anthocyanidins, (detailed description provided in the section 1.3). These compounds have several biological activities. For example, the CP has been recognized for its antioxidant capacity attributed to the presence of phenolic acids such as chlorogenic acid and protocatechuic acid [76][77]. Moreover, it has shown antimicrobial activity against Gram (+) bacteria, which was explained for the presence of quinic acid, malic acid, and chlorogenic acid as well as the important caffeine content in the samples [77].

## **II. Cacao**

### **2.1 Description, consumption and production**

Cocoa is a very important economic crop. For example, it is the third most important beverage after tea and coffee [1]. Moreover, it is used for the fabrication of a wide range of food products, such as the chocolate.

Cacao could be native to tropical regions of the northern part of South America or, according to some reports, of Central America [78]. Actually, the dissemination of this crop could have been from the tropical lands of South America to the southeast of Mexico. However, some theories propose the contrary, which means that the crop extended from Mexico or Central America to the Amazonas basins. Although it exists controversies about its origin, this tree is undoubtedly autochthonous from the American continent [79]. Indeed, the cacao has been cultivated for over 2000 years in Mexico and Central America for the Aztec, Mayas, and Incas cultures. In these cultures, the cacao was already consumed as food, energizer beverage, and as a general tonic to protect against diseases. The cacao beans were used also in religious rites, or as currency in some civilizations to buy slaves and luxury items [80].

After the arrival of Spanish people in Mesoamerica in the middle of 1500, the way to consume the cocoa revolutionized, producing the called “creolization” of chocolate. The “creolization” was characterized by changing the traditional preparation of the cocoa beverage, originally with chili and pepper, for sugar, cinnamon, and vanilla. Immediately, the cocoa was spread to Europe, and then to Asia and Africa [79]. In this last continent, the cocoa seed was planted successfully in Nigeria, Ghana, and Cameroon, in 1854, 1874, and in the period of 1925 to 1939, respectively [81].

Nowadays, there are three economical important species: Criollo, Forastero, and Trinitario.

Criollo’s cultivars dominated the market until the middle of the eighteenth century [81], and were cultivated during the pre-Columbian and Spanish colonial period [82]. Criollo has been recognized for its flavor [83] and quality, but with a low vigor and yield [84]. This type is grown mainly in Central America, where only 5 to 10 % of chocolate is made. Forastero

derives from the subspecies *sphaerocarpum*, which includes several sub-varieties. In general, Forastero species are identified according to the pod morphology, e.g. the Amelonado is characterized by a melon shape [83]. Additionally, the Forastero cacao has a high yield production and is very resistant to diseases; therefore it is the most common commercial specie. Indeed, between 80 to 90 % of world chocolate production is made from the Forastero specie [85], which is grown mainly in Africa.

On the other hand, the Trinitario cacao also known as “fine cacao” is descended from a natural hybridization between Criollo and Forastero (Amelonado variety) [81]. This tree variety has great resistance to damages and diseases. Its plantation started in Trinidad, and was spread to Venezuela and then to Ecuador, Cameroon, Samoa, Sri Lanka, Java, and Papua New Guinea. It is considered as an excellent cacao type and accounts for 10 % to 15 % of the worldwide production, offering of "very fine" chocolate with a fruity aroma and slightly acid, spicy and sharp savor [85][81].

All these cultivars belong to the genus of *Theobroma* from the *Malvaceae* family, and *Sterculioideae* subfamily, which includes 20 different species [86].

*Theobroma cacao* L. is a small (4-8 m) evergreen tree, which grows out in a belt between 10°N and 10°S of the Equator. The cacao tree responds well to relatively high humidity and temperatures (between 18 and 32 °C), and an annual rainfall level between 1500 and 2000 mm. The trees live for up to 100 years, but cultivated trees are considered economically productive for about 60 years. They have dark green egg-shaped or elliptic leaves of 20 to 35 cm long and 7 to 8 cm wide. Cocoa flowers are small, yellowish white to pale pink, and grouped together in clusters arising directly from the trunk (cauliflory). After the pollination of flowers, the fruit (commonly referred to as a “cocoa pod”) start to grow out directly from the trunk. The cocoa pod is green, and turns to yellow, orange, red or purple after maturation. These vary significantly in size, shape and texture, but usually, reach between 15 to 25 cm long, with a more or less knobbly surface and lines from up to down (Figure 4).



a)



b)



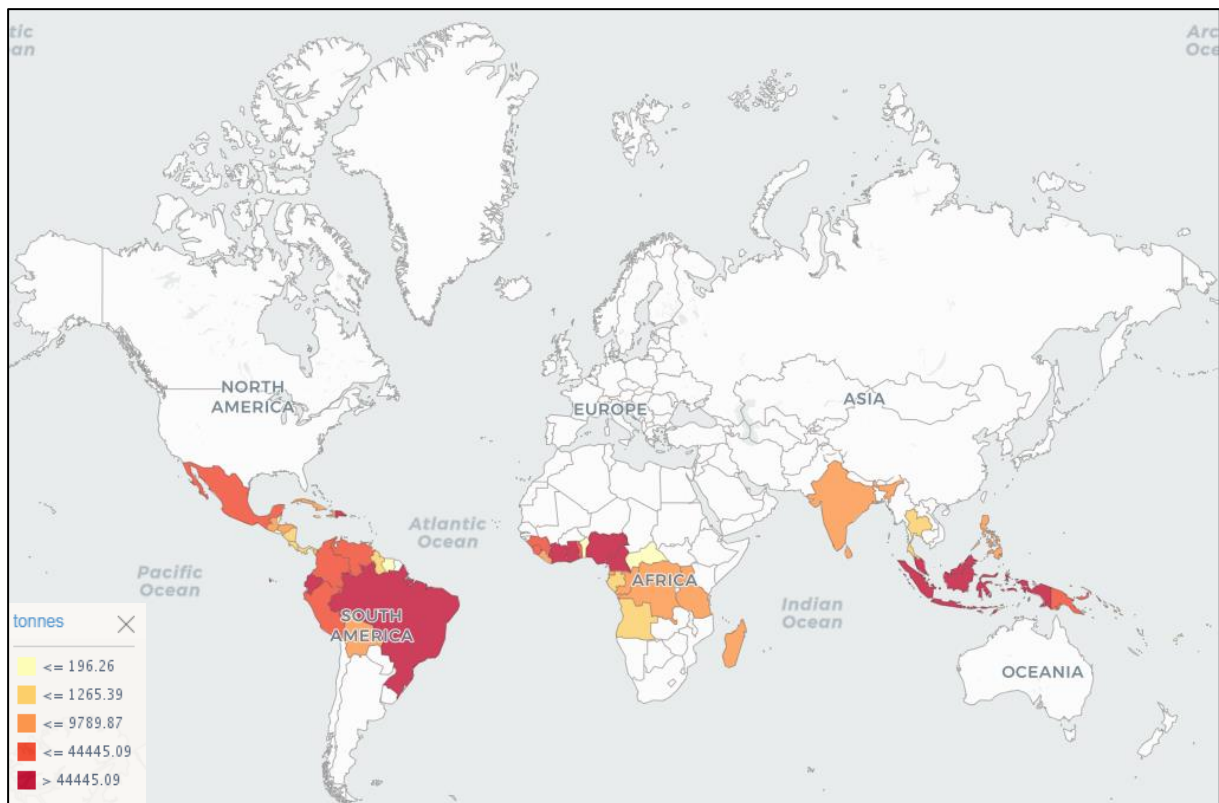
**Figure 4. a) cocoa beans, b) cacao fruit.**

From: [87]

Between 30 and 40 seeds are encased inside the cocoa pod; each one is surrounded by a bitter-sweet white pulp [85] [88] [89]. It is precisely from these seeds that the chocolate is made, and in this form; it widely consumed especially in the European countries. Indeed, in 2015 the top of the consumer countries are Switzerland, Germany, Russia, and United Kingdom, with 8.8, 8.4, 7.3, and 6.8 kg/person/year, respectively [90].

In addition, Netherlands, United States, Malaysia, Germany and Belgium are the main transformation countries, since they are the top importers of cocoa's beans [34].

The main cocoa's producers around the world are Ivory Coast, Ghana, Indonesia, Cameroon, and Nigeria. Together, they produce 3.5 million tons in 2016 and covered the 78.71 % of the worldwide production (Figure 5) [34]. The production in Mexico reached 26,863 tons of cocoa beans, ranking as the thirteenth worldwide producer. Chiapas, Guerrero, and Tabasco are the main producers regions in Mexico [49].



**Figure 5. Cocoa beans production in the world**  
From: [47]

## 2.2 Cacao processing

The cocoa beans process takes place in two stages, known as preprocessing and processing. The first step is carried out on the field, by the cocoa producer, and encompass the (I) harvesting, (II) fruit opening and removal of the seeds, (III) fermentation, (IV) drying of the beans, and (V) storage.

- (I) Harvesting is carried out when the pod is changing color, usually from green to yellow, red or orange. At this moment, they are cut by hands from the trunks or with a machete (cutlass) for the pods that are near to the ground. For the pods on the upper branches, it is necessary to use a special knife fixed on a long pole. The crop does not all ripen at the same time, so harvesting has to be carried out over a period of several months.  
  
Pods are normally harvested every 2 or 4 weeks. In general, the harvested pods are grouped together, and split either in, or at the edge of the plantation. In the best case, this practice can contribute to return nutrients to the soil [87] [89].
- (II) After that, the fruit is opened and the beans are removed, usually within a week or to 10 days after harvesting. In this step, great amount of pods are produced, since the cacao pod husk (CPH) represents of 700 g/kg of the whole matured cocoa fruit. Currently, increases in the production of cocoa beans generates more waste, resulting in environmental problems such as foul odors and propagation diseases (e.g. black pod rot) [2]. Moreover, the CPH can also be infected by the witches broom phytopathogenic fungus *Moniliophthora perniciosa* (formerly *Crinipellis perniciosa*) which causes the “witches broom disease” of the cocoa tree [91].
- (III) The cocoa beans fermentation is the immediate post-harvest process, which is carry out in natural way. This allows complex chemical changes to take place in the bean such as enzyme activity, oxidation, and the breakdown of proteins into amino acids. These chemical reactions contribute to the chocolate flavor and color. The length of fermentation depends on the bean type; Forastero beans require about 5 days, whereas Criollo beans about 2 to 3 days.

- (IV) When fermentation is done, the beans are dried to reduce the moisture content from ~60 to ~7.5 %. Typically, this process is carried out with natural heat from the sun, since it is environmentally friendly, cheap and produces beans of good quality. When the weather conditions are not appropriate for sun drying, artificial methods are adopted, *e.g.* the use of a transparent plastic tent or roof over the cocoa or a heat exchanger.
- Drying should not be too faster in order to ensure the chemical reactions initiated during the fermentation process, preventing acidic and bitter flavor of the beans. Inversely, if the drying is too slow, molds and off flavors can develop [87][89].
- (V) Once the coca beans are dried, they are stored in a cool dry place. Finally, they are transported to the chocolate producing industries or countries.

After that, the second step of the process, known as cocoa processing, begins. It is usually perform in industry or in consumer countries.

During cocoa processing, three main components are produced: cocoa liquor, cocoa butter, and cocoa powder. Cocoa liquor is used with other ingredients to produce confectionery products. Cocoa butter is used in the manufacture of chocolates; whereas cocoa powder has found application as an ingredient in almost any foodstuff including chocolate-flavored drinks or desserts. Thus, cocoa beans are differently transformed, depending on targeted and desired final products of chocolate manufacturing [1] [37].

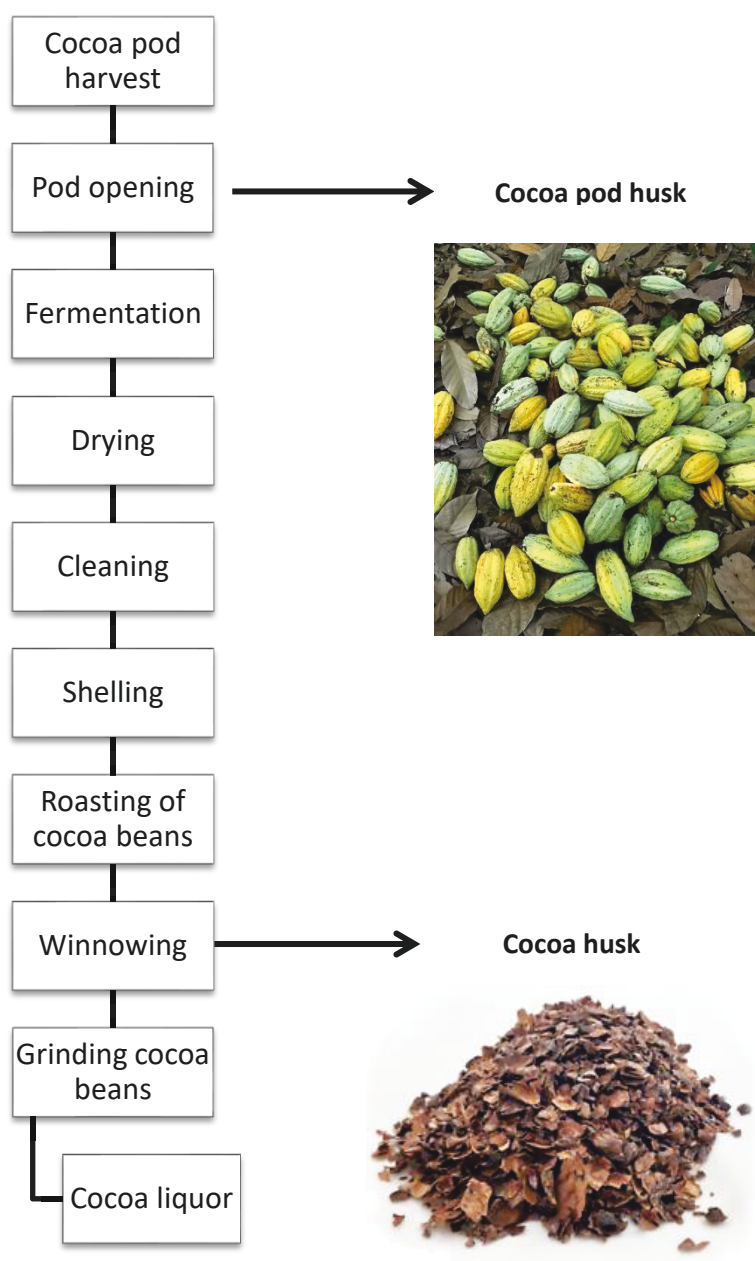
The thee first steps of the cocoa processing is the cleaning of cocoa beans to remove all extraneous material, such as sand, stones, bits of iron, leaf litter or other impurities. Then, depending on the industry, the cocoa beans can be subjected to a thermal pretreatment, followed by breaking, shelling, and winnowing and only later to roasting where the whole beans are roasted with the shells. This step is carried out between 120-150°C during 20 to 40 min. This is the fundamental strategy to bring out the chocolate flavor and color. The roasting promotes the formation of new compounds, such as aldehydes produced from the *Maillard* reaction, as well as alkylpyrazines, alcohols and furanes.

The cocoa husk (CH) can be treated with a micronizator that breaks the integument, and the husks are winnowed; then the raw cocoa beans are roasting. If the beans are roasted with

the intact husks, these are removed immediately afterwards to obtain cocoa nibs [92][32]. During this process large amounts of cocoa husk (CH) are produced as an agro-residue, which represent between 12-15 % of roasted or unroasted beans [93].

Finally, the nibs are ground to extract the greatest amount of fat from the cells within the cotyledons, further leading to the cocoa liquor and then the oblation of cocoa butter.

The temperature and degree of grinding vary according to the type of nib and the final product [87] (Figure 6). Thus, CH and CPH are, respectively, the main by- and co-product from cocoa processing. Besides the economic interest, they represent an environmental and disposal problem in the producer and processing countries. For all those reasons, we found interesting to investigate possible and alternative applications.



**Figure 6. Cocoa bean processing**  
Adapted from [37]

## 2.3 Chemical composition of cocoa husk and cocoa pod husk

The chemical characterization of the CH and CPH expose a richness and abundance in different classes of molecules (Table 3).

**Table 3. Concentration of the major classes of molecules found in cocoa husk (CH) and cocoa pod husk (CPH).**

Components	Content (% dw)	
	CH <sup>a, b, c</sup>	CPH <sup>d, e, f</sup>
Proteins	18.10 ± 8.1	9.14 ± 1.7
Cellulose	13.24	26.15 ± 3.0
Hemicellulose	10.81	12.75 ± 9.6
Lignin	16.43	21.16 ± 2.6
Lipid	1.85 ± 1.2	1.50 ± 0.13
Ash	7.50 ± 1.0	9.07 ± 0.4
Tannins	ND**	1.18 ± 0.2
Theobromine	1.29 ± 0.18	0.40
Total phenolic compounds (GAE*)	5.78 ± 0.29	4.6 ± 0.04

<sup>a</sup> Arlorio *et al.* 2001 [12], <sup>b</sup> Martin-Cabrejas *et al.* 1994 [94], <sup>c</sup> Lecumberri *et al.* 2007 [95], <sup>d</sup> Alemawor *et al.* 2009 [96], <sup>e</sup> Vriesmann *et al.* 2011 <sup>f</sup> Adamafio 2013 [97]. \*GAE: gallic acid equivalents (Folin-Ciocalteu assay). \*\*ND: non detected

CH and CPH have high contents in carbohydrates and an interesting composition in biomolecules such as PC and alkaloid. However, the concentrations, as well as molecular profile, depend on the cocoa's origin and the pretreatment conditions of samples. For example, a comparative study between CH from four origins showed a different concentration in phenolic compounds varying from 3.66 to 5.52 % (dw) [98]. Likewise, Vriesmann and co-workers [2] attribute the variations in the contents of some compounds, affecting by the different geographic origins of the samples.

In CH, Lecumberri *et al.* [95] demonstrated that the PC (especially highly polymerized compounds like condensed tannins) and phenols were bound to proteins and polysaccharides in cell walls. This result was further confirmed by Perez *et al.* [32] in a study dealing with cocoa agro-residues, namely CH and CPH. Indeed, they found the presence of tannins, coumarins, flavonoids, and proanthocyanins in CH. The PC reported in CPH corresponded mainly to flavones and their derivatives, hydroxycinnamic acids and flavonols [16].

On the other hand, methylxanthines (mainly theobromine) have been reported in both agro-residues [37] [97].

In despite the rich content of these raw materials and the several explorative studies that exist, they do not have a definitive use and their application at industrial scale is very limited. In addition, in the specific case of CPH, the applications proposed so far are limited.

## **2.4 Cocoa husk and cocoa pod husk applications**

According to the literature, some researches focus on the potential applications of CH and CPH in food, cosmetic, and health industries as well as in agriculture and in animal nutrition. These applications are described below.

### **2.4.1 Fertilizer and composting/vermicomposting**

The conversion of CPH into fertilizer is a simple alternative approach to reduce the amount of plant waste in an environmentally friendly way. For this, three methods have been reported. In the first one, CPH is burnt in kilns to produce ash; in the second one, CPH can be composted with animal manure; whereas in a third one it can be obtained during biogas production [91]. Some studies have applied the CPH ash in order to increase the crop's growth. For example the agronomic efficiency of potassium ash CPH and the muriate of potash were compared of the maize growth. The best results were obtained with the application of CPH ash at a concentration of 280 kg/ha. The combination of CPH ash with poultry manure also resulted useful in increasing maize yields production, since their combination had cumulative and complementary effect on soil nutrients and nutrients uptake [99][100].

On the other hand, two treatments of brewery waste, or CH, amended with cattle dung, poultry and goat manures at equal rates, were tested with respect to N, P and K concentrations in the leaf and fruit yield of tomato. The results showed that the combinations made with CH increase the fruit yield by 397 % in comparison with the control, and a NPK (Nitrogen, Phosphorus, and Potassium) fertilizer [101]. CH have also been effective in the worm culture, since it contributed to the reproduction of earthworms in the ground. In consequence, the produced vermicompost improves mineralization and



simultaneously produces compounds rich in aromatic groups, finally enhancing the fertility of the soil and the maize production yield [102].

### 2.4.2 Livestock feed

Several studies have explored the possibility of using the cocoa co- and by-products as an ingredient in ruminants, poultries, goats, pigs, and fishes diets. For example, a diet mixing dry brewer's grains and CH, or CPH, in a ratio of 50 % (w/w) was tested in the goats' feed [103]. A better digestibility was observed, especially with CH diet. Thus, the authors recommended CH as an ingredient to complete the diet for ruminants. However, they pointed out the necessity to include other ingredients in order to improve diet's palatability and dilute the possible effect of any toxic substance such as alkaloids [103]. CH was also tested as supplement in the milk cows' diet [104]. They showed acceptability for this diet, and they even gained weight more rapidly in comparison with other proposed diets, but the milk yield production decreased. Moreover, the CH supplement caused constipation in cows. As well, two different diets containing 100 and 200 g/kg of CH were tested in terms of acceptability and digestibility in tilapia *Oreochromis niloticus*. The results showed a good acceptability of the fish, characterized by voracious consumption and positive weight gains. Nevertheless, there were significant reductions in gross feed conversion efficiency [105]. On the other hand, the high nonstarch polysaccharides content in CPH could limit its use in monogastrics. To face this issue, Alemawor *et al.* [96] tested the addition of commercial extracellular exogenous fibrolytic enzymes, and showed an increase of CPH digestive capacity in poultries. Yet, the incorporation up to 300 g/kg of fresh CPH in pig's diet did not produce any adverse effects in their productivity. However, the diet must be supplemented with other nutrients, due to its low rate of available metabolizable energy, leading to a significant increase in the cost of the diet formulation [106]. In addition, earlier studies have found that feeding pregnant pigs with CPH led to spontaneous abortions [107].

### 2.4.3 Adsorbents

The use of CH and CPH as adsorbents of different contaminants, applied to waste treatment or used as biofiltration support was reported. For example, activated carbon prepared with

CH showed a great potential to remove Ni (II) ions from an aqueous solution, and its modification with chitosan enhanced the ability to remove Cr<sup>6+</sup> and Pb<sup>2+</sup> [108][109].

On the other hand, the production of activated carbon from CPH showed capacities to adsorb arsenic (until 80 % in reported experimental conditions) in less than 1 hour [110]. Besides, CPH revealed to be a promising material for the development of low cost biosorption technology that can be used for the removal of Cd<sup>2+</sup> and Pb<sup>2+</sup> from wastewater effluents [111].

Additionally, other study showed the potential of cellulose microfibrils extracted from CH to be used as an adsorbent material for the removal of methylene blue in concentrated aqueous solution. In addition, the CH cellulose microfibrils swelling properties was studied and this material showed a great capacity, therefore finding an application in the wastewater treatment for the removal of basic dyes [112].

#### **2.4.4 Biofuels**

CH and CPH have been studied as a raw material for the production of biogas. For example, it was possible to obtain 199 mL of CH<sub>4</sub>/g from CH; and the production was increased by 14 % when treated with NMMO (*N*-methylmorpholine-*N*-oxide). The biogas production from CPH reached until to 247 m<sup>3</sup> of CH<sub>4</sub>/kg, when this agro-residue was mixed to ruminal fluid and pig manure [113][114].

The high content of polysaccharides (pectins, cellulose, and hemicellulose) in CPH could also be consider as interesting substrates for obtaining second-generation bioethanol, similarly to other lignocellulosic materials that has already shown great potentials (e.g. sugarcane bagasse) [115].

#### **2.4.5 Food additives and products**

Due to its high PC content with potential antioxidant activity, roasted CH is currently use for artisanal purpose or sold as cocoa husk tea and infusions. However, hygiene and food safety standards are not controlled, which rise the question of product qualities due to problems of

contamination [3]. CH have also been added to the production of snacks, or bakes such as muffins and biscuits [116].

Furthermore, CH is a good and inexpensive source of dietary fiber (DF), since it has been reported to contain between 40 to 60 % dw. DF is composed mostly of insoluble fiber comprising of glucose and uronic acid, with smaller amounts of galactose, arabinose, xylose, and mannose. On the other hand, the presence of associated PC may provide fiber materials with intrinsic antioxidant capacity. In addition, the DF can contain protein–Maillard–tannin complexes. However, since this complex is insoluble in acid treatment, generally is meaningless as a measurement of actual lignin. This has great significance for the estimation of DF in such materials, because lignin is included in the definition of DF, whereas tannin/protein complexes and Maillard products are not.

These characteristics make CH a suitable product for food supplements or in the preparation of low-calorie, high-fiber food [117][94][95].

Moreover CH and CPH have been widely studied as pectin sources, which find several application and interest in the food industry, mostly as gelling agents to thicken and to stabilize various products, or in pharmaceutical and cosmetic sectors [1].

Indeed, CH contains approximately 9 % of pectins, specially the high-methoxyl pectins (HMP), which has been extracted free of antinutritive and potentially toxic compounds, such as lindane and ochratoxin A, after sequential washings, including a pre-treatment using 2-propanol [12]. The extracted CH pectins were tested to prepare food products like jam, showing their potential application in the food industry. However, it is yet necessary to optimize the extraction parameters to increase the yield extraction [118].

Regarding the CPH, highly acetylated low methoxyl pectin possessing interesting properties such as microbiological quality, pseudoplastic behavior, and high swelling capacity in aqueous media [94]. Additionally, they can present antimicrobial capacity against Gram positive or negative microorganisms, as well as emulsifier properties, as shown for the sugar-beet pulp pectins. That is why, pectin extracted from CPH has a potential to be used in the nutraceutical and pharmaceutical industries, as a new source of non-structurally modified pectin-derived emulsifier [2][119][38].

### 2.4.6 Source of antioxidants

CH and CPH have been investigated as interesting antioxidants sources since they contain several PC known for their bioactivities. In addition, the cocoa beans and their derived products, such as the chocolate, are also recognized as sources of antioxidants.

The antioxidant activity of CH extracts *versus* synthetic antioxidants (BHT and  $\beta$ -tocopherol) was compared on lipid oxidation of cooked beef stored at 4 °C for up to 14 days. The antioxidant and free radical scavenging activity was significantly greater in CH extracts than that of synthetic antioxidants. The authors attributed the the high oxidative stability of cooked beef treated to the PC's present in CH extracts [120]. In another study, the antioxidant capacity of CH samples from different regions of Venezuela, was evaluated by DPPH and FRAP assays, and showed a positive relationship with the PC content. The FRAP values were similar to the ones obtained with fruits such as red apples and green vegetables (e.g. spinach and artichoke) [3]. Still in the same context, CH and CPH have showed a good antioxidant potential while evaluating through three different *in vitro* methods, FRAP, DPPH and ABTS [121]. This suggests that cocoa by- and co--products may be considered as good natural sources for natural antioxidants. This statement was confirmed by Karim *et al.* [16], who found that CPH had higher efficiency compared with ascorbic acid and pine bark extract in the DPPH assay, while similar results were observed in the FRAP assay. The authors attributed this activity to the presence of carboxylic acids such as citric acid and malic acid, in addition to chlorogenic acid, catechin, quercetin, and other identified PC in CPH.

### 2.4.7 Source of biomolecules

As cacao beans, the CH and CPH could be a great source of a wide quantity of molecules with interesting activities, such as antiviral, anticarcinogenic, antimicrobial, antipercholesteromic, among others. Although PC are suspected to be the main responsible molecules for these activities, some other biomolecules may get involved, either alone or in association.

For example, CH lignin carbohydrate and polysaccharide-rich fractions, prepared by acid precipitation and 50 % (v/v) ethanol showed unexpectedly higher anti-human

immunodeficiency virus (HIV) activity, than that of tannins, flavonoids, and natural lignin from other plants. Moreover, this fraction also showed anti-influenza virus activity [122]. Other study showed the anticarcinogenic potential of CH fractions, which contained 43 % (w/w) of PC. These last ones exerted an excellent protective effect on H<sub>2</sub>O<sub>2</sub>-induced inhibition of GJIC (gap-junction intercellular communication) in rat liver epithelial cells, ten-fold higher than vitamin C. The extracts of CH also suppressed DNA synthesis in all liver, stomach, and colon cancer cells, four-fold higher than that of vitamin C [123].

CH extracts were capable to inhibit dental caries in rats infected with *Streptococcus mutans*. This activity was explained by the presence of polymeric epicatechins in an acetylated form and oleic and linoleic acids, which conferred a strong anti-glucosyltransferase and antimicrobial activity [17]. The extract inhibited the adherence of *S. mutans* to saliva-coated hydroxyapatite and reduced the accumulation of artificial dental plaque by *S. mutans* on orthodontic [124]. On the other hand, fermented CPH extracts showed antimicrobial and antifungal activity against six different strains, especially on Gram-negatives: *Pseudomonas aeruginosa* and *Salmonella choleraesuis*, and *Moniliophthora perniciosa* and *Saccharomyces cerevisiae*, respectively [125]. Furthermore, CPH extracts presented antihypercholesterolemic *in vivo* effect on albino rats, and demonstrated a strong capacity to lower total cholesterol, triglyceride, and LDL, as well as to increase HDL levels in the rats [126].

### III. Phytochemicals

Coffee and cocoa are known for the multiples health benefits, likely due to their wide phytochemicals content. Phytochemicals are defined as chemical compounds produced by plants, which are involved in its development or defense against competitors, pathogens, or predators. They play important role in animals such as substrates for biochemical reactions; cofactors or inhibitors of enzymatic reactions; ligands in intracellular receptors; scavengers of reactive or toxic chemicals; compounds that enhance the absorption and/or stability of essential nutrients; or activities in the gastrointestinal health, among others. This group of compounds includes mainly terpenoids, PC, alkaloids, and other nitrogen-containing plant constituents [127]. Here are a description of the different phytochemicals that focused our attention, as well as their occurrence in the CP, CH and CPH.

#### 3.1 Phenolic compounds

The definition of polyphenols compounds (PC) has been largely debated since it this term can be related to the structure chemical of these compounds, their origin or their biological functions. However, recently, Quideau and co-workers [128] proposed a revisited definition of the term polyphenols:

*The term “polyphenol” should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression*

Phenolic compounds are one of the most widely occurring groups of phytochemicals. They are plant secondary metabolites derivated from the pentose phosphate, shikimate, and phenylpropanoid pathways [129].

PC embraces a wide range of plant substances, which possess one or several aromatic rings, bearing one or more hydroxyl substituents [130] (Figure 7). Thus, they show intense absorption in the UV region of the spectrum. These kinds of compounds are usually located in vacuoles of plant cells, and most of naturally occurring phenolic substances are associated

with mono- or polysaccharides, linked to one or more phenolic groups. Although not exclusive, they mostly tend to be water-soluble. In addition, PC are able to complex with protein by hydrogen bonding. In addition, they are very prone to enzymatic oxidation due to the action of specific “polyphenol oxidase or phenolase (EC 1.10.3.1)” enzymes present in all plants [130].

PC are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known. They can be present as simple molecules such as phenolic acids, or as highly polymerized substances such as tannins [131]. Frequently, these compounds are associated with lignin combined as ester groups or present in the alcohol-insoluble fraction of the leaf; alternatively they may be present in the alcohol-soluble fraction bound as simple glycosides.

Several thousands of PC have been described and classified by their source of origin, biological function, and chemical structure (Table 5)

In plants, PC are involved in growth and reproduction, providing protection against pathogens and predators. Additionally, they are involved in the preservation plant integrity under the continuous threat of environmental stressors, including ultraviolet light, radiation, and high temperatures. Moreover, PC contribute to color and sensory characteristics of fruits and vegetables [131].

PC are an essential part of the human diet since they are distributed in fruits and vegetables, and the high consumption of plant-based food is related to diverse benefits for health. This statement was studied in the 50's, by analyzing the diet of the residents of Mediterranean countries. This diet is characterized by high consumption of vegetables, pulses, fruit, and cereals, but it is relatively high in monounsaturated fat. Despite this, the results showed a much lower incidence of cardiovascular disease, cancer, Alzheimer's disease, and Parkinson's disease, and even a reduction in overall premature death overall. Accordingly, several studies have demonstrated that PC have a great range of physiological properties, such as antioxidant, anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio protective and vasodilatory activity [132][133][134].

Several classification systems for polyphenol have been proposed. In general, flavonoids and some classes of nonflavonoids are usually distinguished [135]. Nonflavonoids are phenolic acids, which include benzoic (based on a phenolic ring and an organic carboxylic acid function, C<sub>6</sub>-C<sub>1</sub> skeleton) and hydroxycinnamic acids (C<sub>6</sub>-C<sub>3</sub> skeleton), and stilbenes; but also other complex derived molecules (e.g. stilbene oligomers, gallotannins, ellagitannins, and lignins). Flavonoids are the most important single group of phenolics; they share a common nucleus consisting of 2 phenolic rings and an oxygenated heterocycle. They are divided into several groups differing in the oxidation state of the heterocyclic pyran ring (e.g., anthocyanins, flavonols, and flavanols) (Table 4)[130][135] Cheynier, 2005). For the purpose of this work, the chemical structure of some of the phenolic compounds present in CP, CPH and CH, will be presented later in this section.

**Table 4. Classification of phenolic compounds <sup>a, b</sup>**

Class and sub-class		Examples
Non flavonoids	Benzoic acids	Gallic acid; protocatechuic acid; p-hydroxybenzoic acid
	Hydroxycinnamic acids	Caffeic acid; chlorogenic acid; ferulic acid
	Hydrolyzables tannins	Pentagalloylglucose
	Stilbenes	Resveratrol
	Lignans	Secoisolariciresinol; matairesinol; lariciresinol
Flavonoids	Flavonols	Quercetin; kaempferol; myricetin
	Flavones	Apigenin; luteolin
	Flavanones	Hesperetin; naringenin; eriodictyol
	Flavanols	Catechins; epicatechins; gallocatechins
	Anthocyanidins	Cyanidin; delphinidin; malvidin
	Condensed tannins	Procyanidin; prodelphinidins
	Isoflavones	Daidzein; genistein; glycitein

<sup>a</sup> Monach *et al.* 2004 [136], <sup>b</sup> Farah *et al.* 2006 [55].

Although several studies exist to describe the occurrence, reactivity, and bioactivity of PC, some works that represent a niche of opportunities remain to investigate. Among them, the research for new vegetal sources of PC, improving extraction methods and emphasizing the importance to develop environmentally friendly processing are of a great challenge.



In this context, the study of PC in agroindustrial wastes represents a very interesting option with the aim at finding inexpensive natural antioxidant sources, nutraceuticals, or preservatives.

PC that belong to hidroxyinnamic acids, flavonols, flavanols, condensed tannins, or anthocyanidins families have been already described in the CP, CH, and CPH. TPC in these residues are shown in Table 5.

In the following section, the main characteristics of each kind of compound and their occurrence in coffee and cocoa by - and co -products will be discussed

### 3.1.1 Antioxidant activity of phenolic compounds

One of the major and better studied properties of phenolic compounds is their radical-scavenging capacity, which is involved in antioxidant properties. Halliwell and Gutteridge [137] defined an antioxidant as a substance which, at low concentration with respect to an oxidizable substrate, such as unsaturated lipids, significantly retards or prevents oxidation of this substrate. Phenolic compounds are mainly free radical scavengers that delay or inhibit the initiation step or interrupt the propagation step of autoxidation of polyunsaturated lipids. Autoxidation involves a free radical chain reaction, which can be described in terms of initiation, propagation, and termination processes and that is generally spontaneously initiated by exposure of lipids to light (photolysis) with or without the intervention of photosensitizers, to heat (thermolysis), to ionizing radiation, to metal ions or metalloprotein catalysts, or to hydroperoxide decomposition [138].

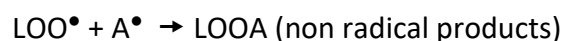
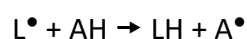
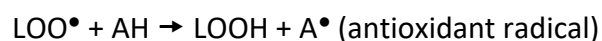
Initiation:  $\text{LH (polyunsaturated lipid)} + \text{initiator} \rightarrow \text{L}^\bullet \text{ (lipid free radical)} + \text{H}^\bullet$

Propagation:  $\text{L}^\bullet + \text{O}_2 \rightleftharpoons \text{LOO}^\bullet \text{ (lipid peroxy radical)}$

$\text{LOO}^\bullet + \text{LH} \rightarrow \text{LOOH (lipid hydroperoxide)} + \text{L}^\bullet$

Termination:  $\left. \begin{array}{l} \text{LOO}^\bullet + \text{LOO}^\bullet \\ \text{LOO}^\bullet + \text{L}^\bullet \\ \text{L}^\bullet + \text{L}^\bullet \end{array} \right\} \rightarrow \text{non radical products}$

As antioxidants (AH), PC react with lipid and peroxy radicals to convert them to more stable, non-radical products. AH donate hydrogen atoms to the lipid radicals and produce lipid derivatives and antioxidant radicals ( $A^\bullet$ ) that are more stable and less readily available to further promote autoxidation [138][139]:

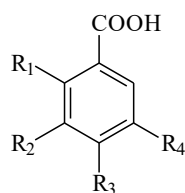


PC that belong to hydroxynamic acids, flavonols, flavanols, condensed tannins, or anthocyanidins families have been already described in the CP, CH, and CPH. In the following section, the main characteristics of each kind of compound and their occurrence in coffee and cocoa by- and co-products will be discussed.

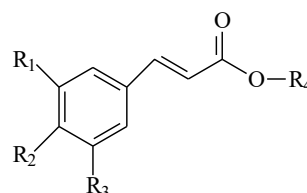
### **3.1.2 Phenolic compounds in coffee pulp**

Phenolic acids possess a phenolic ring and an organic carboxylic acid function ( $C_6-C_1$  skeleton). Two important naturally occurring types of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids, which derive from non-phenolic molecules of benzoic and cinnamic acids, respectively (Figure 7). In plants, these compounds are connected with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy. They are distributed in seeds, leaves, roots, and stems of plants [140].

## Hydroxybenzoic acids



## Hydroxycinnamic acids



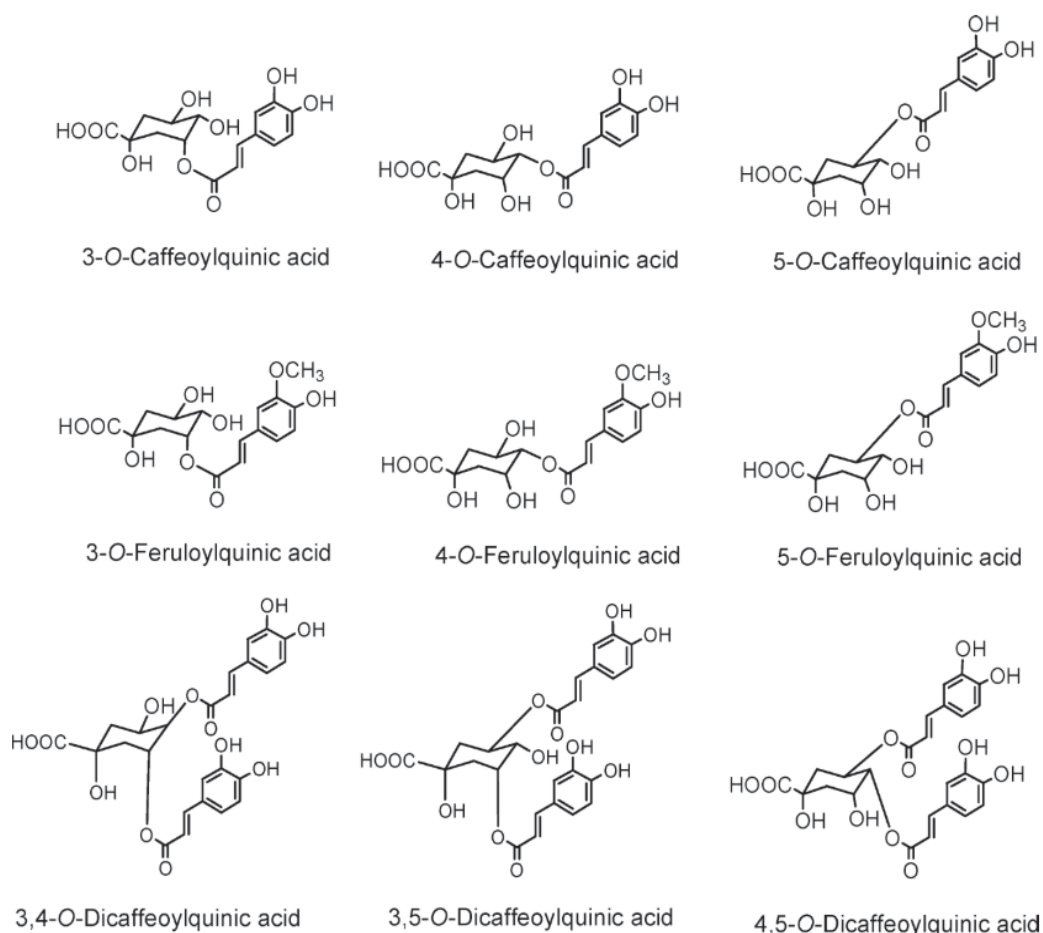
Acid	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	Acid	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
<i>p</i> -Hydroxybenzoic	H	H	OH	H	<i>p</i> -Coumaric	H	OH	H	H
Gallic	H	OH	OH	OH	Caffeic	OH	OH	H	H
Gentisic	OH	H	H	OH	Ferulic	OCH <sub>3</sub>	OH	H	H
Isovanillic	H	OH	OCH <sub>3</sub>	H	Isoferulic	OH	OCH <sub>3</sub>	H	H
Protocatechuic	H	OH	OH	H	Sinapic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>	H
Salicylic	OH	H	H	H	Rosmarinic	OH	OH	H	C <sub>9</sub> H <sub>9</sub> O <sub>4</sub>
Syringic	H	OCH <sub>3</sub>	OH	OCH <sub>3</sub>	<i>p</i> -Coumaroylquinic	H	OH	H	C <sub>7</sub> H <sub>11</sub> O <sub>5</sub>
Vanillic	H	OCH <sub>3</sub>	OH	H	Caffeoylquinic	OH	OH	H	C <sub>7</sub> H <sub>11</sub> O <sub>5</sub>
Vératric	H	OCH <sub>3</sub>	OCH <sub>3</sub>	H	Feruloylquinic	OCH <sub>3</sub>	OH	H	C <sub>7</sub> H <sub>11</sub> O <sub>5</sub>
					Sinapoylquinic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>	C <sub>7</sub> H <sub>11</sub> O <sub>5</sub>

Figure 7. Chemical structures of phenolic acids

The main phenolic acid in coffee belongs to the chlorogenic acids (CGA) family, which is a family esters formed between quinic acid and *trans*-cinnamic acids; including the caffeoylquinic acids (CQA); dicaffeoylquinic acids (diCQA); feruloylquinic acids (FQA); *p*-coumaroylquinic acids (*p*CoQA), and six mixed diesters of caffeoyl- feruloyl-quinic acids (CFAQ) [55] (Figure 8).

Green coffee beans are known to be an important natural source of 5-*O*-caffeoylquinic (5-CQA) [141], it represents 42.2 to 58.7 % dw of identified PC in CP. However, Ramirez-Martinez [142] also described the presence of 3-CQA (5.7 %), 3,4-diCQA (5.7 %), 3,5-diCQA acid (19.3 %), 4,5-diCQA acid (4.4 %), and ferulic acid (1.0 %) in Arabica fresh coffee pulp (Figure 8). Clifford and Ramirez-Martinez [141] analyzed the CGA on different CP derived from two species of coffee (Robusta and Arabica) and two associated hybrids. Total CGA in Arabica CP ranged from 1.01 to 1.16 % (dw), including 0.87-0.97 % dw of monomers, 0.02 % dw of 5-FQA, and 0.14-0.17 % dw of dimers. Three CQA isomers (3-, 4-, and 5-CQA), 5-FQA, and two diCQA isomers (3,4- and 3,5-diCQA) were found. Protocatechuic acid (3,4-

dihydroxybenzoic acid; 0.17-0.47 % dw) and caffeine (1.38-1.67 % dw) were also identified and quantified.



**Figure 8. Structure of main chlorogenic acids detected in coffee pulp**

From: [4]

Flavonols are the most ubiquitous flavonoids in food. Generally, they are associated with a sugar moiety, which is very often glucose or rhamnose, but other sugars may also get involved (eg. galactose, arabinose, xylose, glucuronic acid) [132]. A concentration of 1.6 % of flavonols has been quantified in fresh CP [57]. The major compound that has been identified is rutin (Figure 10), with a content that reaches the 2.2 % of the total PC content in CP [58] (Table 5).

Flavanols are derivatives of flavans, they possess two chiral carbons, meaning that four diastereoisomers occur for each of them. Catechin and epicatechin are the main flavanols found in food, and contrary to flavonols, they are not present as glycosylated

forms. Traces of catechin and epicatechin (0.006-0.44%) have been identified in eight cultivars of Arabica coffee [142].

Condensed tannins (or proanthocyanidins) are dimers, oligomers, and polymers of catechins that are bound between carbons C<sub>4</sub> and C<sub>8</sub> (or C<sub>6</sub>) (Figure 9). They have molecular weights ranging from 578 to at least 3000 g.mol<sup>-1</sup>, reason why their degree of polymerizations has rarely been determined in food [132]. Moreover, tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and proteins. This characteristic is responsible for the astringency of tannin-rich foods. In fresh CP, the condensed tannins have been reported ranging between 2.01-4.63, and 4.57-6.17 % dw, respectively [57][143].

Table 5. Phenolic compounds identified in Coffee pulp, Cocoa husk, and Cocoa pod husk

Phenolic compounds reported	Recoveries (%)		Solvent used	Extraction technique	Reference
	Folin-Ciocalteu	HPLC			
Coffee pulp					
Protocatechuic acid	-	0.026	MeOH 80 % (v/v)	Maceration (room temperature)	[142]
		0.10	Boiling MeOH 70 % (v/v)	Reflux (15 min)	[141]
Catechin	-	0.036	MeOH 80 % (v/v)	Maceration (room temperature)	[142]
Chlorogenic acid (5-CQA)	-	0.50	MeOH 80 % (v/v)	Maceration (room temperature)	[142]
		0.31	Ethyl acetate	Enzymatic extraction by pectinase from <i>A. niger</i> (40 °C/6h)	[144]
		0.83	Boiling MeOH 70 % (v/v)	Reflux (15 min)	[141]
		0.014 - 0.056	MetOH 80 % (v/v) (1 % acetic acid)	HSE <sup>2</sup> (55°C/35 min)	[145]
Epicatechin	-	0.28	MeOH 80 % (v/v)	Maceration (room temperature)	[142]
3,5-DCQ	-	0.08	MeOH 80 % (v/v)	Maceration (room temperature)	[142]
	-	0.34	Boiling MeOH 70 % (v/v)	Reflux (15 min)	[141]
3,4-DCQ	-	0.10	Boiling MeOH 70 % (v/v)	Reflux (15 min)	[141]
4,5-DCQ	-	0.06	Boiling MeOH 70 % (v/v)	Reflux (15 min)	[141]
Rutin	-	0.026	MeOH 80 % (v/v)	Maceration (room temperature)	[142]
Caffeic acid	-	0.20	Ethyl acetate	Enzymatic extraction by pectinase from <i>A. niger</i> (40 °C/6h)	[144]
Ferulic acid	-	0.11			
Coumaric acid	-	0.08			
Procyanidins	-	2.01	Aqueous acetone (2:3)	NS	[57]

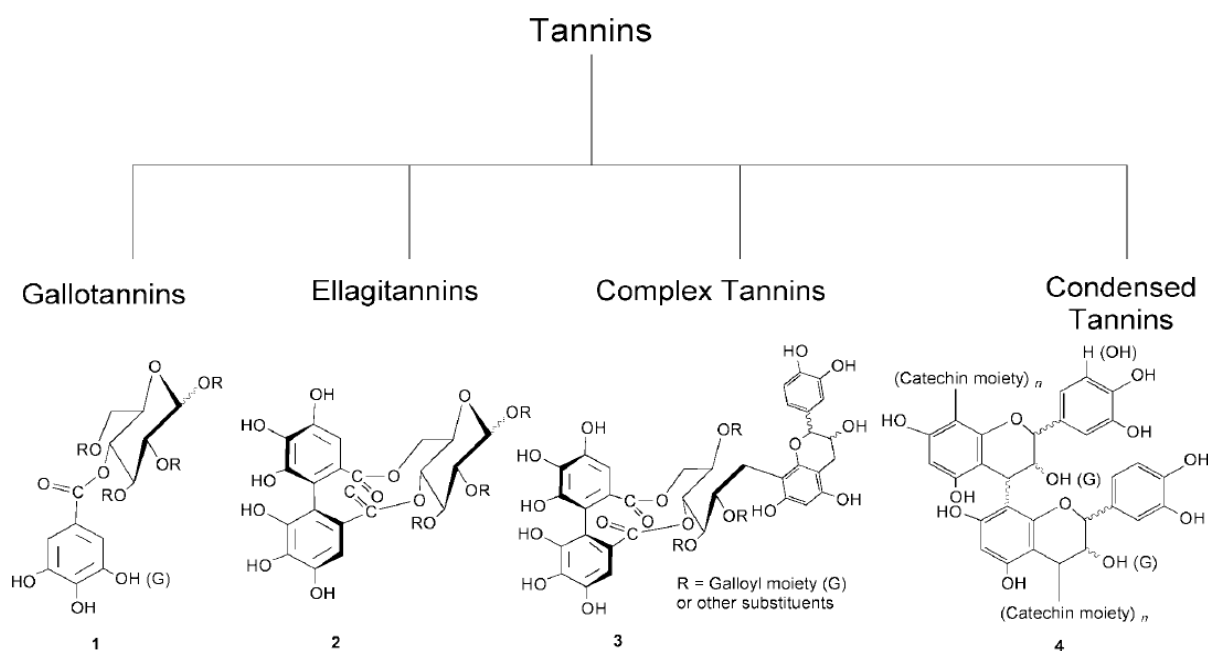
Phenolic compounds reported	Recoveries (%)		Solvent used	Extraction technique	Reference
	Folin-Ciocalteu	HPLC			
Total phenolic compounds (TPC)	33.5	4.71	MetOH 80 % (v/v) (1 % acetic acid)	HSE (55°C / 35 min)	[145]
	2.62	-	NS <sup>1</sup>	NS	[56]
<b>Cocoa husk</b>					
Epicatechin	-	0.53-1.05	EtOH 62.67 % (v/v)	HSE (25°C/75 min)	[98]
	-	0.7	Water acidified (pH 3)	HSE (70°C/1 h)	[146]
	-	1.58	MeOH 80 % (v/v)		
	-	0.9	EtOH/ acidified water (pH 3) 70:30 % (v/v)		
	-	1.8	MeOH/ acidified water (pH 3) 70:30 % (v/v)		
Catechin	-	0.67	Water acidified (pH 3)	HSE (70°C/1 h)	[146]
	-	0.1	MeOH 80 % (v/v)		
	-	0.2	EtOH/ acidified water (pH 3) 70:30 % (v/v)		
	-	0.4	MeOH/ acidified water (pH 3) 70:30 % (v/v)		
	-	0.57	Water		
	-	0.94	Water acidified (pH 3)		
	-	1.46	MeOH 80 % (v/v)		
	-	4.9	EtOH/ acidified water (pH 3) 70:30 % (v/v)		

Phenolic compounds reported	Recoveries (%)		Solvent used	Extraction technique	Reference
	Folin-Ciocalteu	HPLC			
Caffeic acid	-	0.09	MeOH/ acidified water (pH 3) 70:30 % (v/v)	HSE (70°C/1 h)	[146]
Procyanthocyanidins	-	Detected	EtOH 50 % (v/v)	Reflux (50°C/1h)	[17]
Total phenolic compounds (TPC)	1.82	-	MeOH: hydrochloric acid 99:1	Soxhlet (14 h)	[12]
	5.78	-	MeOH 50 % (v/v) / Acetone 70 % (v/v)	HSE (1h/ room temperature)	[95]
	6.04	-	Water	Reflux (5 min)	[147]
	3.66 -5.15	-	EtOH 62.67 % (v/v)	HSE (25°C/75 min)	[98]
	8.6	-	MeOH 50 % (v/v)	Reflux (60 °C/ 5h)	[123]
	13.3	-	EtOH 50 % (v/v)		
	14	-	Acetone 50 % (v/v)		
	2.3	-	Water		
Total phenolic compounds (TPC)	2.52	-	MeOH 80 % (v/v)	MAE <sup>3</sup> (50 °C/5 min)	[148]
	2.34	-	EtOH 80 % (v/v)		
	4.18	-	Acetone 80 % (v/v)		
	1.72	-	Water		



Phenolic compounds reported	Recoveries (%)		Solvent used	Extraction technique	Reference
	Folin-Ciocalteu	HPLC			
Cocoa pod husk					
Rhamnetin	-	Detected	EtOH (80 % v/v)	HSE (40°C/30 min)	[16]
Protocatechuic acid	-				
p-hydroxybenzoic acid	-				
Kaempferol derivate	-				
Luteolin	-				
Chlorogenic acid derivate	-				
Resveratol	-				
TPC	4.6	-	EtOH (80 % v/v)	HSE (50°C/5 h)	[2]
	5.65	-	Acetone 70 % (v/v)	Maceration (Room temperature/ 5 days)	[126]

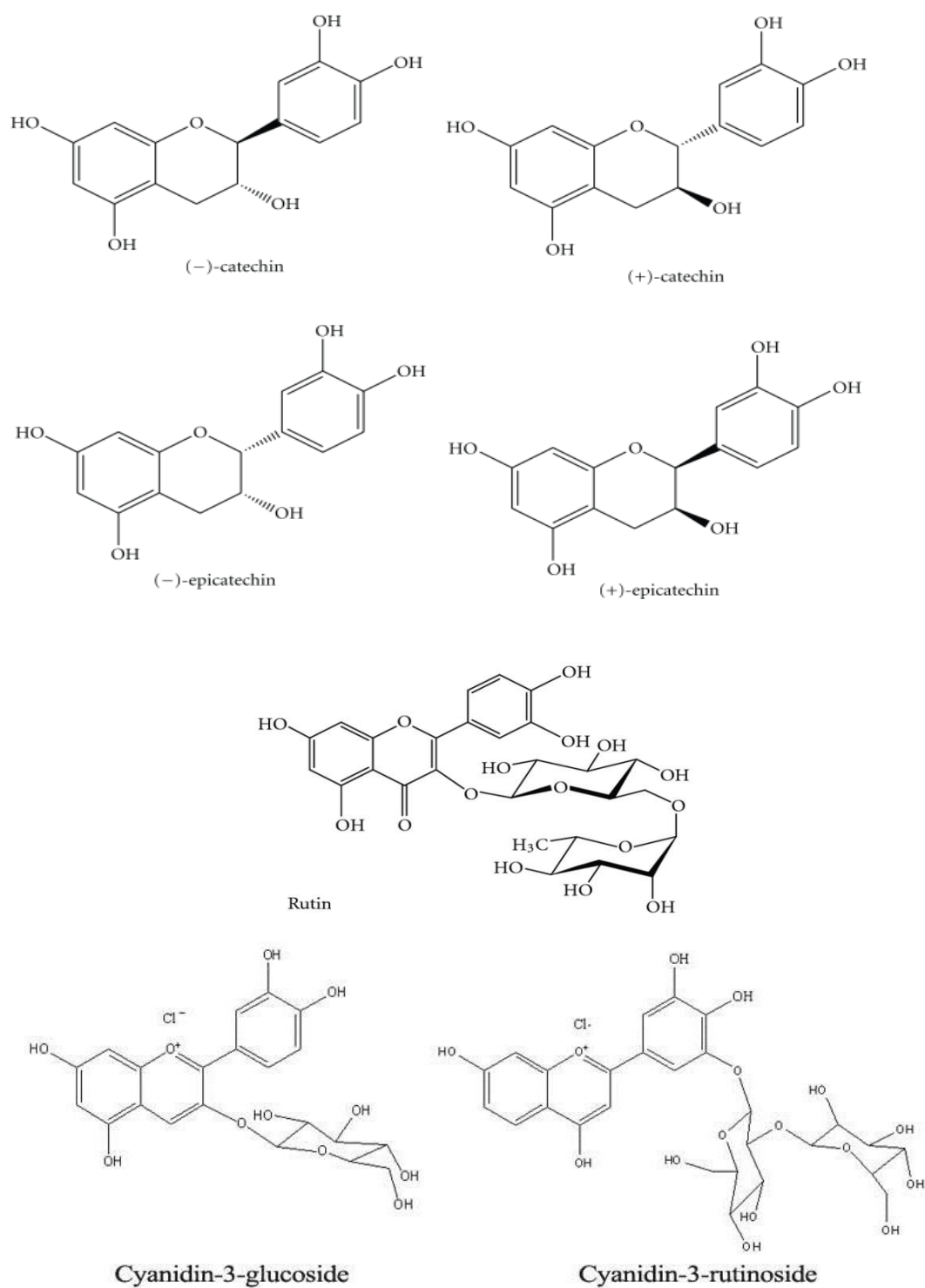
<sup>1</sup> NS: not showed, <sup>2</sup> Heat stirring extraction (HSE), <sup>3</sup> Microwave assisted extraction (MAE).



**Figure 9. Classification of tannins according to Khanbabaee and van Ree (2001)**  
From: [149]

On the other hand, some traces of anthocyanins have been also described in CP in a concentration between 0.05 and 0.88 % [58][57]. These are pigments, dissolved in the vacuolar sap of the epidermal tissues of flowers and fruits, which exist in different chemical forms, both colored and uncolored, according to pH. Although they are highly unstable in the aglycone form (anthocyanidins), while they are in plants, they are resistant to light, pH, and oxidation conditions that are likely to degrade them [132].

Additionally, cyanidin-3-rutinoside and cyanidin-3-glucoside (Figure 10), and their aglycones were identified as the major anthocyanins present in CP, before and after tissue browning [150]. Moreover, the red color of coffee peel was attributed to the presence of cyanidin-3-rutinoside [51]. Thanks to the presence of these compounds, the CP could be used as a source of natural colorants. Especially taking into account that in wet process of coffee, CP are removed and their color is rapidly degraded by the action of enzymes (peroxidases and polyphenoloxidases) [1].



**Figure 10. Chemical structure of some phenolic compounds found in coffee and cocoa**  
From: [151]

### 3.1.3 Phenolic compounds in cocoa husk and pod husk

Cocoa beans are a rich source of PC, where three groups can be distinguished: flavanols (37 %), anthocyanins (4 %), and condensed tannins (58 %) (Figure 10). Among them, the main identified PC is (-)- epicatechin, which represents up to 35 % of the total polyphenol content [152]. The PC content in cocoa residues (CPH and CH) has been explored in some studies, proposing them as an inexpensive source of these compounds. Frequently, their quantification is made by Folin-Ciocalteu method (FC), and the results are expressed in total phenolic content. In this context, several studies have reported a total concentration of PC, ranging from 1.82 to 5.78 % dw, and 0.36 to 4.6 % dw for CPH and CH, respectively (Table 5). This variation is affected by several factors, such as the extraction method and the solvents used, and the variety and origin of the samples [2][95][12][3][121].

Other studies have shown that the dietary fibers obtained from CH are associated with PC (with about 1.32 % and 4.46 % dw of soluble polyphenols and condensed tannins, respectively) [95]. Additionally, the total content of flavonoids and tannins was determined in twelve samples of CH from different origins and varieties. The higher concentrations were found in CH from Honduras, with 4.1 % and 2.8 % dw of flavonoids and tannins, respectively. Regarding the elucidation of the phenolic profile in CH, it has been reported the presence of epicatechin in a maximal concentration of 0.41 % dw [98]. Another study showed that the epicatechin and catechin concentrations in CH increase after the natural fermentation. This phenomenon was also observed in the epigallocatechin quantification, since it was only identified in fermented CH, and not in raw CH. Furthermore, some traces of caffeic acid have been also reported in CH [146].

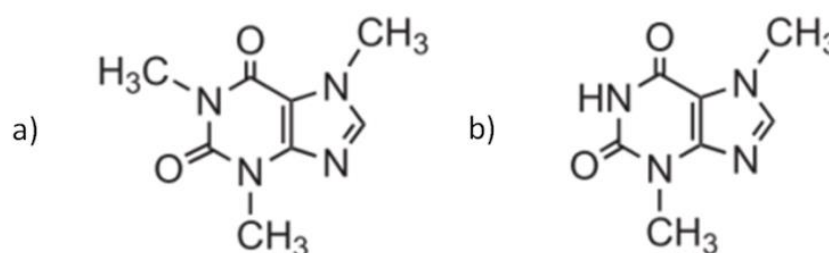
Likewise in CPH, several PC have been also identified by comparing their HPLC spectrum and the database from available literature, among them, phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid, and salicylic acid), flavonols (kaempferol) and flavones (linarin), flavones (apigenin and luteolin), and stilbenes (resveratrol) [16]. In addition, the epicatechin was identified as the main PC in CPH extracts. In the same study, a polymeric proanthocyanidins consisting exclusively of condensed epicatechin units was identified [17].

### 3.2 Alkaloids

It does not exist a unique definition for alkaloids, but from a chemistry standpoint, they have been described as a group of complex heterocyclic nitrogen compounds, with strong and often toxic physiological activity [153].

Alkaloids are a diverse group of low molecular weight, nitrogen-containing compounds, mostly derived from amino acids or from the transamination process. However, similar alkaloids can have quite different biosynthetic pathways and different biological effects [154].

Regarding the alkaloids classification, they can be divided with respect to their biological and ecological activity; their biosynthetic pathway, and their chemical structures. In this last classification, there are true alkaloids (heterocyclic ring with nitrogen/derived from amino acids), protoalkaloids (heterocyclic ring without nitrogen/derived from amino acids) and pseudoalkaloids (basic carbon skeletons/not derived from amino acids) [153]. Caffeine (1, 3, 7-trimethylxanthine) and theobromine (3, 7-dimethylxanthine) belong to this last group. Both compounds are derived from purine, a nitrogenous base of nucleotide, which consists of purine and pentose sugar (D-ribose or 2 deoxy-D-ribose), that is why they are known as purine alkaloids. Additionally, they are the best-known alkaloids in popular non-alcoholic beverages (coffee, cocoa, and tea) [155] (Figure 11).



**Figure 11. Structure of main purine alkaloids in coffee and cocoa**  
a) caffeine, b) theobromine.

The alkaloids are secondary metabolites especially found in the plant kingdom, in about 25 % of higher plants species [154]. However, these compounds were also identified in animals, insects, marine invertebrates, fungus, and microorganisms [153][156].

The alkaloid role in plants is related to enhance reproductive rates, to improve defenses against biotic and abiotic stresses, and defensive strategies against predators.

In animals, certain alkaloids display ichthyotoxicity, antibacterial, antifungal and antiviral activity, and are involved as a component of chemical defense system [153].

On the other hand, alkaloids have been recognized by human beings as high added-value molecules, since they have been used in the fabrication of drugs, potions, medicines, teas, and poisons, for more than 4000 years [156] [157]. Nowadays, the alkaloid properties have allowed their applications in several fields. For example, they have been widely studied in medicine for the clinical application as stimulants, analgesics, antihypertensive, and vasoconstrictors, among others. In the agricultural field, they have been used as pesticides and fertilizers; however, their use has been limited due to their high toxicity for humans. In food industry, some alkaloids are used as additives, the well-known example being the quinine as a bitter in tonic water. Another ancient use of alkaloids is for psychoactive drugs, such as cocaine, cathinone, nicotine, among others. Additionally, in the biotechnology field, alkaloids were involved in cell and root cultures to increase the production of certain compounds *e.g.* quinine, quinidine or serpentine [153].

### **3.2.1 Alkaloids in coffee pulp**

Nowadays, coffee beans are not only the base to prepare one of the most consumed beverages in the world, but they are also the world's primary source of caffeine. Indeed, coffee quality is related (among other factors) to the caffeine content, aroma, and taste. In addition, caffeine has become a major commercial commodity for food additive in many carbonated, sweetened drinks, especially those popular within young people [153].

Nevertheless, caffeine is not the only one alkaloid present in coffee. Actually, the coffee plants contain two different kinds of alkaloid delivered from nucleotides: purine alkaloids (such as caffeine and theobromine), and pyridine alkaloid, represented by trigonelline (nicotinic acid *N*-methylbetaine) [158].

About 40 % of total nitrogen present in CP corresponds to non-protein nitrogen, which includes caffeine and trigonelline [36]. The whole fruit of Arabica and Robusta coffee contain

between 0.52-0.13 % and 0.82-0.49 % of caffeine, respectively [159]. Other study that assessed the caffeine concentration from Robusta coffee and Arabica CP, revealed that this last one had the highest caffeine content (1.38-1.67%) [141].

The characterization of a CP aqueous extract from Arabica coffee highlighted the presence of trigonelline. This alkaloid, together with other compounds such as caffeine, quinic acid, and CQA, showed antimicrobial activity against Gram-positive bacteria [77].

### 3.2.2 Alkaloids in cocoa husk and cocoa pod husk

Theobromine is the main alkaloid in the cacao, concentrated in the seeds, leaves and fruits [160]. It is commercially available and possesses therapeutic uses, such as vasodilatador and diuretic, among others [154].

Caffeine and theophylline (1,3-dimethylxanthine) have been also extracted from Forasteros, Criollos, and Trinitarios cocoa beans [161]. The theobromine content in CPH and CH ranges from 0.15 – 0.4 % and 0.05-2.1 %, respectively [162][146]. Theobromine content in CH can be affected by the cocoa beans fermentation, since during this process, theobromine migrates from the beans to the CH [97].

On the other hand, small amounts of 3- and 7-methylxanthines, and 7-methylxanthosine have been detected in CH and CPH. Additionally, 1-methylxanthine, theophylline, and paraxanthine (Figure 12) have been also detected in CH [163].

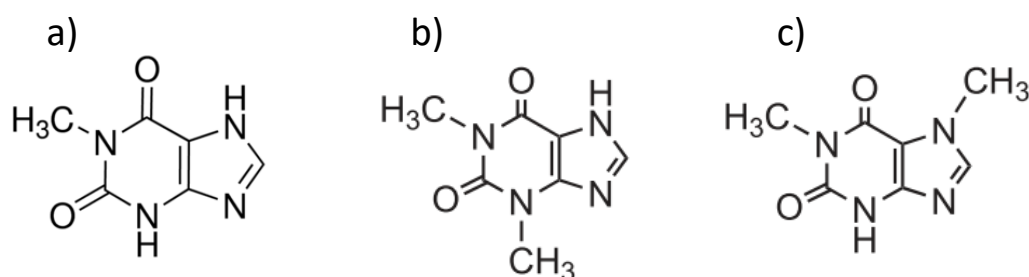
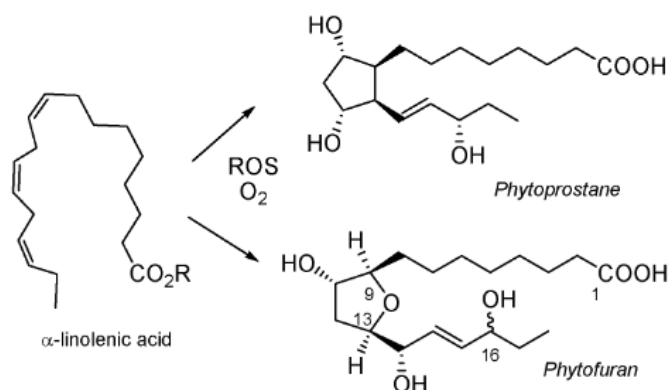


Figure 12. Chemical structures a) 1-methylxanthone, b) theophylline and c) paraxanthine

### 3.3 Phytoprostans and phytofurans

The studies on the phytochemical composition of coffee and cocoa agro-residues have proved to be rich sources of biomolecules. However, the recent discovery of a novel family of plant effectors called phytoprostans (PhytoPs) and phytofurans (PhytoFs) (Figure 13) has generated a new field of research [164], which could lead to new perspectives for the use of CP, CH, and CPH. Indeed, PhytoPs and PhytoFs are homologues of isoprostanes (IsoPs) and isofurans (IsoFs), which are products of free radical-induced peroxidation of polyunsaturated fatty acids (PUFAs), such as arachidonic acid, in animal cells.

Similarly, in plants, the PhytoPs and PhytoFs are formed by non-enzymatic oxidation of  $\alpha$ -linolenic acid (C18:3 n-3, ALA) [24]. The PhytoPs formation starts with the oxidation of linoleates, present in the plant tissues by free-radical to yield racemic peroxy fatty-acid radicals. These radicals may abstract hydrogen from PUFAs in an autocatalytic process, thereby propagating the radical chain reaction. PUFA hydroperoxides and peroxy radicals that have more than two double bonds can be further oxidized to produce hydroperoxides that have a prostaglandin G-ring system. This ring is a precursor of at least six different classes of PhytoPs containing one to six prostaglandin rings ( $A_1$ ,  $B_1$ ,  $D_1$ ,  $E_1$  or  $F_1$ ) or a deoxy-J1-ring system ( $dJ_1$ ) [165] (Figure 14). The most common form in plants are  $D_1$ - and  $F_1$ -PhytoP [166]. Finally, these molecules are formed by a ring and a trine unit substituted by a methyl terminus and carboxyl terminus of the fatty acid. On the other hand, PhytoFs formation is favored by the oxygen tension increases and take a parallel pathway after the initial endoperoxide formation [167] [168] (Figure 13).



**Figure 13. Representative structures of phytoprostane and phytofuran from ALA peroxidation**  
From: [167] and [168]



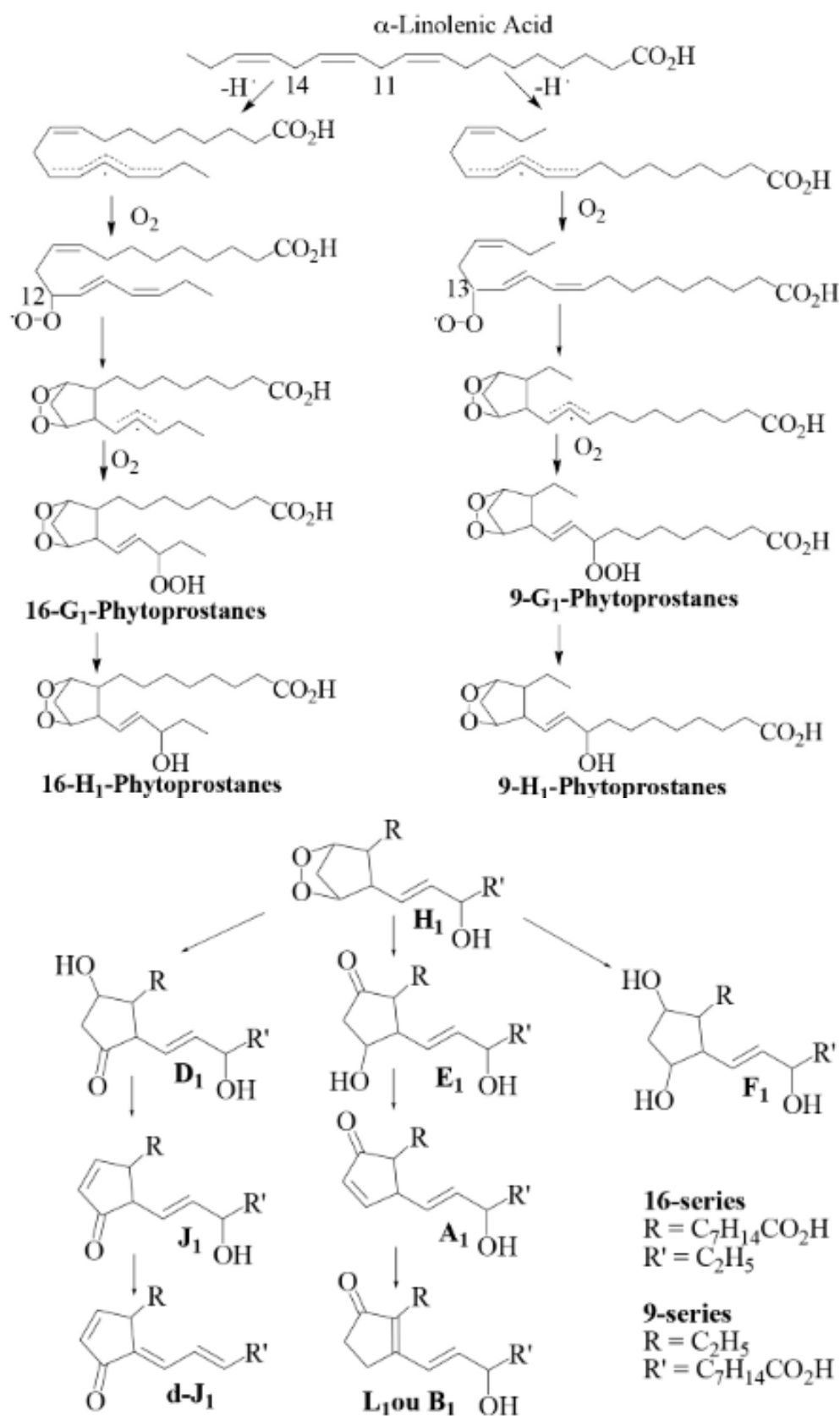


Figure 14. Pathway of phytoprostane biosynthesis by non-enzymatic peroxidation of  $\alpha$ -linolenic acid

From: [169]

PhytoPs and PhytoFs generation in plants could be involved in the prevention of cellular death and gene regulation. However, their exact role and function in plant physiology remain unknown. Additionally, they are considered as excellent biomarkers of oxidative degradation in plant-derived foodstuff [7] [8].

The absorption of PhytoPs and PhytoFs by the human body can be through food intake or pollen aspiration [170]. In animal bodies, these lipid mediators are likely involved in several biological functions; *e.g.* in the brain and central nervous system development, in the prevention of metabolic disorders [167], in cytoprotective responses in immature brain cells such as neuroblasts and oligodendrocytes [171], or mediators of oxidative stress [165]. In addition, they could be involved in anti-inflammatory and apoptosis-inducing activities [7].

Consequently, there is a growing interest in studying the molecular profile and amount of these kinds of compounds in plant foods and their response to cultural and postharvest transformation practices and/or environmental conditions. On the one hand, PhytoPs have already been described in nut kernels [170], olives [172], and macroalgae [173], among others. On the other hand, PhytoFs were found in nuts and seeds [167], and more recently in melon leaves [174] and rice's flour [175]. However, no reports exist on the content of these molecules neither in coffee nor in cocoa by- and co-products. Hence, the identification and quantification of PhytoPs and PhytoFs from CP, CH, and CPH could provide added value to the agro-residues from the coffee and cocoa transformation industries. Indeed, PhytoPs and PhytoFs rich extracts could be obtained from these agro-residues to enrich food or to be used as nutritional supplements due to their interesting biological activities.

### 3.4 Thiol precursors

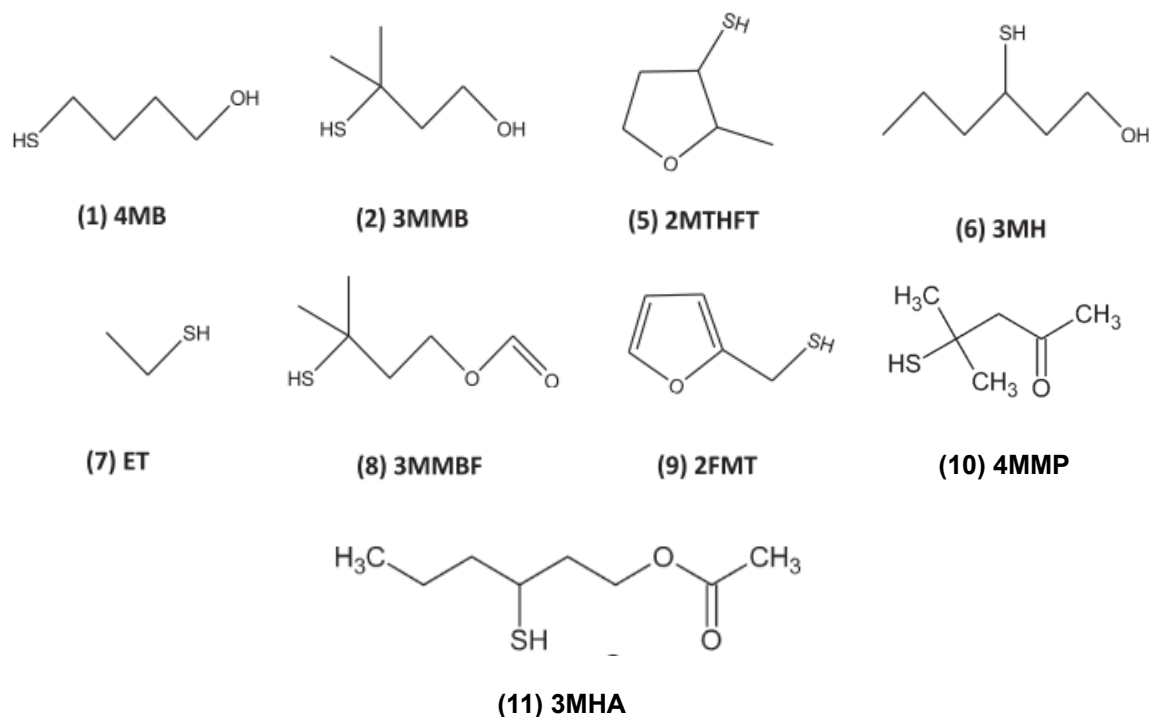
The aroma is one of the most important characteristic in coffee and cocoa products and contributes to guarantee the product quality. For example, volatile sulfur compounds, and especially volatile thiols, are extremely odoriferous molecules, which contribute to the aromas of many fruits, plants, and food [28][176]. Some of these compounds denominated biothiols, realize important roles in vegetables and fruits, such as antioxidant protection [177].

Food thiols occur either naturally or formed as a result of processing and storage conditions (heating, smoking, cooking, frying, roasting, irradiation, enzymatic, etc.). Nevertheless, they are present usually in low concentrations (ppm or ppb).

Around four hundred volatile compounds have been identified in coffee (developed mainly during the roasting), whereas in cocoa, these compounds are formed during fermentation and roasting stages. A study identified 92 and 65 volatile compounds in Criollo and Trinitario cocoa types [178] [179].

Moreover, the presence of thiols described as a type of mercaptan (polyfunctional mercaptans) characterized by a sulfhydryl functional group, were studied by Quintanilla-Casas *et al.* [9] in brewed coffee. They identified and quantified sixteen different thiols, some of them being reported for the first time in coffee brews. Among them, 4-mercapto-1-butanol (4MB), 3-mercapto-3-methylbutan-1-ol (3MMB), 2-methyl-3-tetrahydrofuranthiol (2MTHFT), 3-mercaptohexanol (3MH), ethanethiol (ET), 3-mercapto-3-methylbutyl formate (3MMBF), and 2-furanmethanethiol (2FMT) (Figure 15) were confirmed with their respective standards.

Similarly, in wine industry, various compounds called varietal thiols such as 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexyl acetate (3MHA) (Figure 15), and 3MH, have been identified as key molecules of young wines, and aromatic precursors for many grape varieties [25].



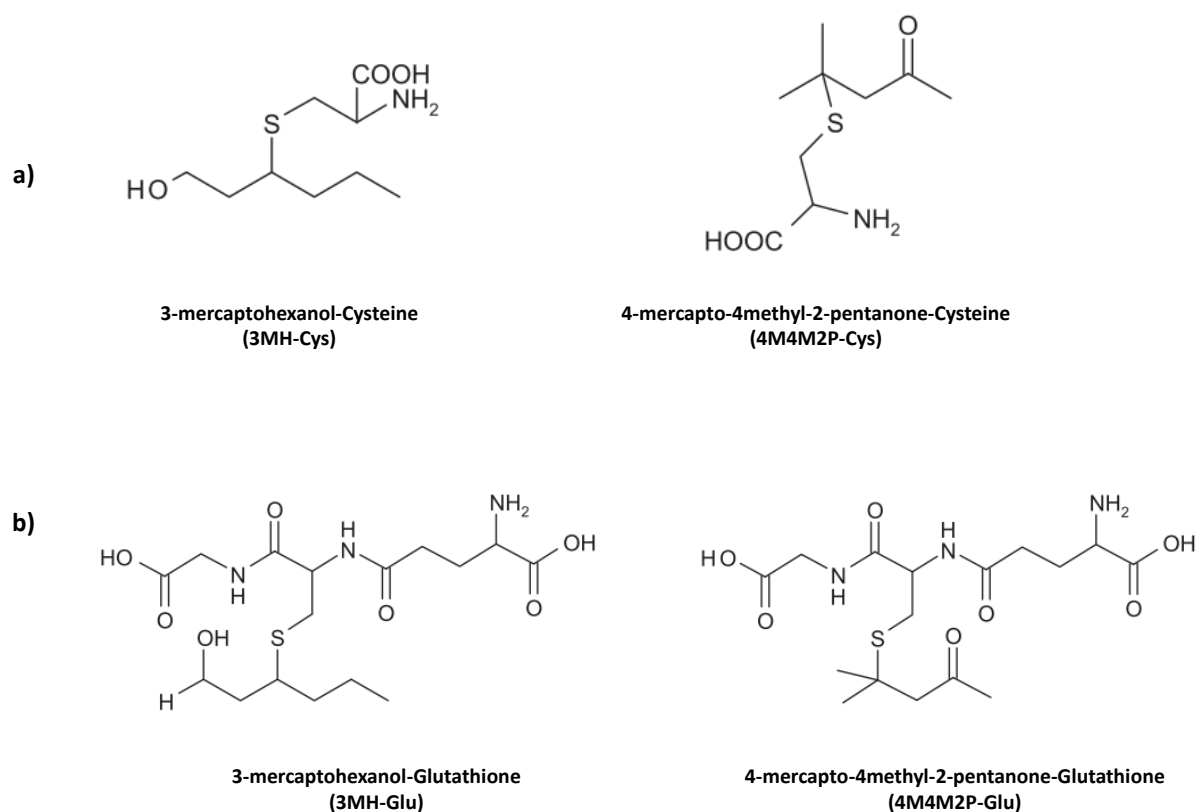
**Figure 15. Chemical structures of thiols**

(1) 4- mercapto-1-butanol (4MB); (2) 3-mercapto-3-methylbutan-1-ol (3MMB); (5) 2- methyl-3-tetrahydrofuranthiol (2MTHFT); (6) 3-mercaptohexanol (3MH); (7) ethane- thiol (ET); (8) 3-mercapto-3-methylbutyl formate (3MMBF); (9): 2-furanmethanethiol (2FMT); (10) : 4-mercapto- 4-methylpentan-2-one (4MMP); and (11): 3-mercaptohexan-1-ol-acetate (3MHA)

From: [9] and [25]

These compounds are very powerful aromas, with detection thresholds typically in the nanograms per liter range, and are characterized by distinctive aroma qualities [180], notably.

Fruity notes (grapefruit, passion fruit, blackcurrant bud, and box tree) that are released from thiol precursors which are odorless conjugates, known as cysteine and glutathione conjugates during the alcoholic fermentation (Figure 16).



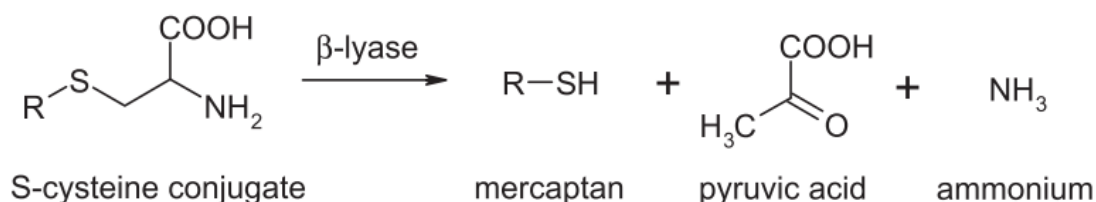
**Figure 16. Structure of a) cysteinylated and b) glutathionylated precursors of 3-mercaptohexanol (3MH) and 4-mercapto-4-methyl-2-pentanone (4M4M2P)**

From: [26]

The study of thiol precursors in different grape varieties has shown that their concentration was dependent on terroir conditions, wine crops management, as well as hydric stress [181]. In addition, other factors such as crop diseases could increase the thiol precursors' concentration in the grapes. For example, a study demonstrated that wine obtained from grapes affected by Noble rot (*Botrytis cinerea*) showed higher concentrations of 3MH than those obtained from healthy grapes, indicating that these grapes probably contain higher concentrations of its precursor [182].

The cysteinylated precursors were initially identified in Sauvignon Blanc grapes and then, in Cabernet Sauvignon and Merlot. The 3MH-Cys precursor presents two chiral centers, whereas the resulting volatile thiol (3MH) has a chiral center with two diastereoisomers. Although the olfaction thresholds for the R and S forms of the 3MH are very similar, their aromas are very distinct. Indeed, R form generates grapefruit notes, while S form liberates passion fruit notes [183].

The cystathionine  $\beta$ -lyase is largely responsible of the conversion of 3MH-Cys into 3MH (Figure 17). This enzyme catalyzes the rupture of the carbon–sulphur bond, and volatile sulphured aromas are released [10]. This enzyme has been identified in bacteria, fungi, and plants, and was already used in cheese and wine flavours [176][26].



**Figure 17. Reaction of breakage of cysteinylated precursor in the presence of the enzyme  $\beta$ -lyase**  
From: [26]

Recent studies confirmed that the cysteinylated precursor of 3MH derives from the catabolism of its own glutathione precursor (3MH-Glu), as it was observed in Sauvignon Blanc juice. To release the 3MH-Cys, the reaction needs the presence of two enzymes:  $\gamma$ -glutamyltranspeptidase, which catalyses the reaction for glutamic acid removal, and carboxypeptidase, which removes glycine [26][184][185].

Glutathione (GSH) is an endogenous tripeptide of L-glutamate, L-cysteine, and glycine. It is the most abundant thiolic compound of low molecular weight in plant, but it is also present in mammalian and prokaryotic organisms. GSH plays a role as antioxidant, cell protector, immunity booster, and it is involved in the detoxification pathway [186][26].

Food processing operations involved the production of wide range of beverages and food, including coffee and chocolate, may cause a loss of their original aroma. Thus, a subsequent supplementation is necessary in order to restore or to improve it in order to assure the food acceptability [187]. As a result, research has been exploring alternatives to produce aroma molecules. Among them, the chemical synthesis showed to be a very effective option. However, the actual trend has shown that the consumer prefers food labeled as natural and safe for health [75]. To face this situation, the production of aromas by fermentation could offer an alternative. In addition, the compounds produced through this process will be, for

example, considered as natural by the US Food and Drug Administration (FDA), as long as the starting materials are natural.

Some examples are soymilk and flour rice productions, where the use of fermentation substrates and fermenting microorganisms from other natural sources is allowed.

Furthermore, these examples have shown that acceptable or pleasant novel odor attributes can be created under suitable conditions [187]. Thus, this alternative could be applied to other fermented foods and beverages, since during fermentation some aromatic precursors are released. Additionally, the presence in fruits of aromatic precursors which can act as sources of potential or latent aroma is well-known for many years [26]. In this context, the agro-wastes of coffee and cocoa could offer an alternative in the development of natural sources of thiol precursors.

## IV. Deep eutectic solvents (DES)

Nowadays, the academic researches, as well as the industrials, are facing a huge challenge in the development of new clean technologies. Indeed, the environmental issues on the planet are increasing exponentially (the contamination, the climate change, the limited resources, and overexploitation).

Thus, in the beginning of the 1990s the term “Green Chemistry” was formulated, with the intention to “design chemical products and processes to reduce or eliminate the use and generation of hazardous substances” [188]. Nowadays, this concept has taken force and became almost an obligation since the EU environmental policy and legislation have declared the reduction of hazardous solvents in the industry for the period 2010–2050, as a priority [189].

However, solvents are used on a daily basis in numerous industrial processes, and it is estimated that they comprise almost 60 % of all industrial emissions and 30 % of all volatile organic compound emissions worldwide.

In this context, the best solution would be the implementation of solvent-free processes; however, solvents are almost unavoidable due to their crucial role to dissolve solids, to promote or allow mass and heat transfer for reactions, or to perform separation and purification steps, among others.

In this context, ionic liquids (ILs) have been described as “green solvents”. They contribute to the development of a first new multimolecular generation of solvent exhibiting unusual properties. ILs are low melting point organic salts ( $<100\text{ }^{\circ}\text{C}$ ) and consist in an association between an organic cation (such as an imidazolium or pyridinium) and a coordinating anion. They possess a very low vapor pressure, they are not flammable, and they are stable at high temperatures (around  $200\text{ }^{\circ}\text{C}$ ). In addition, several possibilities exist to fine-tune their properties. ILs have been used mainly in organic chemistry, electrochemistry [190], analytical chemistry, and chemical engineering research areas. Additionally, they have been applied in the extraction and separation of a wide range of primary and secondary metabolites, as well as macromolecules (e.g. DNA, proteins and polysaccharides) [191], and as alternative media



for enzymatic reactions. In addition, some ILs can dissolve starch and cellulose, which are very difficult to get solubilized in water without heating [192].

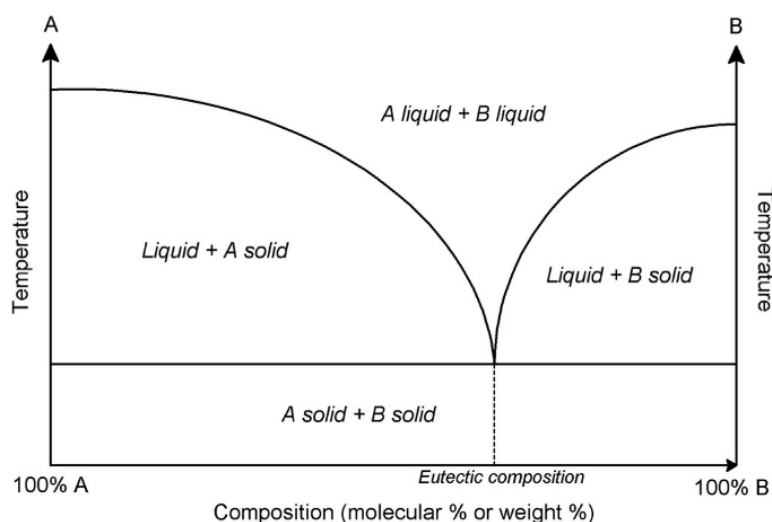
Despite these features, the IL's shortcomings are so significant that they cannot be hindered: high price, water stability, high toxicity, very low atom economy for their synthesis, difficulty to get pure, and poor biodegradability. Considering the aforementioned statements, finding and developing new green and efficient solvents is a great challenge, with high-stakes.

Over the last few years, a new kind of solvents called "Deep Eutectic Solvents" (DES) has been developed, as analogs of ILs. However, they differ from two main aspects: the source of the starting materials and, to a lesser extent, the synthesis. Furthermore compare to related ILs, they offer many advantages such as low price, chemical inertness with water, easy preparation and most of them are biodegradable with low toxicity [193].

## **4.1 DES description**

DES were introduced for the first time by Abbot and co-workers in 2002 [194] while they studied the solvent properties of mixtures of amides with quaternary ammonium salts.

The DES liquid phases are obtained after mixing compounds, able to self-associate, and resulting in a eutectic mixture with a melting point significantly lower than the individual components (Figure 18). Indeed, these interactions lead to energy reduction characterized by a decrease, sometimes very substantial ( $> 100\text{ }^{\circ}\text{C}$ ), of the melting point of the mixture [195]. At the molecular level, the components are able to establish very organized non-covalent bonding, mostly through hydrogen bonds network.



**Figure 18. Schematic phase diagram for binary eutectic mixture**  
From: [195]

The DES are usually formed by association between a halide salt or another hydrogen bond acceptor (HBA), and a hydrogen bond donor (HBD).

The most common DES are formed by choline chloride (ChCl) as HBA, with cheap and safe HBD, the most popular ones being urea, ethylene glycol, and glycerol [196], but other alcohols, amino acids, carboxylic acids, and sugars have also been commonly used [197]. Yet, many others combinations are possible.

## 4.2 Natural Deep Eutectic Solvents (NaDES)

Very recently the term “Natural Deep Eutectic Solvent” (NaDES) has been introduced to describe a category of DES obtained after the combination between molecules that are abundantly present in almost all living organisms. These compounds include sugars, amino acids, vitamins, and some organic acids such as malic acid, citric acid, lactic acid, and succinic acid. Actually, NaDES have been proposed as a different alternative to DES (and IL) for two reasons: their ingredients are sourced from nature and they are believed to have a real physiological or biological role in nature. Indeed, although their accurate role and presence in living cells is still in investigation, the authors [198] hypothesized that in living organisms, the NaDES may be an alternative medium to water and lipids, and would explain numerous biological processes. The biological and physiological role in living organisms is therefore the main feature that differentiates NaDES from other DES or IL made of natural components.

Nature has provided solutions to allow living organisms to inhabit locations with extreme temperature differences, and these living beings have adapted themselves to overcome and prevent potential cellular and metabolism dysfunctions. We can imagine that the NaDES, formed in different organelles such as plastids and vesicles, or attached to protein aggregates and cellular membranes, may get involved in several biological strategies in the living organisms.

For example, several plants secrete non-volatile saps to attract or to catch insects (*e.g. drosera*). Visual appearance between such a secretion and NaDES are very close, especially the viscosity. In addition, the qualitative analysis usually exposed the presence of sugar encountered in NaDES mixtures, with a stoichiometry between the sugars very close to the one observed in NaDES. For example, the nectar of *Cleome hassleoriana* showed a sugar composition very similar to that of a NaDES (fructose–glucose–sucrose), practically equimolar ratios [199]. The same observation may be drawn with components found in the honey (glucose and fructose) or maple syrup (*Acer* spp.) [198].

Many plant metabolites are highly concentrated and sometime non-water soluble, but are synthesized, stored, and transported in plants. Consequently, the role of NaDES could be also to allow or promote the biotransformation of poor water-soluble substances in which both substrates and enzymes would be dissolved. This assumption is actually strongly strengthened by both the solubilization capacity and the activity/stability of enzymes in these media. For example, authors showed that the solubility of flavonoid rutin in various NaDES increased by 50 to 100 times, in comparison with water. Furthermore, they observed that the solubility of macromolecules, such as salmon DNA, increased 1.46 fold in NaDES: malic acid:Proline (1:1 mole ratio), compared to water, or that non-water-soluble polysaccharide or starch, were highly solubilized in NaDES made with ChCl:glucose: (1:1 molar ratio) [198].

In addition, NaDES could also get involved in harsh environmental conditions for cold and/or drought resistance. Indeed, the strategy that living organisms have found to survive extreme temperature amplitudes involves synchronized production of diverse biological metabolites. The self-organizing structure found in NaDES, allow the component to remain liquid and stable over a large temperature range (far below 0 °C, and up to 200 °C). This allows both

water and metabolites to remain liquid, even at extreme temperatures. Thus, NaDES could be a strategy for living organisms to resist or acclimate in extreme temperature conditions. For example, NaDES may be a way to prevent the crystallization of water inside living cells for winter survival. Similarly, in plant, this strategy could explain how a seed can germinate after 30,000 years in permafrost, how varieties of pea (*Pisum sativum*) can resist to intense frost, how lichen could survive drought, and how cacti and resurrection plants that are rich in choline (a molecule abundantly present in the NaDES formation) may survive (e.g. *Selaginella lepidophylla*).

There are some evident connections between DES and NaDES, and some of those mixtures are even labeled with different names in literature whereas they are precisely the same [195]. However, DES and NaDES may, retain special features; this is the case with NaDES which are exclusively formed by non-ionic species, which involve different driving forces to create the liquid since the “Hole theory” [200] (for explanation see below) put forward to explain the molecular motion in DES are difficult to consider. Nevertheless, DES and NaDES share very similar physicochemical properties (strong ability to dissolve protic molecules, low vapor pressure and miscibility with water among other).

### 4.3 DES synthesis and characteristics

Unlike ILs, the synthesis of DES offers the advantage to be fast, simple, and does not require subsequent purification to be used.

Those liquids may be prepared by three different ways that could be adjusted with some modifications according to the nature of the compounds:

- I. Heating and stirring method: the components are placed in a closed bottle, and heated at 60 °C under magnetic agitation until a clear liquid is formed. Importantly, when carboxylic acids are used as HBD in combination with ChCl, heating is not recommended to avoid the formation of impurities as i. e. esters between ChCl and the acid. Instead, grinding DES components in a mortar with a pestle at room temperature is preferred in order to form the liquid phase [201].
- II. Evaporating method: components are dissolved in water and evaporated at 50°C with a rotatory evaporator. The obtained liquid is put in a desiccator with silica gel until it reaches a constant weight.

- III. Freeze drying method: based on the freeze-drying of a mix of the aqueous solutions of the individual counterparts (not frequently used [202]).
- IV. New preparation technique by means of a microwave (MW) assisted preparation: the component was mixed in the proper mass and 200W power for 20 seconds of microwave irradiation was enough to reach the liquid phase. This method dramatically reduces the preparation time from tens of minutes (30-90 min) to seconds (20 s), and the energy consumption (> 500 times) [203].
- V. Mechanochemical synthesis: based in a continuous flow methodology by Twin Screw Extrusion (TSE). This methodology gives products in higher purity, and decrease the reaction time from hours to minutes compared to the batch process, and consequently the avoidance of thermal degradation [204].

The DES melting point temperature is related to the starting molecule properties. For example, the DES based on dicarboxylic acids and ammonium salt (ChCl), have a melting point ranging from 10 to 71 °C [205], whereas, carbohydrate and urea have shown have melting point between 65-77 °C. Moreover, DES were found to be stable at 95 °C for 4 h without any evident decomposition [206].

On the other hand, the DES viscosity is usually higher than conventional solvents, but similar to ILs (>100 cP at room temperature). Thus, this is an important parameter when considering a solvent for phytochemicals extraction (favors mass transfer, processing, handling, etc.). However, the fluidity of the mixture can be adjusted, because of the nature of starting molecules, the size of the species, the mole ratio, the water content, and the temperature. The very low mobility of free species within the DES comes from the presence of an extensive hydrogen bond network between each component, and to a lesser extent, Van der Waals and electrostatic interactions [196].

Fluidity may also be explained by the “Hole theory”, which assumes that, on melting, the ionic material contains empty spaces that arise from thermally generated fluctuations in local density. Thus, the viscosity is related to the free volume and the probability of finding holes of suitable dimensions for the solvent molecules/ions to move into vacant sites [200]. The free volume can be increased by lowering the surface tension with smaller ions. In addition, it has been already shown that the viscosity decreases significantly when the

temperature increases [207][194][208]. For example, the viscosity of glucose:ChCl:water is decreased by 2/3 when the temperature increases from 20° to 40 °C.

The DES solvation capacity is strongly influenced by the capacity to form hydrogen bonding between DES and targeted compounds. Thus, DES can have a high affinity with all compounds capable of donating electrons or protons. As a consequence, protic solvents (e.g. MeOH, EtOH, or water) will tend to be miscible with DES, while aprotic ones (e.g. toluene, hexane, ethyl acetate, acetonitrile, diethyl ether, etc.) will be immiscible. Thus, hexane or ethyl acetate may be used for the extraction of molecules of low or medium polarity, allowing the DES to be reused [200].

Although, DES are usually hydrophilic (Table 6), their properties can be tuned by the proper selection of the individual components. Indeed, newly in 2015 hydrophobic deep eutectic solvents were presented for the first time, and evaluated as extractants for volatile fatty acids (VFAs) [209].

These DES consisted of decanoic acid and a six different quaternary ammonium salt, as HBA. The authors showed that the extraction efficiencies increased with increasing chain length of HBAs, and thus DES' hydrophobicity. The results showed higher extraction efficiencies of the VFAs using hydrophobic DES compared to the conventional trioctylamine (TOA) extraction medium. Similarly, a study investigated the relevant thermophysical properties of hydrophobic menthol-based DES with carboxylic acids. The thermal analysis confirmed the DES characteristic, with lower melting temperatures than the starting components. Moreover, the FTIR study revealed a new hydrogen bond formation in the vicinity of the COOH group [210]. The discovery of hydrophobic DES allowed enhancing their field of applications, especially for liquid–liquid extraction processes. For example, Florindo and co-workers [211] studied the use of hydrophobic DES in the wastewater treatment. The best efficiency for the extraction of four neonicotinoids was reached using DES based on DL-Menthol and organic acids, highlighting their relevant role as sustainable solvents for industrial applications. Additionally, the hydrophobic DES have been also applied as extraction media for artificial pigments from beverages, as well as polycyclic aromatic hydrocarbons [212][213].

Table 6. Solvent polarity of some DES

Solvent composition	Mole ratio	$E_T$ (kcal/mol) <sup>a</sup>	Ref.
Water	-	63.10	[214]
MeOH	-	55.00	[214]
EtOH	-	51.90	[214]
ChCl:glycerol	1:1	58.49	[215]
ChCl:glycerol	1:2	58.07	[215]
ChCl:glycerol	1:3	57.96	[215]
ChCl:1,2-ethanediol	1:2	57.10	[216]
ChCl:xylose	2:1	50.69	[217]
ChCl:glucose	1:1	50.43	[217]
1,2-Propanediol:ChCl:water	1:1:1	50.07	[207]
Sorbitol:ChCl:water	1:2.5:0.3	49.98	[218]
Fructose:ChCl:water	1:2.5:2.5	49.81	[218]
Xylitol:ChCl:water	1:2:3	49.72	[207]
ChCl:citric acid	2:1	48.30	[217]
Proline:malic acid:water	1:1:3	48.05	[207]
Citric acid:glucose	1:1	47.81	[217]
Glucose:tartaric acid	1:1	47.73	[217]
Malic acid:ChCl:water	1:1:2	44.81	[207]

<sup>a</sup>  $E_T$ = energy absorbance transition

From: [6]

As depicted in Table 6, the polarity of DES is very close to water and to the ones of the most polar organic solvents (MeOH, EtOH, etc.).

The organic acid-based DES are the most polar, followed by the amino acids- and sugar-based DES; while polyalcohol-based DES are the less polar [207]. Regarding the polarity of ChCl-based DES, it increases as a function of the quaternary ammonium salt's concentration [215].

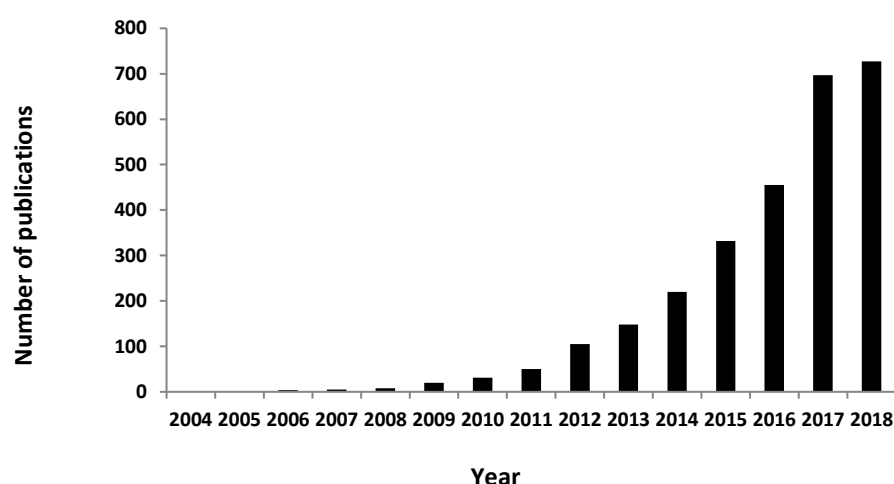
This characteristic is also affected by the addition of water into the composition of DES. However, some studies have been shown that the water addition superior to 50 % v/v provokes a dramatic change in the structure of DES. This feature is due to the rupture of a hydrogen bond between the initial constituents [207] [202]. Additionally, there is an inverse relationship between the viscosity of DES and temperature, as viscosity decreases with increasing temperature [205].

Regarding to DES density, the data on the density reported in the literature are not enough to be comprehensive. However, few studies examined the density of certain DES mixture

showing that most of polar DES exhibit higher densities than water. Besides, the densities of DES decreased linearly with increasing temperature, and increased with increasing DES mole fraction [197] [219]. Regarding the pH value of DES, it depends on the chemical nature of the components and especially on the HBD. Thus, it is possible to find basic, neutral, or acid DESs [200].

## 4.4 DES applications

The DES display interesting characteristics such as their huge chemical diversity, biodegradable properties, sustainability and acceptable toxicity profile [220]. Additionally, the fact that DES are easily prepared by low cost, with the possibility of adjusting their properties has intensified the interest of the scientific community. Indeed, since 2014 the publications related to DES application have increased exponentially (Figure 19).



**Figure 19. Number of publications in DES and NaDES from 2015 to 2017**  
From: [221]

The largest number of research on DES have been focused on Chemistry, material sciences, and Physics [222]. Among these fields, the predominant topics are related to organic synthesis reactions (either enzymatic or chemical) [223][224], metal processing [225], purification and processing in biodiesel production [225], removal of environmental contaminants and separation of azeotropes [226], or isolation and fractionation of compounds [227]. Moreover, DES have shown a great potential, especially in the medicinal, and biotechnology fields. Despite that, there are only a few studies on the toxicological DES



profile. For example, some *in vitro* studies have been made in bacteria, fish, human, and plant cells, while *in vivo* studies have been only performed in mice and some marine organisms [228]. These few studies reported that the toxicity of the DES was significantly higher than the toxicity of the starting materials. This can occur because the characteristic of the DES' components could be altered during the formation of DES [229][230]. However, these results are not conclusive, since other factors can be involved such as the molar ratio, the trial conditions, and/or the nature of DES components. Thus, with the lack of information about their toxicity, studies are still required before implementing DES for food, pharmaceutical or medical applications.

Here is a literature review of the different DES applications.

#### 4.4.1 Chemical and enzymatic synthesis

Organic reactions in green media have attracted considerable attention because it may reduce the use of toxic chemicals. Additionally, DES use as solvent media could offer other advantages, not only as a reaction media, but also as a catalyst [231].

In 2004, an investigation suggested that DES could be used as versatile alternatives to ILs. In this work, DES-based on ChCl and zinc have been successfully employed for a variety of synthetic reactions including Diels–Alder cycloaddition, Fischer indole annulation, and polymerization [205]. Other research groups have described other strategies for using DES in synthesis reactions. For example, the utilization of ChCl:*p*-toluenesulfonic acid (1:1 mole ratio) DES for the selective preparation of  $\alpha$ -mono- or  $\alpha,\alpha$ -dichloroketones at room temperature. The result showed that in comparison with MeOH, the reaction time was shorter, although similar yields were reached [232]. Comparable results were found when ChCl:urea (1:2 mole ratio) DES was used as catalyst and reaction medium for the bromination of 1-aminoanthra-9,10-quinone. The DES allowed a reduction of the reaction time with a high purity yield. Additionally, DES mixture was easily separated and reused, without a loss of efficiency [233]. The same DES combination was also applied for the synthesis of cinnamic acid and its derivatives via a Perkin reaction [234], and for the selective N-alkylation of aromatic primary amines. This method allowed preventing the complex multiple alkylations that are required with polar organic solvents and high reaction temperatures. Other study compared two methods for the process: biocatalysis using lipase-

catalyzed reaction, and DES as a catalyst and recyclable solvent. The best yield was reached using EtOH as organic solvent in combination with lipase. However, the DES ChCl:urea gave products in the same reaction time with slightly lower yields [235].

In this context, one of the first pioneer investigations done in the enzymatic synthesis was carried out by Gilles and co-workers [236]. They demonstrated that enzymes are able to retain their activity when dissolved in eutectic mixtures, providing a better reaction media than the conventional organic solvents. Other investigations were carried out with lipases such as *Candida antarctica* B (iCALB), *Candida antarctica* A (CALA), and *Pseudomonas capacea* lipase (PCL). Among them, iCALB exhibited the highest activities, whatever the DES, and the best results were obtained in ChCl:urea and ChCl:glycerol [200] [237]. In addition, seven DES were evaluated as medium for lipase-catalyzed reactions in the alcoholysis of vinyl laurate with 1-butanol, 1-octanol, and 1-octadecanol, using iCALB as catalyst. The results showed at total conversion of vinyl laurate into the corresponding esters using ChCl:urea and ChCl:glycerol [223]. Gorke and co-workers pointed out that these DES were also suitable co-solvents for lipase hydrolysis of *p*-nitrophenyl esters, or for perhydrolysis reaction [238].

On the other side, DES have been studied as synthesis media for polymers synthesis. For example, the use of ChCl:acrylic acid or ChCl:methacrylic acid DES for frontal polymerization showed superior performance (> 90%) in comparison with organic solvents and ionic liquids [239]. This process provides rapid and high conversion rates and reduces the energy costs associated with the preparation of polymers [228]. In the same research activities, poly (octanediol-co-citrate) elastomers containing high loading of lidocaine were synthesized using 1,8-octanediol and lidocaine based DES. In this study, DES played a role as a reservoir of monomer (1,8-octanediol), as a reservoir of active pharmaceutical ingredient (lidocaine) and as a solvent for the dissolution of citric acid used as an initiator in this polymerization reaction [240]. On the other hand, it has been demonstrated the possibility to use DES as polymers plasticizing agents for the development of 3D porous architectures, that have shown applications in a wide variety of scientific fields, particularly in analytical chemistry [231]. Additionally, DES and lipases were also successfully combined to make polymers by ring opening polymerization of caprolactone [238]. Other interesting application recently

explored is the use of DES in nanoparticle synthesis, since they offer thermal stability, good dispersibility, large ionic conductivity, and wide electrochemical window [207]. After synthesis, these materials are widely applied in the different steps of analytical process like extraction, separation, and detection enhancement [231].

#### **4.4.2 Preparation of materials**

Conventional preparation of inorganic materials often takes place in water or organic solvents, using in many cases thermal steps for crystallization and structure formation.

Recently, a new strategy called ionothermal synthesis has been developed using ILs or DES as solvents [197]. According to the Royal society of Chemistry, ionothermal synthesis is a synthesis method for growing single crystals from an ionic liquid in an autoclave (a thick-walled steel vessel) at high temperature (400 °C) and pressure. In 2004, Cooper et co-workers [241] developed a novel method for preparing microporous solids at ambient pressure using ChCl:urea as both the solvent and template (or structure-directing agent that guides the synthesis pathway towards particular structures) in ionothermal synthesis of zeolite analogs. During this study it was possible to tune the solvent properties of the DES to match the system requirements.

DES based on ChCl and renewable carboxylic acids have been also used as an alternative medium to ILs in the ionothermal strategy [197]. In 2009, the Morris group reported the use of ChCl:1,3-dimethylurea to prepare a borate-containing material [242].

In resume, the ionothermal synthesis using DES have many benefits over hydrothermal synthesis. Hydrothermal method relies on the forced hydrolysis of the reactants in order to produce new materials including single crystals, zeolites and related microporous materials, ionic conductors, complex oxides and fluorides, inorganic–organic hybrid materials, and nanomaterials. This is achieved usually in water at moderate temperatures (<200 °C) and high pressures [243]. On the contrary, ionothermal synthesis can take place at ambient pressure, due to the DES low vapor pressure even at elevated temperatures, eliminating safety concerns with high solvent pressures. In addition, DES can play multiple roles during synthesis, including the classical role of solvent, templating agent, reactant, etc. [197].

The potentially millions of eutectic mixtures available compared to only ~600 molecular solvents used for hydrothermal synthesis, offers more opportunities to conduct ionothermal synthesis [241]. Thus, many inorganic materials with valuable properties and structures, including zeolite-type materials, metal–organic frameworks (MOFs), metal oxides, nanomaterials, carbon materials, among others, have been synthesized using the ionothermal synthesis strategy [197].

#### **4.4.3 Electrochemistry**

Recently, the ILs have been used to the modification of electrodes to be used in the electrochemical analysis. Although these studies have revealed good results, the preparation involved complex procedures, that are usually expensive and take considerable time [244]. In this context, DES are an attractive option since they have interesting electrochemical properties, such as higher ionic conductivity and wider electrochemical windows, just like ILs. The literature shows that the main DES applications in electrochemistry are in the field of electrodeposition (58.18%), electrochemical behavior (20%), and enhancing performance of electrochemical properties (14.55%). However, others few studies such as electrowinning and electropolymerization have been also developed with the DES [231].

In this context Abbott and co-workers were the first to describe the electrolytic deposition of copper and copper composites from a solution of metal chloride salt, either in ChCl:urea or ChCl:ethylene glycol (EG) [194]. In fact, choline chloride based DES have been successfully assessed for electrodeposition of different metals (Cr, Mn, Cu, Ag, etc.) [245], alloys (Zn/Cr, Zn/Sn, etc.) [246][247], or electrowinning of metals from complex oxide matrices [248] with enhancing electrochemical properties [249].

#### **4.4.4 Extraction solvent**

As previously described, some phytochemicals fulfill important functions in plants such as growth, reproduction, defense, and detoxification process, among others. However, in the last decades, their study has been focused on the beneficial effects on the human health, since they can help in the prevention of various diseases. For example, they possess

important biological properties such as anti-inflammatory, antiviral, analgesic, anticarcinogenic, antifungal and antibacterial activities, and antioxidant [250][136].

Plant metabolites differ in many aspects such as chemical structure, polarity, stability, quantity among others. Thus, to achieve efficient extraction of phytochemicals, solvents are required. Since there is not a universal solvent, one of the preliminary and critical step before foresee any application lies in their extraction from plant tissues [251].

The most common extraction techniques are liquid–liquid extractions (LLE) or solid-phase extractions (SPE) [252]. They employ solvents, mostly organic, such as acetone and ethyl acetate, or alkyl alcohols like methanol (MeOH), ethanol (EtOH) and propanol; or their mixtures [253]. Even if organic solvents have an excellent ability for dissolution and extraction of different structure of molecules, they show many intrinsic drawbacks, such as accumulation in the atmosphere (low boiling points), flammability, high toxicity, non-biodegradability, and cost [254]. Additionally, from an environmental standpoint, these solvents can no longer meet the trend of Green Chemistry. For all those reasons, new alternative green methodologies and solvents are investigated to extract metabolites from plants.

Supercritical fluids (SCF), mainly CO<sub>2</sub> [255], or water [256] could be an option, but their restricted range of molecule solubility, the high intrinsic reactivity, the high cost of equipment for obtaining supercritical conditions and the prized pure water (especially while contamination could happen, making water purification very difficult and expensive), do not offer real prospects for the future. Biomass-derived solvents, such as EtOH, limonene, ethyl lactate, glycerol, or 2-methyltetrahydrofuran, could also be used as another alternative. They are bio-renewable, innocuous, and not very expensive, but their versatility and solvation properties are rather restricted.

Interestingly enough, the DES properties, such as negligible volatility, adjustable viscosity, sustainability, biodegradability, acceptable toxicity profiles, and high solubilization power offer interesting perspectives as green smart solvent.

Additionally, DES have the ability of donating and accepting protons and electrons, allowing them to strongly interact with various functional groups such as hydroxyl groups, carboxylic

acids, and amines [19]. Thus they can extract both polar and non-polar compounds [199], with great potential in the extraction of valuable secondary metabolites of plants. In addition, DES may be used as, first, extracting media, and, second, formulation system, thus bypassing the problem of solute recovery. Indeed, depending on the nature of the starting components, DES may be fully compatible with the targeted application, and the extracts (composed of DES with enhanced concentration of bioactive molecules) allow for better phytoactive profiles in comparison with other extraction procedure [257]. For example, some DES, especially those containing organic acids, have been suggested to possess antimicrobial activity, which may be related to their ability to solubilize a wide range of solutes (e.g. components in the bacterial membrane), pH, osmolality or chelation of membrane-bound divalent cations [258][259][260]. Such property may be advantageous when DES are used as both solvent for extraction and for formulation of systems containing molecules possessing antimicrobial activity, *e.g.* PC.

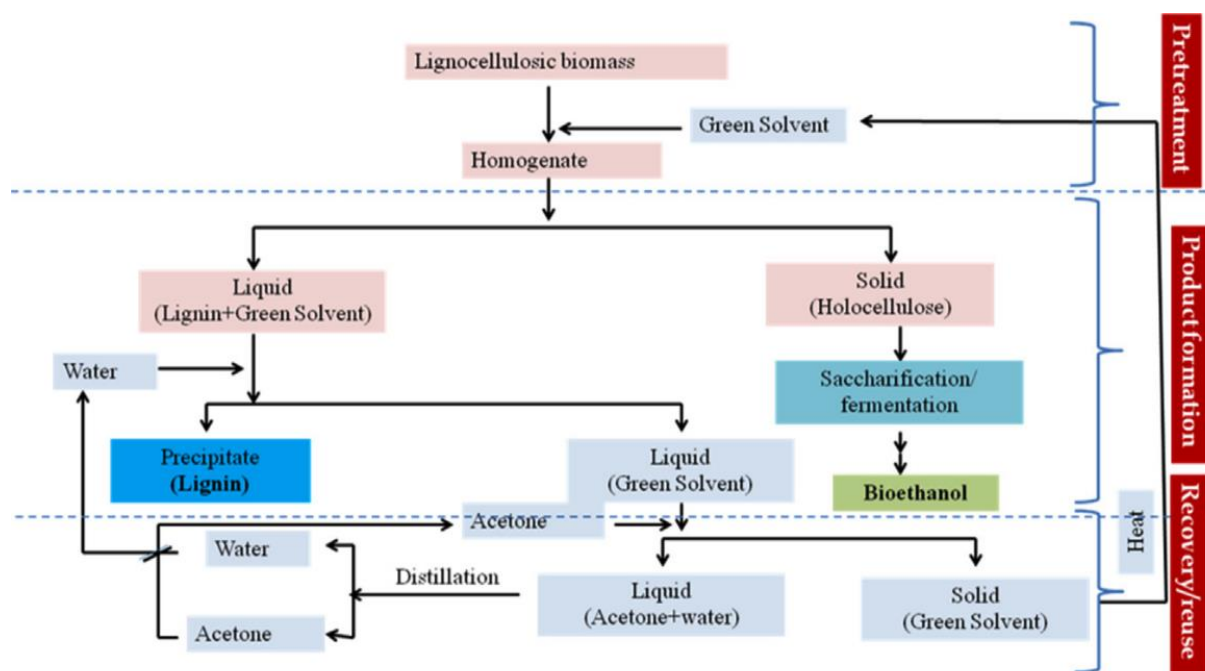
Actually, bioactive compounds isolated by DES and NaDES have been increasingly investigated over the last years, since plant metabolites show a great potential as nutraceuticals.

In the field of food industry and agro-forest waste valorization, through treatment by DES of the waste, by-products, and natural materials, several types of compounds were obtained. Among them the flavonoids, stilbenes, tannins and anthocyanins, and other PC extraction has been studied [6], as well as the biomass treatment [35].

Biomass is a very important source of diverse materials, biofuels, and high value chemicals. Fractionation on raw materials is an essential operation for almost all processes acquiring other products [261]. However, the applications of standard technologies produce toxic and dangerous contaminants which are released into the air or into the water [262]. In this context, ILs appeared to be efficient and promising solvents in the pretreatment of lignocellulosic biomass. Nevertheless, their industrial application is still facing major issues, such as high cost and hazardous toxicity [263]. On the contrary, DES seem an interesting alternative due to the aforementioned physico-chemical characteristics. In addition, they presented other advantages such as inhibition of the water reactivity, stabilization of

carbohydrates and furanic derivatives through hydrogen-bond interactions, decrease of polyol viscosity, and compatibility with few enzymes, among others [19].

In 2012, Francisco and co-workers [192] reported for the first time the promising solubility of wheat straw biomass in DES. In this study, the authors showed that the lactic acid and ChCl mixture were efficient in lignin solubility but was negligible for cellulose. Inversely, malic acid:proline showed much higher capacity to solubilize starch and cellulose. Several studies followed up and went to investigate the impact of DES treatment on the composition of treated biomass. For example, it was found very low cellulose solubility when using malic acid:alanine (1:1 mole ratio) and malic acid: proline (1:1 mole ratio). However, solubility of starch was significantly higher especially using mixtures of glycine:malic acid (1:1 mole ratio) and ChCl:malic acid (1:1 mole ratio). Additionally, the solubility of lignin in DES was very promising, especially for DES containing lactic acid, with a solubilization considerably higher than the cellulose and starch [264]. These results were confirmed by Kumar and co-workers [265] who studied the ethanol production through the pretreatment of rice straw by five DES based on ChCl and betaine. They observed that cellulose and xylan have insignificant solubility. According to their results, 500 mg of lignin was 100 % soluble in ChCl:lactic acid (1:2 mole ratio), (1:5 mole ratio) and (1:9 mole ratio) whereas only 12 % and 38 % were soluble, in betaine:lactic acid (1:2 mole ratio) and betaine:lactic acid (1:5 mole ratio), respectively. The authors showed that the addition of small amounts of water during pretreatment significantly enhanced the total lignin solubility. Finally, they proposed an integrated zero-waste process for cellulosic ethanol production including three main stages: (i) DES lignocellulosic biomass pretreatment, (ii) lignin and ethanol production, and (iii) solvent recovery and reuse (Figure 20).



**Figure 20. Flowchart of an integrated zero-waste cellulosic ethanol production technology**  
From: [265]

DES have also received a particular attention for the dehydration of hexoses and pentoses to furanic derivatives such as 5-hydroxymethylfurfural (HMF) and furfural, respectively [19]. In this field, three main strategies have been used: catalytic conversion of carbohydrates in neat DES, catalytic conversion of carbohydrates in an aqueous solution of DES, and catalytic conversion of carbohydrates in a supported-DES phase [19]. For example, it was possible to strongly solubilize fructose using ChCl based DES in combination with citric acid or oxalic acid. Moreover, when the reaction was heated at 80 °C for 1 h, HMF was produced with 76 % yield [266]. In another study, after 15 and 25 min of reaction at 140 °C, furfural was obtained with 59 % and 54 % from xylan and xylose, respectively, using the same mixture based on ChCl and citric acid containing  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  [267]. The formation of HMF and furfural has been reported as products of biomass degradation after DES acid pretreatment [268]. Other strategy used carbohydrates as both reactant and component of the DES (always in combination with ChCl). This approach allows a highly concentrated solution of carbohydrates (40-50 wt%), a significant advantage with respect to industrial transfer [19].

The DES ability to deconstruct the biomass seems to be encouraging for different fields of application, as well as to the production of value-added chemicals.



Additionally, DES are particularly attractive for bioconversion of substrates having limited solubility in common organic solvents, or for processes that cannot be performed in water owing to competitive hydrolysis reaction. They also have the potential to play an important role as a solvent, or co-solvent with water, in various stages of the conversion of lignocellulosic biomass. However, beyond their price and its ecological footprint, it is important to consider the DES physical properties (for example, viscosity, conductivity, polarity, and boiling point) as well as the separation process, for their application in this field.

Alternatively, the interest of DES (or NaDES) for metabolites extraction from plant materials is increasing, because they are very good solvents with rare solvation properties. The main striking example was obtained with rutin solvation, which is a heteroside with sugar residues, but paradoxically almost insoluble in water [198]. It was found that hydrated DES (or NaDES) had a strong ability to dissolve the rutin, as well as other cellular metabolic compounds, indicating 50 to 100-fold higher concentration than in water. On the one hand, in these media, the hydrogen interactions between DES (or NaDES) and a solute are so strong (stronger than water-solute) that they prevail over the rest of the solute-solute electrostatic forces. On the other hand, this phenomenon may be explained by the presence of flat aromatic hydrophobic groups that are able to establish "pi-stacking" interactions stronger than solvation forces. The stability of the building block created by the pi-stacking of a large number of aromatic rings is strengthened by the surrounding hydrogen bonds of their polar hydroxyl groups (phenols). This leads to the formation of aggregates which can reach high sizes that, at room temperature, become insoluble and precipitate in the aqueous phase. Interestingly, it is by masking the polar groups (phenolics -OH), by transformation into glycol ethers, that the water insolubility is overrode (e.g. troxerutin is totally water-soluble). The transformation of free -OH groups into ethers destabilizes the stacks in favor of a solvation and solubilization by water molecules. In other words, the van der Waals interactions become weaker than the steric hindrance of the ether groups, leading to a dispersion of the aromatic rings in the aqueous phase.

The great diversity of phytochemicals including PC, added to inherent properties of plant material in term of structure and composition, make difficult the selection of the right material-solvent system and methodology to extract those molecules [6]. Hence, the use of

DES for the extraction can offer a wide range of possibilities. For example, the researches focused on the extraction of PC by different combinations of DES and vegetables sources are very important (Table 7). For example, different phenolic acids such as 5-CQA, CA, 3,5-DCQA, 3,4-DCQA, and 4,5-DCQA, were successfully extracted from *Lonicera japonica* with 1,3 ChCl:butanediol (1:6 mole ratio) [269]. In a very similar study, Park and co-workers [270], investigated the phenolic acids (5-CQA and CA) extraction from *Herba artemisiae scopariae* using DES and MeOH/H<sub>2</sub>O (60:40, v/v) as a binary mixture. Twelve DES were tested, and the best result was obtained with tetramethylammonium chloride:urea (1:4 mole ratio). The extraction yields were similar when 50, 65, and 80 % of DES were added in a mixture with MeOH/H<sub>2</sub>O (60:40, v/v). Likewise, flavonoids extraction by DES has also been performed. For example catechin, ECG, EGCG, EGC, EC, wogonoside, baicalein, baicalin, and wogonin from *Camellia sinensis* and *Radix scutellariae* were recovered using lactic acid:ChCl (2:1 mole ratio) [271][272]. In a similar study EGCG, EC, and EGC from *Camellia sinensis* leaves were extracted by different ChCl-based DES made of HBDs that belong to the family of alcohols, organic acids, and saccharides. The results showed that lactic acid allowed extracting three catechins at higher yields [271]. In 2015, Qi *et al.* [273] evaluated the extraction of nine flavonoids from *Equisetum palustre* L. using nine different ChCl- or betaine-based DES. The best extraction yield of the target compounds was reached when ethylene glycol was used as HBD.

Table 7. DES as solvent for PC extraction from different vegetables sources

DES Composition			Source	Mole ratio	% Water (v/v)	Target compounds	Ref.
ChCl	1,3-Butanediol	-	<i>Lonicera japonica</i>	1:6	10	5-CQA, CA, 3,5-DCQA, 3,4-DCQA and 4,5-DCQA.	[269]
ChCl	Maltose	-	<i>Cajanus cajan</i>	1:2	20	Pinostrobin chalcone, pinostrobin, longistylane C, cajaninstilbene acid, cajanuslactone, cajanol, apigenin-6,8-di-C- $\alpha$ -L-arabinoside, and apigenin-8-C- $\alpha$ -L-arabinoside.	[274]
ChCl	Betaine hydrochloride	Ethylene glycol	<i>Equisetum palustre</i> L.	1:1:2	20	Kaempferol-3-O- $\beta$ -D-glucopyranoside-7-O- $\beta$ -D-glucopyranoside, kaempferol-3-O- $\beta$ -D-rutinoside-7-O- $\beta$ -D-glucopyranoside, luteolin-7-O- $\beta$ -D-glucopyranoside, quercetin-3-O- $\beta$ -D-glucopyranoside, apigenin-5-O- $\beta$ -D-glucopyranoside, genkwanin-5-O- $\beta$ -D-glucopyranoside, luteolin, genkwanin, and apigenin.	[273]
ChCl	1,4-Butanediol	-	<i>Pyrola incarnata</i> Fisch, <i>Chamaecyparis obtusa</i>	1:4 1:5	30	Hyperin, 2'-O-galloylhyperin, quercitrin, quercetin-O-rhamnoside, chimaphilin, amentoflavone and myricetin	[275] [276]
ChCl	Lactic acid	-	<i>Camellia sinensis</i> , <i>Radix scutellariae</i>	1:2	20	Catechin, ECG, EGCG, EGC, EC, wogonoside, baicalin, baicalin and wogonin	[271] [272]
ChCl	Ethylene glycol	-	Green tea, <i>Prunella vulgaris</i> L.	1:5 1:4	30	Catechin, EGCG, ECG, rosmarinic acid, and salviaflaside	[277] [278]
ChCl	Laevulinic acid	-	<i>Salviae miltiorrhizae</i> , <i>Platycladi cacumen</i>	1:2	25 10	Rosmarinic acid, salvianolic acid, anlithospermic acid, myricitrin, quercitrin, amentoflavone, and hinokiflavone	[279] [280]
ChCl	Oxalic acid	-	Grape skin	1:1	0.25:1 <sup>a</sup>	(+)-Catechin, delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside, and quercetin-3-O-glucoside	[281]
ChCl	1,6-hexanediol	-	Pigeon pea roots	1 : 7	30	Genistin, genistein, and apigenin	[282]
Citric acid	D-(+)-Maltose	-	Grape skin	4:1	3:7 <sup>a</sup>	Total anthocyanins content	[283]
L-Proline	Glycerol	-	<i>Flos sophorae</i>	1:2.5	3:7 <sup>a</sup>	Quercetin, kaempferol, and isorhamnetin glycosides	[284]

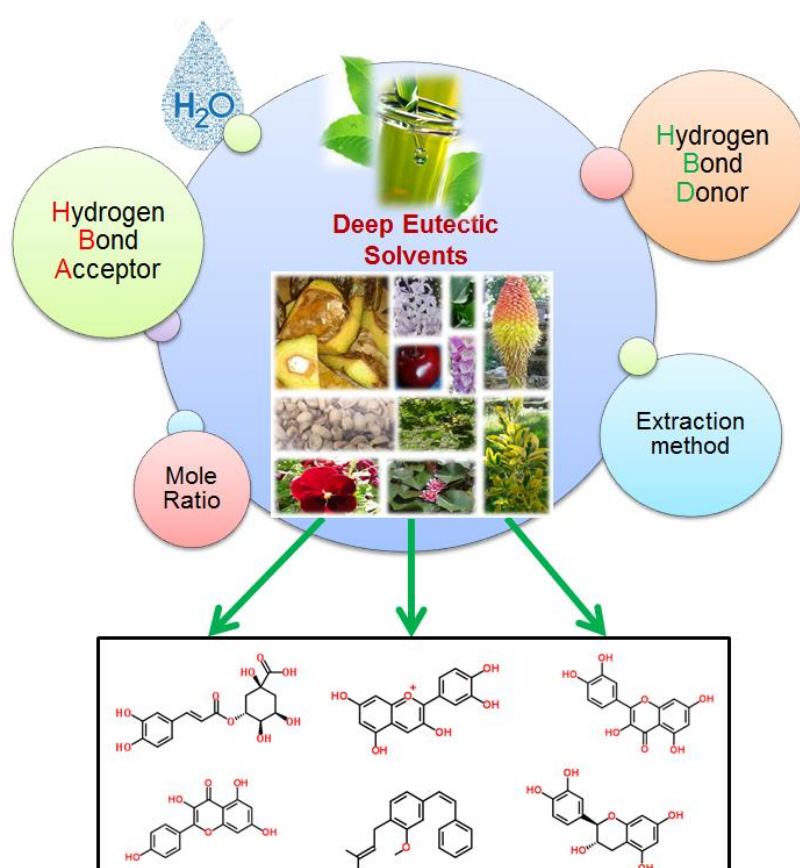
<sup>a</sup> Water:DES % w/w  
From: [6]

In addition, ethylene glycol in combination with ChCl showed the best extraction yield for higher polar flavone compounds, whereas associated with betaine it showed better extraction for the relatively weak polar ones. This result pointed out the importance of the correct selection of the DES for extracting the desired compound(s).

In other study, 43 DES with different physical properties were used for extraction of five different types of bioactive natural-occurring compounds (flavonoid, alkaloid, anthraquinone, saponin, and phenolic acid) from Chinese herbal plant. The authors showed that icariine prenylated flavonol glycoside was highly extractable with L-proline based DES, and the results emphasized that 14 DES exhibited higher yields for icariine extraction than MeOH, with the best results obtained when lactic acid was used in association with L-proline (1:1 mole ratio). In addition, compounds such as isoquinoline alkaloids from *Berberidis radix* were effectively extracted with different carboxylic acid-based DES [279].

The anthocyanin extraction from grape pomace was carried out with 10 different ChCl-based DES in association with different classes of HBDs including carboxylic acids (D- and L-malic acids and citric acid), sugar alcohols (glycerol and maltitol), monosaccharides (D-(-)-glucose, D-(-)-fructose, D-(-)-galactose, and D-(-)-ribose), and disaccharides (sucrose and D-(+)-maltose). The combinations with citric acid, maltitol, fructose, and D-(+)-maltose showed significantly higher levels of total anthocyanins, in comparison with MeOH:H<sub>2</sub>O (80:20, v/v). To improve the extraction yields, the authors evaluated the association of citric acid with fructose, maltose, or maltitol. Citric acid:maltose (4:1 mole ratio) in binary mixture with water (DES:water 3:7 (w/w)) showed the best extraction yield [283]. Likewise, delphinidin-3-O-monoglucoside, cyanidin-3-O-monoglucoside, petunidin-3-O-monoglucoside, peonidin-3-O-monoglucoside, malvidin-3-O-monoglucoside, malvidin-3-O-acetylmonoglucoside, peonidin-3-(6-O-p-coumaroyl)-monoglucoside, and malvidin-3-(6-O-p-coumaroyl)-monoglucoside were extracted from the grape skin using five ChCl-based DES [281]. According to the results, the best extraction yields were obtained with ChCl:oxalic acid (1:1 mole ratio) followed by ChCl:malic acid (1.5:1 mole ratio). The results suggest that acidity of the solvents could be the key in the efficient extraction of anthocyanins, since the pH is very important in the anthocyanin equilibrium forms and stability.

The application of DES as solvents for stilbenes extraction has been also studied by Wei and co-workers [274]. Indeed, Longistylane C and cajaninstilbene were extracted from *Cajanus cajan*'s leaves using 14 different DES made of ChCl, citric acid, and lactic acid. Although EtOH (80 % v/v) and ChCl:maltose (1:2 mole ratio) showed similar amounts of extraction for the compounds, this DES was the best in comparison with the other DES mixtures. In addition, the efficiency of DES as extraction solvent can be affected by experimental conditions and the nature of the liquid. Among them, the most important are viscosity, water content (generally between 20 and 30 % (v/v)), DES constituents' mole ratio, liquid/solid ratio between the solvent and the sample (optimal between 10 and 20 mL/g), temperature (between 60 and 70 °C), etc. Thus, it is recommended to fine-tune the conditions in order to enhance the extraction yields (Figure 21) [6].



**Figure 21. Influence of extraction conditions using DES as extraction solvents**

From: [6]

An important challenge in the use of DES as extraction solvent is the target molecules recovering after extraction, since this can involve expensive and complicated steps. One of

the simplest procedures is combining evaporation and separation by chromatographic techniques. Paradoxically, in the particular DES case, their very low vapor pressures are both a drawback and a benefit to performing the extraction. This is an asset during extraction processing because the temperature may be increased without considering solvent evaporation, but at the same time, it will make difficult nonvolatile solute separation. Moreover, higher DES extraction capacity means a better interaction between PC and DES; as a result, more problems to isolate PC from DES. Four main strategies have been studied to recover molecules, especially PC, from DES: liquid–liquid extraction (LLE), solid–liquid extraction (SLE), or precipitation by addition of antisolvents (water preferentially). However, both LLE and SLE would involve the use of a non-negligible amount of alternative (e.g., organic) solvents that could, in the end, counterbalance the initial DES' interest. The addition of antisolvents (EtOH or water) is probably the most valuable method because, unlike SLE and LLE, it does not require specific equipment or additional fees or investments and allows the development of sustainable processing. Yet, it cannot be used for EtOH- or water-soluble DES' molecules [6]. Facing this problematic, a perfect option would be to use DES as, first, extracting media, and, second, formulation system. Indeed, depending on the nature of the starting components, DES may be fully compatible with cosmetics applications, pharmaceutical formulations, or even food manufacturing. Moreover, some studies have shown that DES can promote the biodisponibility of some drugs [285][286][287].

# *Materials and methods*



## Materials and methods

### V. Reagents

The solvents used in the development of experimental part are described in the Table 8.

**Table 8. List of chemicals, with grade, CAS number, and supplier.**

Reagent/Solvent	Grade	CAS Number
<b>Supplier : ACROS ORGANIC (Geel, Belgium)</b>		
Butylated hydroxytoluene (BHT)	99%	128-37-0
<b>Supplier : Avanti (Alabama, USA)</b>		
1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC)	Analytical	18194-25-7
1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Analytical	63-89-8
<b>Supplier : Biolabo (Maizy, France)</b>		
Uric acid Kit	-	80231 800001 87601
<b>Supplier : Biosentec (Auzeville-Tolosane, France)</b>		
Glycerol Kit	-	25
<b>Supplier : Cayman Chemical (Ann Arbor, MI, USA)</b>		
d <sub>4</sub> -15-F <sub>2</sub> t-IsoP (8-isoPGF <sub>2</sub> α-d <sub>4</sub> )	≥ 99 %	211105-40-7
<b>Supplier : Elitech (Puteaux, France)</b>		
ALT/GPT 4+1 SL	-	ALSL-0250
AST/GOT 4+1 SL	-	ASSL-0250
ELICAL 2, calibrator	-	CALI-0550
<b>Supplier : Fischer Scientific (Loughborough, UK)</b>		
Acetonitrile	HPLC	75-05-8
Chloroform	HPLC	67-66-3
<b>Supplier : Honeywell (Muskegon, MI, USA)</b>		
Ethanol (EtOH)	Reagent	64-17-5
Methanol (MeOH) CHROMASOLV ®	HPLC ≥ 99.9 %	67-56-1
Water CHROMASOLV ®	HPLC	77732-18-5
<b>Supplier : Larodan (Solna, Sweden)</b>		
sn-Glycero-3-phosphocholine (GPC)	>99%	28319-77-9
<b>Supplier : Merck (Darmstadt, Germany)</b>		
Glacial acetic acid	Reagent 99-100 %	64-19-7



Reagent/Solvent	Grade	CAS Number
Sulfuric acid	99%	7664-93-9
<b>Supplier : Prolabo- VWR (Fontenay-sous-Bois, France)</b>		
Acetic acid	99-100 %	64-19-7
<b>Supplier : Sigma-Aldrich (Saint Louis ,USA)</b>		
1,2 Propanediol	≥ 99.5 %	55-57-6
1,4 butanediol	Reagent Plus <sup>®</sup> 99 %	110-63-04
2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)	97%	2997-92-4
Acetic acid	Reagent Plus <sup>®</sup> ≥ 99.9 %	64-19-7
Acetic anhydride	Reagent Plus <sup>®</sup> 99 %	108-24-7
Ammoniac	≥ 99.95 %	7664-41-7
Arachidic acid	≥ 99 % (GC)	506-30-9
Behenic acid	99%	112-85-6
Betaine (perchloric acid titration)	≥ 98 %	107-43-7
Caffeine	Reagent Plus <sup>®</sup>	58-08-2
Chlorogenic acid	≥ 95 %	327-97-9
Chloroforme	Reagent ≥ 99 %	67-66-3
Choline chloride (ChCl)	≥ 98 %	67-48-1
D-(-)-Ribose	≥ 99 %	50-691
D-(+)-Galactose	≥ 99 %	59-23-4
D-(+)-Glucose	≥ 99.5 % (GC)	50-99-7
D-(+)-Mannose	≥ 99 %	3458-28-4
D-(+)-Xylose	≥ 99 %	58-86-6
Dimethylsulfoxide	≥ 99.5 % (GC)	67-68-5
Erucic acid	Analytical standard	112-86-7
Folin-Ciocalteu	Reagent	ND
Furfural	99%	98-01-1
Gallic acid	≥ 97.5 %	149-91-7
Glycerol	≥ 99 %	56-81-5
Heptadecanoic acid	99%	506-12-7

Reagent/Solvent	Grade	CAS Number
L-(+)-Arabinose	≥ 99 %	87-72-9
Lactic acid	~ 90%	50-21-5
Lauric acid	≥ 98 % (GC)	143-07-7
Lignin alkali	Reagent	8068-05-1
Linoleic acid	≥ 99 %	60-33-3
Malic acid	Reagent Plus <sup>®</sup> 99 %	6915-15-7
Methyl imidazol	≥ 99 %	616-44-7
Myristic acid	≥ 98 % (GC)	544-63-8
Oleic acid	≥ 99 % (GC)	112-80-1
Palmitic acid	≥ 99 %	57-10-3
Palmitoleic acid	≥ 98.5 % (GC)	373-49-9
Pentadecanoic acid	~ 99 % (GC)	1002-84-2
Phosphate buffer pH 7.2		ND
Rhamnose	≥ 99 %	10030-85-0
Sodium borohydride	≥ 96 %	16940-66-2
Sodium carbonate anhydride	Reagent	497-19-8
Stearic acid	≥ 98.5 % (capillary GC)	57-11-4
Theobromine	≥ 98 %	83-67-0
Trolox	97%	53188-07-1
α-Linolenic acid	Analytical standard	463-40-1
β-D-Allose	-	7283-09-2
<b>Supplier : Sigma-Aldrich (Saint Quentin Fallavier, France)</b>		
5,5'-Dithiobis(2-nitrobenzoic acid)	BioReagent, ≥ 98 %	69-78-3
Ammonia	≥ 99.95 %	7664-41-7
Bovine serum albumin (BSA)	≥ 96 %	9048-46-8
Chloramine T trihydraté	Reagent	7080-50-4
DCC (N, N' -dicyclohexylcarbodiimide)	HPLC	538-75-0
EDTA disodium salt	Molecular biology	39-33-33
Ethanol absolute	Spectrometric 95 %	64-17-5

Reagent/Solvent	Grade	CAS Number
Formic acid	Reagent $\geq 95\%$	64-18-6
Hexane	Analytical standard	110-54-3
Potassium hydroxide (KOH)	$\geq 85\%$	1310-58-3
Potassium iodide	Reagent $\geq 99.5\%$	7681-11-0
PPyr32 (4-pyrrolidinopyridine)	HPLC	2456-81-7
Rat CRP/C-Reactive Protein ELISA Kit	-	RAB0097
Sodium chloride	$\geq 99.5\%$ (AT)	7647-14-5
Tung Oil	HPLC	8001-20-5
<b>Supplier : Sobioda, MontBonnot (Saint-Martin, France)</b>		
Cholestérol HDL Kit	-	W1W412-72395 W1W412-72495
Creatinine LT Kit	-	W1W419-08199 W1W419-08299
Glucose LD M Kit	-	1306132-1306200E 1306122-1306143E
Triglycerides LQ GPO-POD Kit	-	WTRIG050
<b>Supplier : VWR (Fontenay-sous-Bois, France)</b>		
Di-potassium phosphate (K <sub>2</sub> HPO <sub>4</sub> )	EMERURE® for analyses	04-11-7758
Potassium phosphate (K <sub>2</sub> HPO <sub>4</sub> )	EMERURE® ISO for analyses	7778-77-0
Ethyl acetate	HPLC	141-78-6
<b>Supplier : Wako Chemical (Neuss, Germany)</b>		
2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN)	95%	44-19-11-8

The external standards d<sub>4</sub>-10(R)-10F<sub>4t</sub> NeuroP, C<sub>21</sub>-15-F<sub>2t</sub>-IsoP, and C<sub>19</sub>-16-F<sub>1t</sub>-PhytoP were synthesized by the *Institut des Biomolécules Max Mousseron* at the Faculty of Pharmacy, according to the previously described procedure [288][289].

## VI. Material plant

Green coffee beans (GCB) (*Coffea arabica* var. *sachimor*) and coffee pulp (CP) were collected in Huatusco (Veracruz, Mexico), while Criollo cocoa husk (CH) *Theobroma cacao*) and cocoa pod husk (CPH) were collected in Comalcalco (Tabasco, Mexico), both in 2016. CP and CPH

were chopped and dried in a fluidized bed dryer (GRACO, TGA101, Coatepec, Veracruz, Mexico) for 90 min at 60 °C. GCB were sun-dried, and milled in a grinding mill IKA-WERKE M20 (Wilmington, United States), sieved to particle size of 630 µm, and then stored at -20 °C before *in vivo* experiments. CH were obtained as by-products of the conventional industrial roasting process from the collection center of AMCO Company located in Tabasco, Veracruz, Mexico. The dried raw material was milled in a Knife Shredder (RETSCH SM100, Haan, Germany) using sieves of 5 mm. The final material was stored under vacuum in plastic bags in the dark at 4 °C until the experiments.

In the case of the PhytoPs and PhytoFs extraction, dried raw material was milled in a kitchen-type grinder (Moulinex Deluxe Stainless Steel Mill 843, France), sieved in particle sizes between 315 and 630 µm, and then stored at -20 °C before use.

## **VII. Analytical methods**

### **7.1 Chemical characterization of different classes of molecules**

Before starting the extraction experiments, the chemical composition of each sample (CP, CH, and CPH) was determined. Each determination was made by triplicate.

Total ash, moisture, and protein (N×6.25) were determined according to the Association of Official Analytical Chemists guidelines (AOAC) (2005) [290], and expressed on dry weight (dw) basis.

The lipid determination was performed by Soxhlet extraction with hexane as solvent according to the AOAC method [290], and the extracts were converted to methyl esters (FAMES) according to NF T60-233 method [291]. The fatty acid profile was performed on the GC Agilent 6890 (Bios Analytique, France) series, equipped with an Innowax capillary column (SGE, Courtaboeuf, France), with the following characteristics: length, 30 m; internal diameter, 0.32 mm; film thickness 0.25 µm. Helium was used as carrier gas with a flow of 1 mL/min, splitting ratio: 1/80. The temperature of the injector was 250 °C and flame ionization detector was set at 270 °C. The oven was heated from 185 °C to 225 °C at 5 °C/min

and held at 225 °C for 20 min. Fatty acids methyl esters (FAMES) were identified by comparison with commercially available standards (Table 8). The fatty acid content was expressed as mg/g dw of each plant material, according to the relative amount (% w/w) of this fatty acid in the lipid fraction.

The water content of CP, CH, and CPH was determined by measuring the weight loss of a sample after heating the material. For this, 3 g of each raw material was heated at 105 °C, and the difference between the sample weight prior the heating and after reaching a steady-state mass determines the moisture content.

Fiber fractions were determined by the Van Soest sequential analytical method [292]. The results are expressed as NDF (neutral detergent fiber), ADF (acid detergent fiber), and ADL (acid detergent lignin). This method allows an estimation of fiber fractions: hemicellulose (NDF-ADF), cellulose (ADF-ADL) and lignin (ADL). Analyses were made at CIRAD-UMR SELMET (Système d'élevage méditerranéennes et tropicaux) (Montpellier, France).

The sugar determination was carried out by the modified method reported for Blakeney and co-workers [293], through the preparation of aditol acetates from monosaccharides.

In the case of our samples (CP, CH, and CPH) a sulfuric acid pre-hydrolysis (26 N, room temperature; 0.625 mL/ 200 mg of each sample) kinetic was tested at 0 (without hydrolysis) 1, 2, 3 and, 4 h. Then all samples were hydrolyzed during 2 h at 100°C by adding 1 mL of sulfuric acid (3 N). The monosaccharides were reduced for 90 min at 35 °C by adding 1 mL of solution previously prepared as follows: 0.5 g of sodium borohydride were dissolved in 25 mL of anhydrous dimethyl sulphoxide at 100 °C, and then cooled to room temperature. After reduction, the excess of sodium borohydride was decomposed by the addition of 100 µL glacial acetic acid, and then the acetylation of the samples was carried out adding 150 µL of methylimidazole followed by 2 mL acetic anhydride and mixing. After 20 min at room temperature, water (6 mL) was added to decompose the excess of acetic anhydride. Finally, chloroform (2 mL) was added and the mixture was agitated on a vortex mixer. After the phases were separated by decantation, and the aqueous phase was discarded. This step was repeated two times washing the samples with 8 mL of water, stirring and discarding the aqueous phase. Finally, 1 mL of the organic phase was recovered and concentrated until

dryness (by evaporation). Before analysis, the obtained aditol acetates were diluted in 350  $\mu$ L of chloroform.

Anhydrosugar determination and quantification was done by gas–liquid chromatography (GC) equipped with DB 225 capillary column (50 % cyanopropylphenyl-dimethylpolysiloxane) (30 m x 0.32 mm) (Macherey-Nagel, Strasbourg, France) at 255 °C, using hydrogen as carrier gas and allose (1 mg/mL was added for each sample)) as internal standard. The oven temperature was 215 °C and the injection port and detector were heated to 220 and 260 °C, respectively. The calibration curves of rhamnose, ribose, arabinose, xylose, mannose, galactose, and glucose were used to identify and quantify each monosaccharide.

The content of each monosaccharide was expressed as % on dry weight.

## **7.2 Biomolecules extraction by deep eutectic solvents (DES)**

According to the literature CP, CH, and CPH are vegetal samples rich in interesting biomolecules. The earlier objective of this research thesis was the extraction and quantification of PC and alkaloids, since they are the most described compounds in these vegetal samples, with interesting bioactivities.

After that, the vegetal materials were evaluated in terms of isoprostanoids (PhytoPs and PhytoFs), and thiols precursors content, using classic organic solvents for their extraction.

### **7.2.1 Preparation of DES**

DES solvents (CCLA to BeB) (Table 9) were prepared by mixing, stirring, and heating the constituents (HBA and HBD) in inert glass flasks closed by caps according to the already published procedure [224]. The mixtures were stirred under orbital shaker (250 rpm, 60 °C) in an orbital shaker incubator (STUART Scientific S150, Legallais, Montferrier-sur-Lez, France) until a colorless liquid was formed (typically 2 hours). Then 10 % (v/v) of the total volume of HBA+HBD of water were added after 2 h. Finally, the hydrated DES were heated and stirred (250 rpm, 60°C) for another 10 min to get a perfect phase mixing. The pH was measured at room temperature on a sample of freshly prepared DES with an EcoScan pH 5 Palmtop pH-

meter (Legallais, Montferrier-sur-Lez, France), and the water content was determined by Karl Fischer titration GRS 2000 (Legallais, Montferrier-sur-Lez, France).

**Table 9. Name and composition of DES used for extraction**

Name	HBA <sup>1</sup>	HBD <sup>2</sup>	Mole ratio (HBA/HBD/water)	pH	Water content (%)
CCLA	ChCl	Lactic acid	1:2:1.4 <sup>4</sup>	0.78 ± 0.18	13.61 ± 0.01
BeLA	Betaine	Lactic acid	1:2:1.4 <sup>4</sup>	3.80 ± 0.17	15.18 ± 0.10
CCGly	ChCl	Glycerol	1:2:1.5 <sup>4</sup>	4.34 ± 0.27	13.90 ± 0.09
BeGly	Betaine	Glycerol	1:2:1 <sup>4</sup>	8.78 ± 0.44	9.61 ± 0.07
CCB	ChCl	1,4-Butanediol	1:2:1.1 <sup>4</sup>	5.36 ± 0.58	10.29 ± 0.07
BeB	Betaine	1,4-Butanediol	1:2:1 <sup>4</sup>	8.22 ± 0.72	11.62 ± 0.05

<sup>1</sup>Hydrogen bond acceptor; <sup>2</sup>Hydrogen bond donor; <sup>3</sup>Choline chloride; <sup>4</sup>Water represents 10 % v/v of the total volume of HBA+HBD

### 7.2.2 Phenolic compounds and alkaloids extraction

DES (CCLA to BeB, Table 9) extraction was carried out by HSE (heating stirring extraction) performed under magnetic stirring at 3500 rpm and 60°C for 2 h on amber glass flasks closed by caps. The objective of this experiment was to compare, on the one hand, the influence of ChCl or betaine as HBA on the DES phytochemical extraction, and on the other hand to study the influence of DES extraction assisted by HSE or UPAE (ultrasonic probe assisted extraction).

For HSE assisted DES extraction, phytochemicals were extracted from 0.5 g of each plant material (CP, CH, and CPH) by adding 2.5 mL of DES (DESA1 to DESC2) or EtOH 70 % (v/v) for the control. Extraction was performed in triplicate for 1 h and 3500 rpm at 60°C on amber glass flasks closed by caps.

For UPAE, a BRANSON sonifier SF-250 equipped with an ultrahigh probe of 1/8" was used, and the extraction was carried out in triplicate with 1 g of agro-residues (CP, CH, or CPH) and 5 mL of DES or EtOH 70 % (v/v), during 3 min with a constant duty cycle (200 W).

### 7.2.3 HPLC analysis

After extraction, 1 mL of each DES or EtOH 70 % (v/v) extraction media were combined with 1 mL of distilled water and centrifuged at 2600 rpm for 5 min at room temperature. The supernatant was collected, diluted 2-fold with deionized water, and then filtered through a 0.45  $\mu\text{m}$  cellulose filter (Minisart Legallais, Montferrier-sur-Lez, France) before being analyzed by HPLC.

The HPLC analysis was performed with a Shimadzu LC-20AD, equipped with a SPD-M20A photo-diode array detector and a CTO-10AS<sub>VP</sub> column oven from Shimadzu (Noisel, France). The chromatographic separation was carried out with a Kinetex 5  $\mu\text{m}$  C-18 column (100 Å, 4.6×250 mm, Phenomenex, Le Pecq, France). The elution solvents were (A) water (0.1 % acetic acid, v/v) and (B) methanol (0.1 % acetic acid, v/v). The elution gradient program was performed as follows (time (min), solvent B (%)): (3, 5), (13, 5), (23, 100), (33, 100), (34, 0), (40, 0), (45, 5). The flow rate, injection volume, and detection wavelength were 1.0 mL/min, 20  $\mu\text{L}$ , and 280 nm, respectively.

The total phenolic compounds (TPC) assessment by HPLC was obtained after integration of all peaks detected at 280 nm. A calibration curve (peak area at 280 nm) of standard reference gallic acid was constructed using concentration in the range of 0-600  $\mu\text{g/mL}$ , and the TPC were calculated using the linear regression equation obtained from the standard plot of gallic acid. On the other hand, in the molecules analysis extracted from CP, CH, and CPH, results were expressed in grams of CAE (chlorogenic acid (5-CQA)), or caffeine, or theobromine, or furfural equivalents (dw). The regression equations are  $y=46529x$ ,  $R^2= 0.999$  for gallic acid,  $y=30040x$ ,  $R^2= 0.995$  for chlorogenic acid,  $y=-37.54x^2+49244x$ ,  $R^2= 0.995$  for caffeine,  $y=-49.97x^2+56552x$ ,  $R^2= 0.997$  for theobromine and  $y=178823x$ ,  $R^2= 0.998$  for furfural.

### 7.2.4 UPLC-MS analysis

LC-MS analysis of extracted molecules was carried out on Acquity H-Class ultrahigh performance liquid chromatography (UPLC) Acquity H-Class system (Waters Corp., Milford, MA), equipped with a Kinetex EVO C18 column (1.7  $\mu\text{m}$ , 100 Å, 100 × 2.1 mm (Penimex)).



The mobile phase was A: water (0.1 % formic acid, v/v); and B: ACN (0.1% formic acid, v/v). The flow rate was set at 0.5 mL/min. The HPLC gradient (min/%B) was 0/2; 5/5; 15/40; 20/100; 21/100, and the injection volume was 1  $\mu$ L. The UV spectra were recorded at 280 nm. The mass spectrometer Synapt G2-S (Waters Corp., Milford, MA) equipped with an electrospray ionization (ESI) interface was operated in positive (ESI+) and negative (ESI-) mode between 100 and 150 Da. The capillary and cone voltage were 3000 V and 30 V, respectively. The source and desolvation temperatures were 140 °C and 450 °C, respectively. Mass spectra were recorded of  $m/z$  between 100 and 1500.

### **7.2.5 Total PC quantification by Folin-Ciocalteu**

TPC content was analyzed by triplicate according to Folin-Ciocalteu method, adapted to microplate assay [294]. Each sample obtained after the extraction procedure was diluted 400-fold in dionized water and 200  $\mu$ L was added to 1800  $\mu$ L of the Folin-Ciocalteu medium reaction. Then, 300  $\mu$ L was transferred to a 96-well sterile microplate (ThermoFischer, Courtaboeuf, France) and immediately analyzed at 755 nm with a microplate reader (agitation orbitate 3 sec at 37 °C) (Tecan, Gröedig, Austria) equipped with Magellan software. The results were expressed as grams of gallic acid equivalent (GAE)/100 g of each plant material in dw.

## **7.3 Deep eutectic solvents: *in vivo* study**

The *in vivo* effect of a DES green coffee (GCB) extract rich in CGA on rats was studied in collaboration with the joint research unit of Muscular Dynamics and Metabolism (UMR 866 DMEM, INRA) from Montpellier SupAgro.

### **7.3.1 Phenolic compounds extraction from green coffee beans**

In order to obtain a green coffee beans (GCB) extraction rich in chlorogenic acids (CGA), a screening of six DES (Table 7) were tested as solvent using HSE assisted extraction under the following conditions: 60 °C, 250 rpm, 3 h, in sealed flasks protected from light using 0.4 g of ground GCB/mL of DES or EtOH 70 % v/v for the control.

The TPC concentration in both extracts (DES and EtOH 70 %) was quantified by HPLC and expressed as mg of chlorogenic acid equivalents (CAE)/ mL.

BeGly DES was chosen to prepare the GCB extract to be used in the *in vivo* study. For this, a total of 562.5 mL of GCB extract were obtained as follow: the extract was obtained by five different lots, using 0.4 g of GCB/mL of BeGly. The extraction was carried out at 60 °C for 3 h. All the BeGly extracts were pooled together and diluted 3-fold with the same DES used for extraction, to reach a final concentration in PC of ~10 mg CAE/mL prior to rat feeding.

### **7.3.2 HPLC analysis of GCB extact**

Each GCB extract was diluted 400-fold in deionized water, in duplicate. The solution was then filtered through a 0.45 µm cellulose filter (Minisart Legallais, Montferrier-sur-Lez, France) and analyzed by HPLC.

The HPLC analysis was carried out as described at the section 7.2.3. The results are expressed in grams of CAE or caffeine equivalents per 100 g of dry weight GCB (g CAE/ 100 g dw). In both cases, a calibration curve of the standard molecules 5-CQA (327 nm) and caffeine (280 nm) was used to assess the quantification in the extracts, using concentration in the range of 0-150 µg/mL, and 0-600 µg/mL, respectively. The regression equations are  $y=57797x$ ,  $R^2=0.991$  for CGA, and  $y=-37.54x^2+49244x$  for caffeine.

### **7.3.3 Animals and diet description**

Twelve 6-week-old male Wistar rats ( $210 \pm 6$  g/rat) (Charles River, L'Arbresle, France) were housed (2 per cage) under conditions of constant temperature (20–22 °C), humidity (45– 50 %), and a standard dark cycle (20.00–08.00 h). The rats were randomized into 2 groups of 6 animals each and fed by gavage for 14 days at 9:00 and 17:00 h either with 3 mL of water or 3 mL of DES4 extract (containing 28.01 mg of PC expressed in CAE and 5.89 mg of caffeine). Rats were fed with a standard pellet diet (UAR A2) and given free access to food and tap water. Rat body weight, food consumption, and drinking were measured every two days. The institution guidelines of the DMeM team for the care and use of laboratory animals were observed. All experimental procedures were approved by the national ethical committee in France (reference 2016080911004286).

### 7.3.4 Sampling

Rats fasted for 9 h (23:00–08:00 h) were anesthetized with pentobarbital (40 mg/kg body weight) (Ceva Santé Animale, Libourne, France); blood was drawn from the abdominal artery with slightly heparinized syringes to prevent possible coagulation during the blood sampling and distributed into dry and heparinized tubes. Blood tubes were centrifuged at 1000 x g for 10 min at 4 °C, and plasma or serum were collected and stored at -80 °C until analysis. Beforehand, for GSH (reduce glutathione) analysis, 200 µL aliquots of heparinized blood sample were mixed with 1800 µL of ice-cold 6 % (w/v) meta-phosphoric acid, incubated for 30 min on ice, and centrifuged for 10 min at 1000 g at 4 °C. The supernatants were collected and stored until analysis. The liver was quickly removed, rinsed, weighed, and frozen in liquid nitrogen, and then kept at -80 °C. Gastrocnemius and soleus muscles, heart, kidney, spleen, and adipose tissue were also removed, rinsed, and weighed.

### 7.3.5 Plasma/serum routine biochemical analyses

The following analyses were performed using commercial kits (Table 8): plasma levels of glucose, uric acid, and urea, serum levels of creatinine, total cholesterol and triglycerides, free fatty acids, glycerol, aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT) activity. Plasma and liver supernatant protein levels were measured by the Bradford technique [295].

### 7.3.6 Blood and liver oxidative stress measurement

Lipid peroxidation levels represented by the thiobarbituric acid-reactive substances (TBARS) were measured in plasma and liver homogenates according to the method described by Sunderman *et al.* [296]. Protein oxidation was assessed in plasma and liver by measurement of sulfhydryl groups (–SH) [297] and advanced oxidation protein product (AOPP) [298]. Glucosylation or advanced glycation end products (AGE) were measured in plasma by fluorescence according to the method described by Munch *et al.* [299].

Liver homogenates were prepared on ice in a ratio of 1 g of wet tissue for 9 mL phosphate buffer (50 mM, pH 7) using a Polytron homogenizer (VWR, Fontenay sous Bois cedex, France). Homogenates were divided into three parts: part 1 was used directly for the

assessment of lipid oxidation using the TBARS assay; part 2 was mixed with identical volume of ice-cold 12 % (w/v) meta-phosphoric acid, incubated for 30 min on ice, centrifuged for 10 min at 1000 x g at 4 °C, and then supernatants were collected and stored until analysis of total GSH; part 3 was centrifuged at 1000 x g for 10 min at 4 °C, and supernatant was collected and stored at -80 °C until analysis of other oxidative and antioxidative stress markers.

### **7.3.7 Lipids content in liver**

The liver samples were homogenized in NaCl (9 g/L) and Triton X-100 (0.1 %) using an Ultra Turax homogenizer. Hepatic levels of triglycerides, free fatty acids, and total cholesterol were quantified directly in the liver homogenate using enzymatic colorimetric methods.

### **7.3.8 Glycogen content**

The liver glycogen content was determined from a slice of the main lobe of the liver, according to Burcelin *et al.* [300]. Briefly, about 50 mg of the liver was dissolved in NaOH, 1 M (ratio 1/6) at 55 °C for 1 h, neutralized with HCl (1 M), and spun down. A sample of the supernatant was incubated in the presence of amyloglucosidase for 2 h at 37 °C in a shaking incubator (750 rpm). After centrifugation, the glucose present in the supernatant was evaluated by a glucose oxidase method.

### **7.3.9 Statistical analysis**

Results were expressed as means  $\pm$  SD (standard deviation) with n = 5. Statistical analyses were based on Student's t test. The limit of statistical significance was set at  $p < 0.05$ . Statistical analyses were performed using the StatView program (SAS Institute, Cary, NC, United States).

## **7.4 Isoprostanoids extraction**

PhytoPs extraction was performed in triplicate from approximately 0.1 g of each sample. Plant material was mixed with 2 mL of MeOH containing 1 % (v/v) of butylated hydroxytoluene (BHT). Then, the mixture was vortexed and 1.5 mL of phosphate buffer (pH

2) saturated in NaCl, and 6 ng of each internal standard (IS) were added. This solution was stirred at room temperature for 1 h at 100 rpm. Then, the extract was centrifuged at 4000 rpm for 5 min at 25 °C, the supernatant was separated, and 4 mL of cold chloroform were added. This mixture was vortexed for 30 s and then centrifuged at 1500 rpm for 5 min at room temperature. Subsequently, the chloroform phase was separated and concentrated under a nitrogen flow at 40 °C for 2 h. Afterward, 950 µL of KOH were added, the mixture was incubated for 30 min at 40 °C, and 1 mL of formic acid (40 mM, pH 4.6) was added before running the SPE (Solid Phase Extraction) separation. SPE Oasis MAX cartridges were conditioned with 2 mL of MeOH and equilibrated with 2 mL of formic acid (20 mM, pH 4.5). After loading the sample, the cartridges were successively washed with: 2 mL of NH<sub>3</sub> 2 % (v/v), 2 mL of a mixture of formic acid (20 mM):MeOH (70:30, v/v), 2 mL of hexane, and finally 2 mL of hexane:ethyl acetate (70:30, v/v). Targeted metabolites were eluted with 2 mL of hexane:ethanol:acetic acid (70:29.4:0.6, v/v). Lastly, all samples were evaporated to dryness under a nitrogen flow at 40 °C for 30 min, and 100 µL of H<sub>2</sub>O/ ACN (0.1 % formic acid (v/v)) (83:17, v/v) were added prior to injection in the micro-HPLC-QTRAP system.

#### 7.4.1 Isoprostanoids identification and quantification

The PhytoPs and PhytoFs analyses were carried out in collaboration with the Pharmacy Faculty (CNRS-Université Montpellier-ENCS).

The PhytoPs, extracted from CP, CH, and CPH, were analyzed using a micro liquid chromatography equipped with CTC Analytics AG (Zwingen, Switzerland), as described by Yonny *et al.* [174]. The autosampler vial tray was kept at 10 °C and the column (HALO C<sub>18</sub> 0.5 x 100 mm, 2.7 µm, Eksigent Technologies, CA, USA) was set at 40 °C. The mobile phase consisted of water (0.1 % formic acid (v/v), (A) and acetonitrile:methanol (80:20, v/v; with 0.1 % formic acid (v/v)) (B). The gradient elution program was performed as follows (min/%B): 0/17; 1.6/17; 2.85/21; 7.27/25; 8.8/28.4; 9.62/33.1; 10.95/33.3; 15/40; 16.47/95; 18.92/95; 19/17; 21/17. Each sample (5 µL) was injected and eluted at a flow rate of 0.03 mL/min. Isoprostanoids were analyzed using an AB SCIEX QTRAP 5500 mass spectrometer (Sciex Applied Biosystems) equipped with ESI source, operated in the negative mode (ESI-). The source voltage was kept at -4.5 kV and nitrogen was used as curtain gas. The multiple reaction monitoring (MRM) analysis was conducted by monitoring the precursor ion to

product transitions. Sensitivity was evaluated in terms of detection and quantification limits of LOD ranged from 0.16 to 0.63 pg/  $\mu$ L, and LOQ between 0.16 to 1.25 pg/  $\mu$ L.

The isoprostanoids quantification was carried out using six PhytoPs and PhytoFs concentration levels (16, 32, 64, 128, 256 and 512 pg/ $\mu$ L) for the analyte to IS (internal standard) ration, and the linear equations were calculated (Table 10).

**Table 10. PhytoPs and PhytoFs quantification process by Micro-HPLC-QTRAP**

Analyte	Calibration curve <sup>1</sup>	R <sup>2</sup>	Weighting
ent-16-F1t-PhytoP	$y = 1.06857x$	0.997	1/x
ent-16-epi-16-F1t-PhytoP	$y = 0.87312x$	0.998	1/x
9-F1t-PhytoP	$y = 0.79830x$	0.998	1/x
9-epi-9-F1t-PhytoP	$y = 0.71005x$	0.998	1/x
ent-16B1t-PhytoP	$y = 1.92920x$	0.991	1/x
ent-9L1t-PhytoP	$y = 2.87653x$	0.983	1/x
ent-16(RS)-9-epi-ST- $\Delta$ 14-10-PhytoF	$y = 1.71852x$	0.998	1/x
ent-9(RS)-12-epi-ST- $\Delta$ 10-13-PhytoF	$y = 0.72222x$	0.998	1/x
ent-16(RS)-13-epi-ST- $\Delta$ 14-9-PhytoF	$y = 1.95197x$	0.997	1/x

<sup>1</sup>Six data points (n = 3); y: the peak area of analyte; x: the concentration of analyte (pg/ $\mu$ L). The deuterated form of IsoPs (D<sub>4</sub>-15F<sub>2t</sub>-IsoP) and C19-16-F<sub>1t</sub>-PhytoP were used as internal standards for quantification of samples in this study.

The isoprostanoids identification was carried out through the comparison between analyte retention times in solvent (mobile phase) and in samples (Table 11). Data processing was performed by MultiQuant 3.0 software (Sciex Applied Biosystems).

**Table 11. Multiple reaction monitoring (MRM) of PhytoPs and PhytoFs determinations**

Analyte	Retention time (t <sub>R</sub> )	Precursor ions (m/z)	Precursor ions (m/z)
<i>Ent</i> -16-B <sub>1t</sub> -PhytoP _T1	10.50	307.2	235
<i>Ent</i> -16-B <sub>1t</sub> -PhytoP _T2	10.50	307.2	223
9/16-F <sub>1t</sub> -PhytoP _T1	5.58-6.27	327.2	283.2
9-F <sub>1t</sub> -PhytoP _T2	5.75	327.2	171.2
9- <i>epi</i> -9-F <sub>1t</sub> -PhytoP _T2	6.27	327.2	171.2
16-F <sub>1t</sub> -PhytoP _T2	6.04	327.2	251.2
16- <i>epi</i> -16-F <sub>1t</sub> -PhytoP _T2	5.58	327.2	251.2
<i>Ent</i> -9-L <sub>1t</sub> -PhytoP _T1	10.67	307.2	185.1
<i>Ent</i> -9-L <sub>1t</sub> -PhytoP _T2	10.67	307.2	197
<i>Ent</i> -16( <i>RS</i> )-13- <i>epi</i> -ST-Δ <sup>14</sup> -9-PhytoF _T1	4.68	343.2	201
<i>Ent</i> -16( <i>RS</i> )-13- <i>epi</i> -ST-Δ <sup>14</sup> -9-PhytoF _T2	4.68	343.2	127
<i>Ent</i> -16( <i>RS</i> )-9- <i>epi</i> -ST-Δ <sup>14</sup> -10-PhytoF _T1	4.68	343.2	209.1
<i>Ent</i> -16( <i>RS</i> )-9- <i>epi</i> -ST-Δ <sup>14</sup> -10-PhytoF _T2	4.68	343.2	199

## 7.5 Thiols precursors analyses

The extraction and analysis of thiols precursors were carried out at Nyseos (Montpellier, France).

CP, CH, and CPH samples were extracted mixing 100 mg of each one with 2 mL of a mixture of water/methanol (1:1 v/v). The resulting solution was stirred at room temperature for 1 h, then centrifuged (12000 rpm) for 15 min. The internal standards (1 mL): 3MH- Cys-d<sub>2</sub>: 111 µg.L<sup>-1</sup>, 3MH-Glu-d<sub>2</sub>: 197 µg.L<sup>-1</sup>, 4MMP- Cys-d<sub>10</sub>:22 µg.L<sup>-1</sup> and 4MMP-Glu-d<sub>10</sub>:20 µg.L<sup>-1</sup>, were added to centrifuged samples. Extracts were then purified according to the methodology described by Roland and co-workers [301]. Then the final extracts were analyzed following the protocol reported by Bonnafeux *et al.* [27] using a UPLC chromatography system hyphenated to a triple quadrupole mass spectrometer (Thermo TSQ Quantiva), equipped with Hypersil gold AQ (1.9 mm, 100 x 2.1 mm) column. The mobile phase was composed of (A) water with 0.1 % of formic acid and (B) acetonitrile with 0.1 % of formic acid. The gradient elution was performed as follow: (time (min), solvent B (%): (7, 0), (12.5, 1.4), (17.5, 1.4), and then the gradient return to initial conditions. The flow rate and temperature were, respectively, 0.6 mL / min, and 50 °C.

### 7.5.1 Statistical analysis

The statistical analysis (one-way analysis of variance ANOVA) was performed using the Minitab software version 18 (Minitab Inc., US). The level of significance was set at  $p < 0.05$ . Results are expressed as mean of triplicates (mean values  $\pm$  standard deviations). Tukey's test was used to determine which mean values were different

## 7.6 *In vitro* antioxidant activity: VesiCAT assay

The antioxidant capacity of ethanolic (70 % v/v) extracts of CP, CH, and CPH was estimated, in order to evaluate the potential use of these agro-residues, as source of natural antioxidants. VesiCAT assay was conducted according to already the published paper [11]. The synthesis of the phospholipid containing eleostearic acids (an octadecatrienoic acid with a conjugated triene) was carried out through the extraction and purification by recrystallization from Tung oil. Then, the phospholipid synthesis (DEPC) was achieved through nucleophilic substitution using sn-glycero-3-phosphocholine (GPC).

### 7.6.1 Extraction and purification of $\alpha$ -eleostearic acid from Tung oil

The eleostearic acid was isolated from the adapted method of Durand *et al.* 2015 [302]. Tung oil (34 g) (Figure 22) and 40.5 g of potassium hydroxide were dissolved in 250 mL of absolute ethanol in a 500 mL round-bottom flask under an argon atmosphere in the dark. The reaction mixture was stirred and refluxed for 3 h.

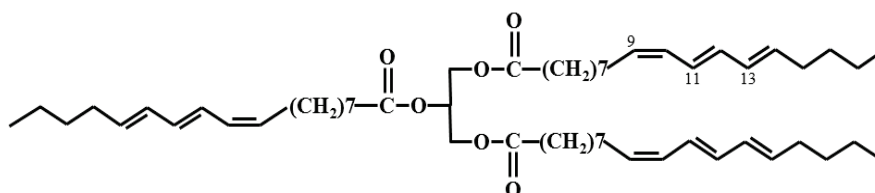


Figure 22. Tung oil

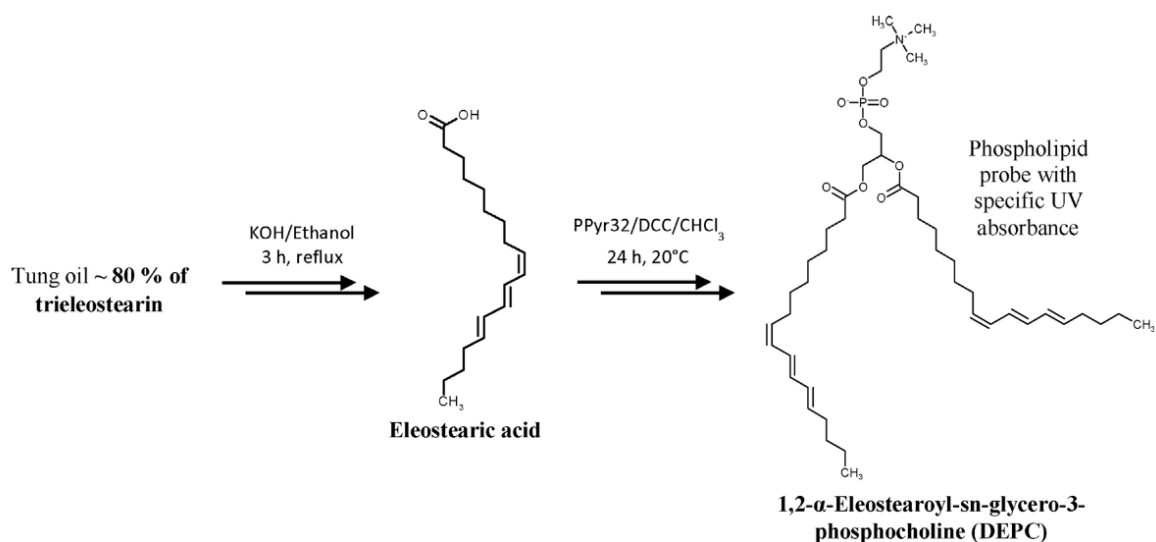
Then, the mixture was cooled by adding 200 mL of distilled water, and the aqueous phase was washed with hexane (3 X 150 mL). The aqueous phase was then acidified (pH 2) with



diluted sulfuric acid solution (50 % v/v, 9 M) and extracted three times with diethyl ether (3 × 200 mL). The combined diethyl ether solution was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure at 15 °C in the dark (Buchi Rota- vapor® R-210, Germany). Then, the obtained crude powder was dissolved in acetone at room temperature and recrystallized twice at -20 °C, in order to purify eleostearic acid. After vacuum filtration, a white powder of pure eleostearic acid (8.4 g) was obtained and dried under vacuum for further esterification. The product was identified by 1D NMR (<sup>1</sup>H and <sup>13</sup>C). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 9.244 (s, br, 1H), 6.36–6.24 (dd, 1H), 6.12–5.97 (q, 2H), 5.96–5.85 (dd, 1H), 5.68–5.56 (dt, 1H), 5.36–5.25 (dt, 1H), 2.30 (t, 2H), 2.16–2.01 (m, 4H), 1.60 (q, 2H), 1.41–1.20 (m, 12H), 0.83 (t, 3H) ppm. <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 179.66 (COOH), 135.1–125.42 (5 CH), 33.8–22.1 (10 CH<sub>2</sub>), 13.9 (1 CH<sub>3</sub>) ppm.

### 7.6.2 Synthesis of 1,2- $\alpha$ -eleostearoyl-sn-glycero-3-phosphocholine (DEPC)

The method to synthesize DEPC (Figure 23) was adapted from Alaoui *et al.* 2014 [303]. To a solution of sn-glycero-3-phosphocholine (GPC) (0.100 g, 0.4 mmol), pure  $\alpha$ -eleostearic acid (0.556 g, 2 mmol) was added, in alcohol-free and anhydrous CHCl<sub>3</sub> (6 mL), under stirring and inert atmosphere. A solution of freshly recrystallized PPyr32 (4-pyrrolidinopyridine, 0.296 g, 2 mmol) and DCC (N, N'-dicyclohexylcarbodiimide, 0.388 g, 2 mmol), in 2 mL of CHCl<sub>3</sub>, was then added dropwise. After 24 h at 20°C, the reaction mixture was filtered on a Sartorius mini-sart filter syringe (0,45  $\mu$ m), concentrated and re-diluted in 6 mL of ethanol/water (9:1 v/v).



**Figure 23. Synthesis pathway of the 1,2- $\alpha$ -eleostearoyl-sn-glycero-3-phosphocholine (DEPC) probe**  
From: [11]

The product was purified, by HPLC semi-preparative chromatography (Thermo Ultimate 3000, Thermo Fisher Scientific, France) equipped with a fraction collector (AFC-3000), and a Hypersil Gold C18 column (5  $\mu$ m, 175 Å, 21.2  $\times$  150 mm; Thermo Fisher Scientific, France). Flow rate and detection were set at 20 mL/min and 273 nm, respectively. Eluent gradient was a binary mixture of MeOH/H<sub>2</sub>O, starting with 50 % MeOH from 0 to 15 min, then 100 % MeOH from 15 to 40 min).

Fractions containing the product were collected, and identified by 1D NMR (<sup>1</sup>H and <sup>13</sup>C) and 2D NMR (<sup>1</sup>H-<sup>1</sup>H and <sup>1</sup>H-<sup>13</sup>C). 135 mg of pure compound was obtained (45 % yield). <sup>1</sup>H NMR (300 MHz CDCl<sub>3</sub>):  $\delta$  0.87 (t, 6H, J = 6.9, C18-CH<sub>3</sub>, C36-CH<sub>3</sub>), 1.21–1.43 (m, 24H, C(4–7, 16, 17, 22–25, 34, 35)-CH<sub>2</sub>), 1.47–1.63 (m, 4H, C(3, 21)-CH<sub>2</sub>), 1.95–2.19 (m, 8H, C(8, 15, 26, 33)-CH<sub>2</sub>), 2.22–2.29 (m, 4H C(2, 20)-CH<sub>2</sub>), 3.32 (s, 9H, N-(CH<sub>3</sub>)<sub>3</sub>), 3.68–3.83 (m, 2H, CH<sub>2</sub>-N), 3.84–3.99 (m, 2H, CH<sub>2</sub> sn-3), 4.03–4.11 (m, 1H, CHsn-1), 4.22–4.42 (m, 3H, PO-CH<sub>2</sub>, CH sn-1), 5.12–5.25 (m, 1H, CH sn-2), 5.31–5.40 (m, 2H C(9,27)-CH), 5.56–5.72 (m, 2H, C14, C32-CH), 5.92–6.17 (m, 6H, C10, C11, C13, C28, C29, C31-CH), 6.30–6.38 (m, 2H, C12, C30-CH). <sup>13</sup>C NMR (100 MHz CDCl<sub>3</sub>):  $\delta$  14.1 (C18,36), 22.4 (C17, 35), 25.1 (C15, 33), 28.0 (C8, 26), 29.25, 29.32, 29.41, 29.51, and 29.85 (C 4–7, 22–25), 31.7 (C16,34), 32.7 (C3, 21), 34.3 and 34.4 (C2,20), 54.7 (N(CH<sub>3</sub>)<sub>3</sub>), 59.5 (C–N, JC–P = 5.1), 63.1 (C sn-1), 63.7 (PO–C, JC–P = 5.1), 66.6

(C sn-3, JC-P = 6.6), 70.7 (Csn-2, JC-P = 7.3), 126.0 (C12, 30), 128.9 (C10, 28), 130.7 (C13, 31), 131.9 (C9,27), 133.0 (C11, 29), 135.4 (C14, 32), 173.3 and 173.6 (C1, 19).

### **7.6.3 VesCAT assay with AAPH-induced oxidation**

The liposomes preparation started by combining 8 mg of DPPC T<sub>c</sub> (phase transition temperature)= 42°C) or DLPC (T<sub>c</sub> = -1°C) and 0.8 mg DEPC (800 µL from DEPC stock solution at 1 mg.mL<sup>-1</sup>) in 2 mL of anhydrous CHCl<sub>3</sub>. The mixture was put in a 25 mL round bottom flask and the solvent was then slowly removed, in the dark (aluminum foil), at room temperature by rotary evaporator to form a thin lipid layer at the bottom of the flask. Then, the round bottom flask was put out under nitrogen flow for 10 min, in order to eliminate any rest of solvent. The phospholipids were resuspended for 30 min with 10 mL of phosphate buffer (PBS, pH=7.2) at a temperature above the T<sub>c</sub> (50°C for DPPC, 20°C for DLPC) of the lipid, at 300 rpm (IKA 4000). This suspension was sonicated in a bath sonicator (5 min, 35 KHz, Transonic T 425/H, Elslloo, The Netherlands) and put back in the incubator for another 30 min at 50 °C of stirring and returned to the bath sonicator for an additional 5 min. The suspension was subsequently subjected to 10 cycles of extrusion in an Avanti mini-extruder apparatus (Alabama, USA) with 100-nm polycarbonate filters, keeping the solution at 50°C. After extrusion, the 10 mL of liposomes were diluted with 10.5 mL of PBS. All liposome samples were freshly prepared the day of the experiments. Particle size distribution was assessed with a nanoparticle size analyzer (Nicomp N3000 DLS System, Port Richey, USA). The Dynamic Light Scattering analyses showed LUV to be monodisperse, with mean diameter = 133.8 ± 20.6 nm.

A total of 240 µL of these suspensions was transferred to a 96-wells microplate (Greiner, Frickenhausen, Germany) (only 80 wells (rows from 2 to 11) were filled up with the liposome solution, and the rows 1 and 12 were filled up with PBS solution).

For assays, the liposomes were treated with different concentrations of each extract: 10, 15, 20, and 30 µg/mL for CP and CH, and 20, 40, 80, and 100 µg/mL for CPH. Additionally, the possible influence of theobromine and caffeine on antioxidant activity was tested at their maximum concentration of solubility: 9 and 19.4 µg/mL, respectively.

Chlorogenic acid concentrations were 0.35, 0.71 and 1.42  $\mu\text{g/mL}$ , while the trolox (reference) concentrations were 0.5, 1 and 2  $\mu\text{g/mL}$ .

Finally, a total of 30  $\mu\text{L}$  of a solution of AAPH in PBS (20 mM) (27.12 mg / 5 mL), prepared immediately before reading, was added to wells to induce oxidation. The progress of reactions was immediately monitored by recording the decrease in absorbance at 273 nm. Measurements were performed every 2 min for 7.5 h, with a 5 s agitation before each measure at 37 °C, using an Infinite M1000 microplate reader (TECAN, Gröedig, Austria) equipped with Magellan software. All measurements were performed in triplicate and reported as the average  $\pm$  standard deviation (SD).

#### **7.6.4 VesiCAT assay with AMVN-induced oxidation**

The preparation of liposome started by dissolution of 16 mg of DLPC ( $T_c = -1^\circ\text{C}$ ) and 0.8 mg DEPC (800  $\mu\text{L}$  from DEPC stock solution at 1 mg/mL in anhydrous  $\text{CHCl}_3$ ), and 1.4 mg of AMVN prepared in 2 mL of anhydrous  $\text{CHCl}_3$  in a 25 mL round bottom flask. Then the solution was put out under nitrogen flow for 5 min. Then, the solvent was slowly removed at room temperature in the dark (aluminum foil) by rotary evaporator, to form a thin lipid layer at the bottom of the flask (around 2 h). The phospholipids were resuspended with 10 mL of PBS at 20°C (30 min in the incubator, 300 rpm IKA 4000). This suspension was sonicated in a bath sonicator (5 min, 35 KHz, Transonic T 425/H, Elsloo, The Netherlands) and put back in the incubator for another 30 min of stirring and returned to the bath sonicator for an additional 5 min. The suspension was subsequently subjected to 10 cycles of extrusion in an Avanti mini-extruder apparatus (Alabama, USA) with 100-nm polycarbonate filters. After extrusion, the 10 mL of liposomes were diluted with 11 mL of PBS. All liposome samples were freshly prepared the day of the experiments. Particle size distribution was assessed with a nanoparticle size analyzer (Nicomp N3000 DLS System). The Dynamic Light Scattering analyses showed LUV to be monodisperse, with mean diameter =  $132.3 \pm 11.3$  nm.

A total of 240  $\mu\text{L}$  of these suspensions was transferred to a 96-wells microplate (Greiner, Frickenhausen, Germany) (only 80 wells (rows from 2 to 11) were filled up with the liposome solution, and the rows 1 and 12 were filled up with PBS solution).

For assays, the liposomes were treated with different concentrations of each extract: 20, 50, 100, and 150 µg/mL for CP; 10, 25, 50, and 75 µg/mL for CH, and 50, 100, 150, and 200 µg/mL for CPH. The theobromine and caffeine were also tested at the same concentration that in AAPH assay.

Chlorogenic acid concentrations were 3.54, 7.08, 14.17 and 21.25 µg/mL, while the trolox (reference) concentrations were 0.37, 0.75 and 1.25 µg/mL

To induce oxidation, the temperature in the microplate wells was increased to  $43 \pm 0.5$  °C, using an orbital agitation with a control temperature at  $43 \pm 0.5$  °C in the microplate wells (temperature setting 47 °C, equipment) before each measure was performed using a Grant Bio PHMP thermoshaker for microplates. The progress of reactions was immediately monitored by recording the decrease in absorbance at 273 nm. Measurements were performed every 15 min for 7.5 h using an Infinite M1000 microplate reader equipped with Magellan software. All measurements were performed in triplicate and reported as the average  $\pm$  SD.

In both analysis (AAPH and AMVN) trolox was used as control, and additionally it was tested the capacity antioxidant of caffeine, theobromine and CGA, since they were identified in CP and CH.

### **7.6.5 Expression of VesiCAT values**

To normalize data, the raw absorbance signal was transformed in relative absorbance according to the Equation 1.

$$\text{Relative absorbance} = \text{Abs}_t / \text{Abs}_0 \quad (1)$$

Where  $\text{Abs}_t$  and  $\text{Abs}_0$  are absorbances measured at times  $t$  and 0 min, respectively. It is worth mentioning that if the measurement is not rapid enough after initiating the oxidation, the  $\text{Abs}_0$  for the blank (without antioxidant) may be lower than the sample containing the antioxidant. In this case, to normalize  $\text{Abs}_0$ , the experimental  $\text{Abs}_0$  of blank can be artificially replaced with the  $\text{Abs}_0$  of samples in Equation (1). The area under curve (AUC) corresponding to relative absorbance decay was then calculated as follows

$$AUC = 1 + Abs_{t1}/Abs_0 + Abs_2/Abs_0 \dots + Abs_{299}/Abs_0 \dots + Abs_{300}/Abs_0 \quad (2)$$

The net protection area provided by an antioxidant sample was then calculated using the difference between the AUC in the presence of an antioxidant sample ( $AUC_{Sample}$ ) and the AUC of the blank ( $AUC_{Control}$ ), the latter consisting of the same mixture without antioxidant. Trolox was used as a calibrator for antioxidant capacity measurements. Thus, the antioxidant capacity of a sample relative to Trolox (VesiCAT value) is given as:

VesiCAT value =

$$[(AUC_{Sample} - AUC_{Control}) / (AUC_{Trolox} - AUC_{Control})] \times [(moles\ of\ Trolox / moles\ of\ sample)] \quad (3)$$

A VesiCAT value was calculated for both AAPH- and AMVN-induced oxidation. Regarding the AMVN-induced oxidation,  $AUC_{80}$  corresponding to the area under the curve obtained after 80 % of DEPC oxidation was calculated. The net protection area provided by the antioxidants and the VesiCAT values were calculated in the same way using equations (2) and (3).

# *Results and discussion*



## Results and discussions

### VIII. Chemical characterization of samples

Before exploring the possibility of an integral valorization of coffee and cocoa by- and co-products, the determination and characterization of different classes of molecules, including fibers, proteins, carbohydrates, lipids, ash, were performed.

#### 8.1 Proximate composition

The amount of protein, hemicellulose, cellulose, lignin, lipids, and ash for each plant material are presented in Table 12. The water content of CP, CH, and CPH, was  $17.69 \pm 0.16$ ,  $9.64 \pm 0.06$ , and  $16.02 \pm 0.78$  %, respectively.

**Table 12. Proximate composition of coffee pulp (CP), cocoa hush (CH), and cocoa pod husk (CPH)**

Component	Content (g/100 g dw)*		
	CP	CH	CPH
Proteins	$10.74 \pm 0.01$	$17.81 \pm 0.15$	$9.05 \pm 0.09$
NDF (neutral detergent fiber)	$38.68 \pm 0.33$	$39.43 \pm 0.29$	$56.97 \pm 1.32$
ADF (acid detergent fiber)	$28.01 \pm 0.57$	$31.13 \pm 0.15$	$47.06 \pm 1.17$
ADL (acid detergent lignin)	$10.46 \pm 0.33$	$13.90 \pm 0.14$	$21.28 \pm 1.13$
Cellulose (ADF-ADL)	$17.55 \pm 0.25$	$17.23 \pm 0.29$	$25.78 \pm 0.04$
Hemicellulose (NDF-ADF)	$10.68 \pm 0.90$	$8.30 \pm 0.44$	$9.92 \pm 0.16$
Lipids	$2.18 \pm 1.19$	$3.89 \pm 0.68$	$1.98 \pm 0.33$
Ash	$4.63 \pm 0.05$	$7.61 \pm 0.09$	$3.10 \pm 0.11$

\*Results are expressed on dry weight as the mean of triplicates (mean values  $\pm$  standard deviations).



The results showed a high content of fiber and lignocellulosic material in CP, CH, and CPH.

Overall, the results were close to the ones reported in the literature (Table 2 and Table 3, *cf.* page 24 and 38, respectively). Yet, some differences were observed. This is possibly the result of several factors, for example, difference in the varieties or species, the edaphoclimatic characteristics of the crop, and/or harvest and post-harvest treatments.

## 8.2 Monosaccharide analysis

The determination of the monosaccharides profile was carried out in duplicate for each sample, and quantification was made by gas chromatography (GC) as reported by Blakeney and co-workers [293]. This analysis presents the advantage to be very sensitive and required very small amount of samples [304].

Originally, this methodology was standardized for the wheat straw and includes a 2 h pre-hydrolysis step. However, the optimization of this step, according to physical structure and chemical composition of a determinate sample is essential to ensure the monosaccharides released. Thus, it was decided to carry out a kinetic pre-hydrolysis at different time, from 1 to 4 h additional to the 2 h hydrolysis step. The concentration and composition in monosaccharide are shown in Figure 24.

The monosaccharides composition of our samples was for:

- CP: glucose > arabinose > mannose > xylose > ribose > galactose
- CH: glucose > mannose > galactose > xylose > arabinose > ribose
- CPH: glucose > xylose > mannose > galactose > arabinose > ribose

These results suggest a predominance of cellulose. The presence of xylose and arabinose may be due to the presence of xyloglucans or arabinogalactans. According to the monosaccharide composition, other hemicelluloses such as mannans, galactomannans or (galacto) glucomannans could also be present as reported by Viesmann *et al.* [2]. Galactose, rhamnose, and arabinose can also derive from pectin hydrolysis.

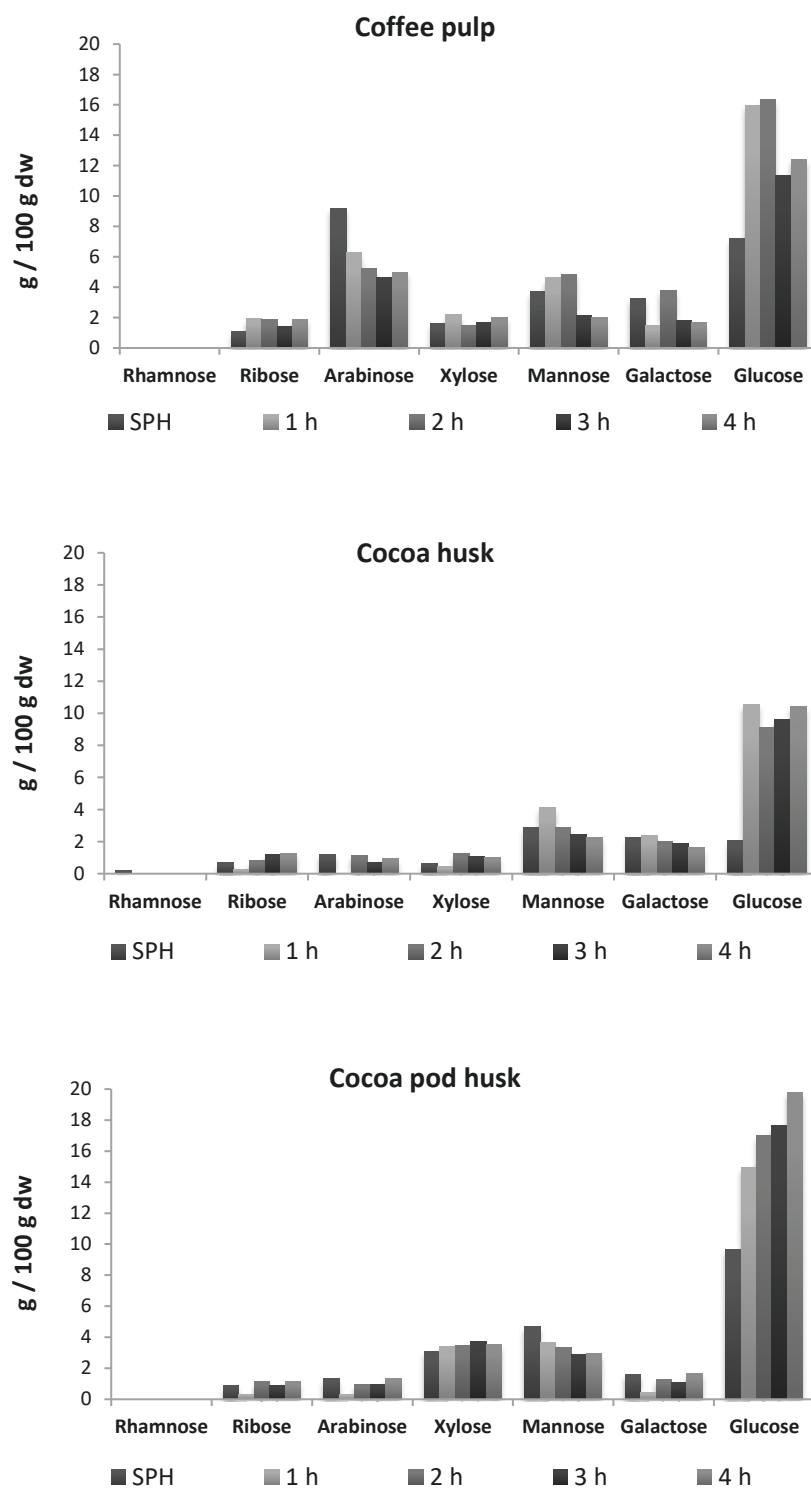


Figure 24. Pre-hydrolysis kinetics for coffee pulp (CP), cocoa husk (CH), and cocoa pod husk (CPH)

The goal of this kinetic pre-hydrolysis was to allow a complete hydrolysis of polysaccharides (principally cellulose), without degrading the released monosaccharides. Results showed that total carbohydrates hydrolysis was reached for CP and CH after, respectively, 1 h and 2 h of pre-hydrolysis, followed by 2 h of hydrolysis (Table 13). In contrast, more than 4 h of pre-hydrolysis was necessary for CPH, since the total monosaccharides amount continued to increase and did not reach a plateau, especially for glucose. This is possibly due to the CPH reported cellulose content that is almost 2-fold higher (26.2 % dw) than CP (17.7 % dw) or CH (13.2 % dw) (Table 2 and Table 3 *cf.* page 24 and 38 ).

**Table 13. Monosaccharide composition of CP, CH, and CPH**

Monosaccharide	g/100 g dw*		
	CP <sup>1</sup>	CH <sup>2</sup>	CPH <sup>3</sup>
Ribose	1.93	0.81	1.14
Arabinose	6.26	1.14	1.37
Xylose	2.20	1.26	3.51
Mannose	4.62	2.86	2.97
Galactose	1.47	2.01	1.68
Glucose	15.96	9.09	19.77
Rhamnose	ND	ND	ND
Total monosaccharides	32.44	17.17	30.44

\*Results are expressed as dry weight (dw) as g monosaccharides/100 g of CP, CH or CPH. n=2. Determined by GC column DB 225 capillary column (50 % cyanopropylphenyl-dimethylpolysiloxane) (30 m x 0.32 mm) at 255 °C; injector temperature: 220°C; detector temperature: 260 °C; flow: 1 mL/min. <sup>1</sup> Coffee pulp after 1 h of pre-hydrolysis and 2 h of hydrolysis. <sup>2</sup> Cocoa husk after 2 h of pre-hydrolysis and 2 h of hydrolysis; <sup>3</sup> Cocoa pod husk after 4 h of pre-hydrolysis and 2 h of hydrolysis.

Vriesmann and co-workers [2] quantified the total monosaccharides as alditol acetates by GC. They reported a total content of 32.3 % dw on CPH after 1 h of pre-hydrolysis with H<sub>2</sub>SO<sub>4</sub> 72 % (w/w) (12 M) at 0 to 4 °C, then diluted to 8 % (w/w) (1.3 M) and kept at 100 °C for 15 h. In our case, we applied, respectively, a longer and shorter pre- and hydrolysis, and obtained a similar result, without reaching a total constant glucose release. We propose thus to extend the pre-hydrolysis time of CPH, in order to achieve a complete hydrolysis of cellulose.

The main monosaccharide presents in CP (15.96 ± 2.09% dw), CH (9.09 ± 0.44 % dw), and CPH (19.77 ± 1.22 % dw) was glucose. It was also reported in the literature as predominant

in such biomasses, with 30 % dw, 13.6 % dw, and 8.6 % dw, respectively [305][117][2]. Moreover, the amount of total monosaccharides quantified in CH (17.17 % dw) and CPH (30.44 % dw) are similar to those reported in the literature (17.49 % dw for CH and 32.3 % dw for CPH) [95][2]. The CP total monosaccharides (32.44 % dw) found in this study was superior to that reported by Urbaneja and co-workers [306] (19.07 %), but inferior to the amount reported by Gurran *et al.* [307] (40 %). This could be due to the different analytical methods and origin and preparation of the samples. On the one hand, Urbaneja and co-workers [7] used dinitrosahcyclic (DNS) acid colorimetric method for the quantification of reducing sugars, using glucose as internal standard. The reducing sugar absorption was measured in a spectrophotometer UV-Vis at 584 nm. However, it was pointed out that the major weakness in this test was the loss of reducing sugars. This can be due to the reagents involved in the enhancing of color, which can contribute to the oxidation of sugars [308]. On the other hand, Gurram and co-workers [8] determined the total monosaccharides concentration by a classic acid hydrolysis ( $\text{H}_2\text{SO}_4$  (72% (w/w), 1 h, 121 °C), and quantification was carried out using a high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD), using fucose as internal standard. HPAE-PAD allows highly selective separations of nonderivatized carbohydrates [309].

### 8.3 Fatty acid profile analysis

The fatty acid characterization revealed an interesting composition in the agro-residues of coffee and cacao (Table 14). The main fatty acids found in our samples were:

- Palmitic acid (16:0) (53.02 %), linoleic acid (18:2) (20.42 %), oleic acid (18:1) (15.90 %), and stearic acid (18:0) (12.74 %) for CP.
- Oleic acid (36.27 %), palmitic acid (29.94 %), stearic acid (26.31 %), and linoleic acid (10.26 %) for CH.
- Palmitic (36.41 %), oleic acid (29.54 %), and linoleic (25.43 %) for CPH.

Regarding the coffee residue, three of the main fatty acids found in CP (palmitic, linoleic, and oleic) are also present in Arabica green coffee beans at 34.25 %, 44.29 %, and 12.77 %, respectively [14]. For the cacao raw materials, the predominant fatty acids were oleic and palimitic acids. The presence of oleic acid (and linoleic acid to lesser extent) was already

reported in CH by Osawa and co-workers [17]. They found that the presence of these fatty acids in CH contributes to the antibacterial activity against *Streptococcus mutans* MT8148R. Additionally, oleic acid is the major fatty acid of many vegetable oils, especially in olive (80 %) and rapeseed oils (60.1 %), and the main monounsaturated fatty acid in food [15]. In cocoa beans, the main fatty acids reported in the literature [310] were oleic (34.73 %) > stearic (33.78 %) > palmitic acid (27.61 %), > linoleic (2.43 %). These four fatty acids are also the four most important in both, CH (oleic > palmitic > stearic > linoleic) and CPH (palmitic > oleic > linoleic > stearic) (Table 14). Karim and co-workers [16] also identified the presence of the oleic acid in CPH ethanolic extracts. Likewise, the cocoa butter is also mainly composed of palmitic (25.1 %) and oleic acid (36.5 %) [311].

**Table 14. Fatty acid composition (% w/w)<sup>1</sup>**

Fatty acid	Coffee pulp (CP)	Cocoa husk (CH)	Cocoa pod husk (CPH)	Arabica green coffee grains [14]	Cocoa beans [310]
12:0	0.15	0.07	0.42	ND	0.02
14:0	1.51	0.87	2.10	0.08	0.06
15:0	0.49	0.13	0.65	ND	0.03
16:0	53.02	29.94	36.41	34.25	27.61
16:1	1.73	1.36	0.88	0.07	0.32
17:0	0.90	0.32	0.49	ND	0.27
17:1	0.57	ND	ND	ND	0.02
18:0	12.74	26.31	5.02	7.44	33.78
18:1	15.90	36.27	29.54	12.77	34.73
18:2	20.42	10.26	25.43	44.39	2.43
18:3	6.57	1.51	4.18	1.90	0.14
20:0	6.04	1.34	0.78	3.09	1.09
20:1	ND <sup>1</sup>	0.15	ND	0.35	0.05
22:0	1.37	0.92	ND	0.41	0.15
22:1	ND	0.13	ND	ND	ND

<sup>1</sup>Results are expressed on dry weight. <sup>1</sup>ND: not detected. Determined by GC column Supelcowax de 30 m x 0,25 mm x 0,25 mm. Initial temperature: 185 °C, temperature rate 4 °C/min, final temperature 225 °C (for 5 min); injector temperature 250°C ; detector FID, temperature: 270 °C, helium; flow: 1 mL/min.

The CP, CH, and CPH fatty acid profile revealed an interesting composition, which prompted us to explore the presence of non-enzymatic oxidized PUFAs, obtained from  $\alpha$ -linolenic acid (18:3) [18], such as phytoprostanes (PhytoPs) and phytofurans (PhytoFs) [7] (*cf.* 3.3). Indeed, these compounds could be biomarkers of the oxidative degradation of plant derived food

products and they display a wide range of biological actions (*cf.* 3.3). In addition, and to the best of our knowledge, these compounds have never previously been reported in our samples (*cf.* 11.2 and 11.3). As it was aforementioned, CP, CH, and CPH are very rich vegetal materials, with an interesting and wide molecular profile. Besides, they constitute available renewable and cheap resources that remain under evaluated regarding their chemical composition. Although several investigations have been carried out in order to add value to the coffee and cocoa by- and co-products (*cf.* section 1.4 and 2.4), it is worth exploring new possibilities since each year the coffee and cocoa production and consumption increase. The linolenic acid represents, respectively, the 6.57, 1.51, and 4.18 % of the total fatty acid identified in CP, CH and CPH. Yet, the PhytoPs and PhytoFs study revealed interesting results that are described in chapter XI of the present manuscript.

## **IX. Bioactive molecules extraction from coffee and cocoa by- and co-products using Deep Eutectic Solvents**

As described in previous chapter CP, CH, and CPH are sources of a wide range of molecules with interesting applications. Phenolic compounds are one of the most studied phytochemical groups extracted from vegetal samples, since they are widely distributed in the kingdom plant. Besides, numerous studies have demonstrated that they are involved in the prevention of several degenerative diseases. Additionally, they possess antimicrobial activities, and represent the main phytochemicals with antioxidant properties found in higher plants. Likewise, alkaloids are one of the largest groups of secondary metabolites present in several economically relevant plants, such as coffee and cocoa, among others. Their use is involved in the medical, agro-industrial, and recreational fields [157].

In order to evaluate the physicochemical and biological properties of these phytochemicals, they need to be extracted, analyzed, and purified from the vegetal matrix. This critical step can be carried out through a liquid extraction that usually involves the use of solvents such as water, EtOH, acetone, MeOH, or propanol, sometime in binary mixture [312]. However, the use of these solvents (except for water, and EtOH) is restricted due to their environmental hazards and high toxicity. In addition, the use of organic solvents obtained from carbon fossils contributes to almost the 60 % of all industrial emissions, and 30 % of all volatile organic compound emissions worldwide [188].

Extraction and purification of biomolecules from plant biomass using green processes is a major challenge that could be achieved using deep eutectic solvents (DES). Moreover, in an extraction process, DES can be re-used, and most important, they could serve for the production of rich-plant extracts for direct use in food, pharmaceutical, cosmetic [313], and agrochemical applications [207].

In this context, the ability of different DES formulations in HSE and UPAE assisted extraction of bioactive molecules from CP, CH and CPH, was studied.

## 9.1 Extraction and estimation of total phenolic compounds (TPC) obtained after HSE (1 h, 60 °C) in CP, CH, and CPH

The extraction was performed using six different combinations of hydrated DES (10 % v/v). Water addition to DES mixtures is well-known to affect their structure and thus, their extraction capacity [202]. Yet, the aim of this study was not to assess the water content effect, already discussed elsewhere. Here, the DES were all hydrated at ~10 % (v/v) of water, because we consider this content as a well-balanced when the aim is to compare their extraction efficiency. Indeed, the viscosity is one of the main issues of DES for extraction processing, and 10 % (v/v) water addition allows the strongest drop in viscosity in most of the DES, thus minimizing the mass transfer difference between medium [207]. In addition, such a value allows maintaining the superstructure, characteristic of DES, which may disappear for higher amounts [198][314][315].

First, a quantitative estimation of phenolic compounds carried out by HSE (1 h, 60 °C) in CP, CH, and CPH was performed. The total phenolic compounds (TPC) were quantified by HPLC and Folin-Ciocalteu assay (FC), as described in the materials and method section.

Regarding the values obtained from HPLC measurements, the most efficient solvents were EtOH 70 % (v/v) and CCLA (no significant difference for CP), whereas BeLA, CCGly, BeGly, CCB, and BeB exposed very similar results (Table 15). Yet, no PC were detected in CPH with the liquid chromatography analyses, whatever the solvents. The HPLC results were contrasted with those obtained by FC method. FC is the most common quantification method of bioactive molecules from vegetable extracts since it is a simple and a rapid colorimetric assay [18]. The highest amount of extracted molecules was reached with CCLA in CP ( $44.2 \pm 1.09$  g GAE/100 g dw), and CH ( $52.86 \pm 0.78$  g GAE/100 g dw), followed by EtOH 70 % (v/v) ( $39.79 \pm 2.99$  and  $19.55 \pm 1.05$  g GAE/100 g dw, respectively). For CPH, the best extraction was obtained with EtOH 70 % followed by BeLA. The TPC quantified with FC assay was 5 to 85.5-fold higher than those obtained by HPLC. For example, the extracted amounts in CP and CH with CCLA were 46.1 and 85.3-fold higher.



**Table 15. Total phenolic compounds (TPC) quantification with HPLC ( $\lambda=280$  nm) and Folin-Ciocalteu assay (FC).**

SOLVENT	g GAE/100 g dw*					
	CP		CH		CPH	
	FC	HPLC	FC	HPLC	FC	HPLC
EtOH	39.73 $\pm$ 2.99 <sup>a</sup>	1.10 $\pm$ 0.06 <sup>a</sup>	19.55 $\pm$ 1.05 <sup>a</sup>	0.74 $\pm$ 0.02 <sup>a</sup>	21.73 $\pm$ 0.15 <sup>a</sup>	
70% (v/v)						
CCLA	44.21 $\pm$ 1.09 <sup>a</sup>	0.96 $\pm$ 0.13 <sup>a</sup>	52.86 $\pm$ 0.78 <sup>b</sup>	0.62 $\pm$ 0.08 <sup>b</sup>	7.18 $\pm$ 0.29 <sup>c, d</sup>	
BeLA	11.33 $\pm$ 0.57 <sup>c, d</sup>	0.65 $\pm$ 0.08 <sup>b</sup>	12.12 $\pm$ 1.02 <sup>c</sup>	0.45 $\pm$ 0.04 <sup>c</sup>	10.16 $\pm$ 0.23 <sup>b</sup>	
CCGly	14.89 $\pm$ 2.54 <sup>b, c</sup>	0.63 $\pm$ 0.08 <sup>b</sup>	6.39 $\pm$ 0.215 <sup>d</sup>	0.46 $\pm$ 0.01 <sup>c</sup>	5.00 $\pm$ 1.69 <sup>e</sup>	ND
BeGly	17.69 $\pm$ 0.50 <sup>b</sup>	0.67 $\pm$ 0.05 <sup>b</sup>	12.69 $\pm$ 2.65 <sup>c</sup>	0.37 $\pm$ 0.04 <sup>c</sup>	5.07 $\pm$ 0.98 <sup>d, e</sup>	
CCB	13.62 $\pm$ 0.52 <sup>b, c, d</sup>	0.64 $\pm$ 0.02 <sup>b</sup>	10.21 $\pm$ 1.62 <sup>c, d</sup>	0.46 $\pm$ 0.02 <sup>c</sup>	4.68 $\pm$ 0.08 <sup>e</sup>	
BeB	9.95 $\pm$ 1.58 <sup>d</sup>	0.57 $\pm$ 0.08 <sup>b</sup>	9.16 $\pm$ 2.10 <sup>c, d</sup>	0.42 $\pm$ 0.02 <sup>c</sup>	7.25 $\pm$ 0.42 <sup>c</sup>	

\*Total phenolic compounds expressed as gallic acid equivalents (GAE) on dry weight. Results are the mean of triplicates (mean values  $\pm$  standard deviations). CP: coffee pulp. CH: cocoa husk. CPH: cocoa pod husk. CC: choline chloride; LA: lactic acid; Be: betaine, Gly: glycerol; B: 1,4 butanediol. Letters a to e: TPC extraction in function of the solvent, values followed by the same letter are not significantly different in Tukey's test using 95 % confidence level.

Few studies in literature have reported the TPC quantification by FC method from coffee residues (e.g. spent coffee grounds or spent filter coffee) using DES as extraction solvent [316][317]. For example, Yoo *et al.* [318] found a phenolic concentration average of 2.05 g GAE/ 100g d.w in spent coffee grounds after ultrasound-assisted extraction (60 °C/10 min) with ChCl-based DES. The spent coffee ground is the remaining residue of several water extractions that may strongly decrease the content of biomolecules and thus, explain such a low value. The higher estimation with FC was even more important in CPH since no PC were detected with HPLC, while up to 21.73 g GAE/ 100 g dw using EtOH 70 % (v/v) was reached with the FC method. In general, the TPC concentration found in this research for all samples was higher than those found in the literature (Table 5). For example, in the case of CP and CH, the TPC quantified using EtOH 70 % (v/v) is slightly superior to those reported in the literature (between 2.62-33.5 and 1.82-13.3 g/100g, for CP and CH respectively). On the other hand, while the TPC reported in CPH is around 6 g/100g, in this work the TPC concentration reached 21.73 g/100g. These differences can be attributed to the extraction

conditions, the solvent used and of course the characteristics of the sample. Additionally, although FC is often used to assess the TPC in plant extracts, an overestimation has been already reported as the main drawback. This phenomenon was attributed to the presence of compounds such as sugars, vitamins, organic acids, and other non-phenolic substances that may react with FC reagent leading to higher estimations, whereas they do not necessarily absorb at 280 nm [18]. Additionally, products derivate from plant food sugars are generated under acid conditions extraction. These compounds can interference with colorimetric quantification assays such as FC method. Thus, considering the chemical composition of our vegetal matrices, which are rich in polysaccharides and proteins (Table 12 *cf.* chapter VIII), and the pH of CCLA (Table 9 *cf.* section 7.2.1), it is not surprising to observe such a result. It is also probably that the absence of TPC detected with HPLC in CPH was a result of rapid PC oxidation by polyphenol oxidase produced by endophytic fungus during the transport and conditioning stages of the samples [319]. Oxidized PC can form oligo and polymers that will not be identified in HPLC, but it is possible that they are partly quantified with the FC assay.

It is important to note that the composition of the DES can seriously affect the FC assay. Indeed, when using ChCl-based DES, the formation of a precipitate was observed, this may also interfere with the vertical spectroscopy lecture in the microplate reader. This phenomenon could be ascribed to ion exchanges between ChCl and potassium carbonate, leading to the formation of insoluble salts. According to above, the TPC quantification of plant biomass extracts by FC method can lead to misinterpretation (mostly overestimation), so caution must therefore be taken, especially when working with ChCl-based DES as extracting solvent.

## 9.2 Qualitative study of the extracted molecules from CP, CH and CPH

The extracts obtained after HSE extraction was recorded at 280 nm corresponding to the  $\lambda_{\max}$  wavelength of PC in order to characterize the CP, CH and CPH extracts (Figure 25). Regardless of the solvent used for the extraction, very similar profiles were obtained from the different plant materials. HPLC analysis showed the presence of three main compounds in CP (C and D) and CH (B and D) extracts, whereas no major peak was observed in CPH in

the tested extraction conditions. A minor compound named A ( $t_R = 9.5$  min,  $\lambda_{max}=277$  nm) was detected in CPH. By comparing the elution time ( $t_R$ ) in HPLC and the UV spectra of commercial standards (Figure 26), compounds B ( $t_R=18.4$  min,  $\lambda_{max}=273$  nm), C ( $t_R=21.01$  min,  $\lambda_{max}=324$ ), and D ( $t_R=21.4$  min,  $\lambda_{max}=273$ ) were assigned to theobromine, chlorogenic acid, and caffeine, respectively, and their structure was confirmed by UPLC-ESI-MS analysis.

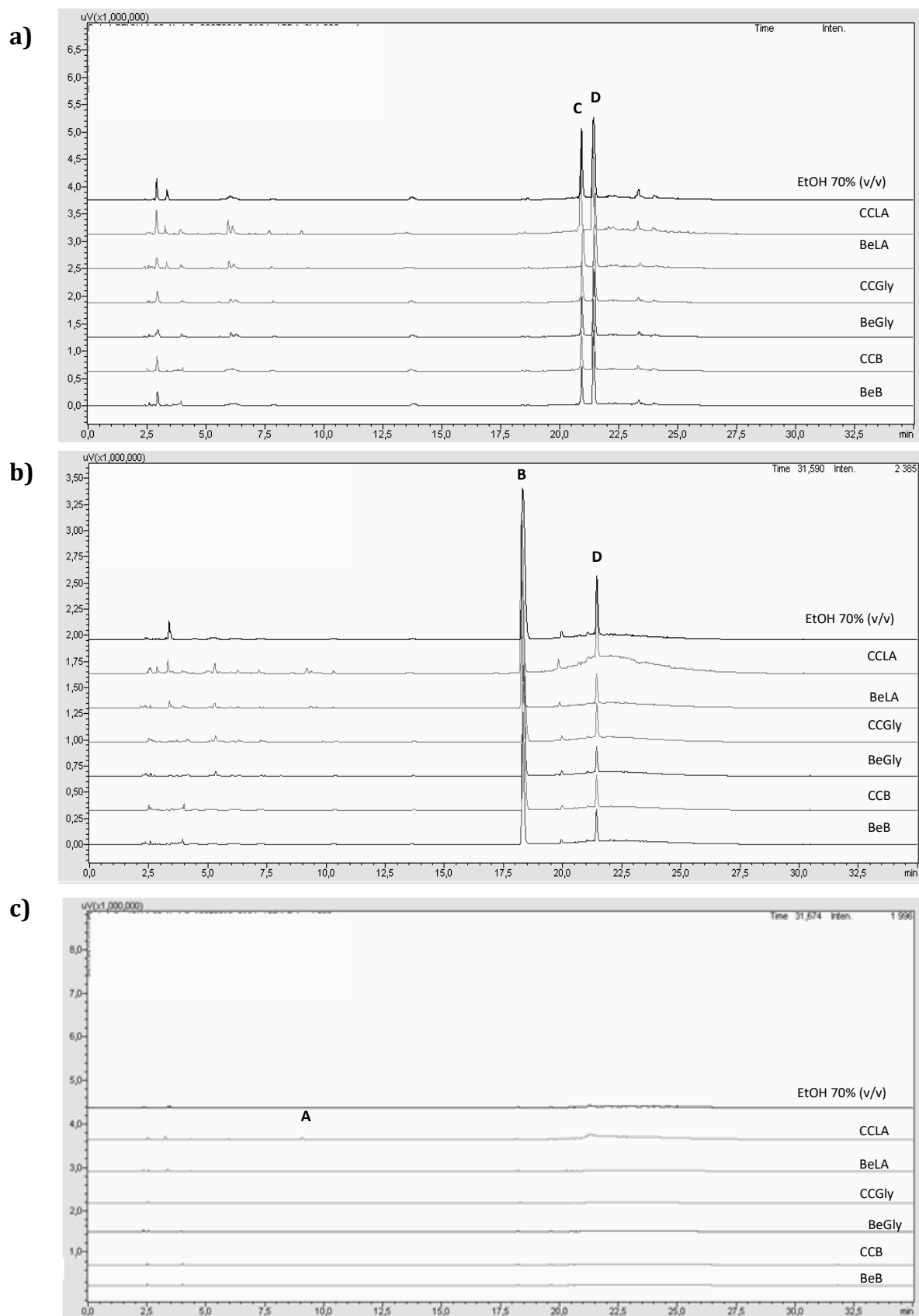
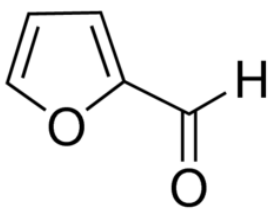
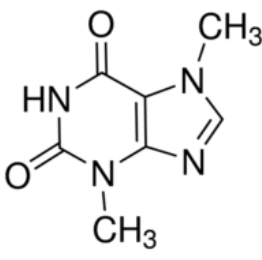


Figure 25. HPLC chromatograms at 280 nm obtained after 1 h of extraction at 60°C of a) coffee pulp (CP), b) cocoa husk (CH), and c) cocoa pod husk (CPH) using different DES and EtOH 70 % (v/v). A) furfural, B) theobromine, C) chlorogenic acid and D) caffeine

									
Compound A	Molecular formula	$\lambda_{\max}$	$[M-H]^-$	$[M+H]^+$	Compound B	Molecular formula	$\lambda_{\max}$	$[M+H]^+$	
Furfural	$C_5H_4O_2$	276.81	-	-	Theobromine	$C_7H_8N_4O_2$	272.81	181.07	

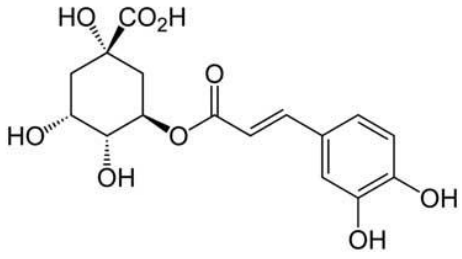
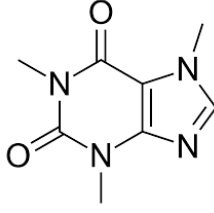
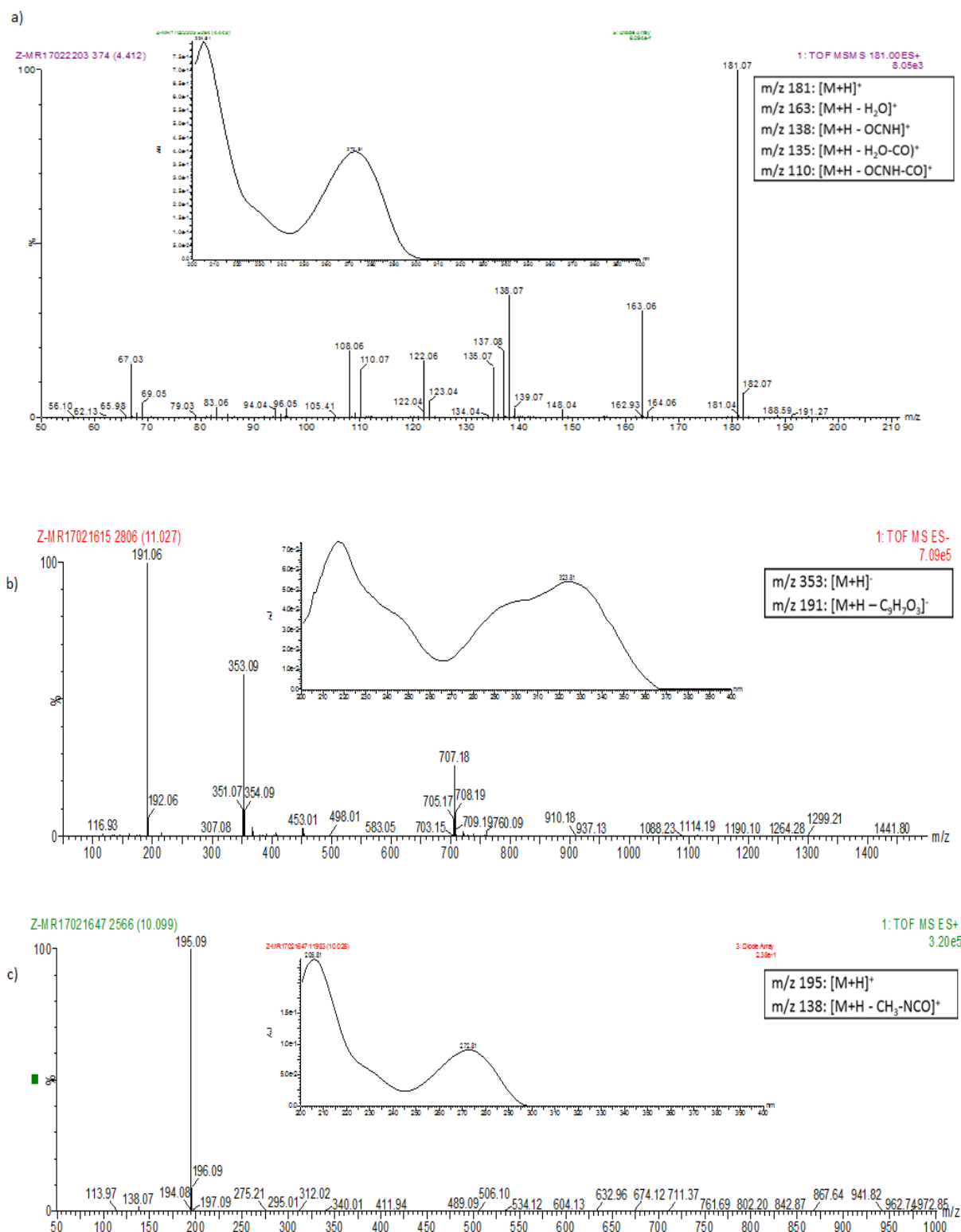
									
Compound C	Molecular formula	$\lambda_{\max}$	$[M-H]^-$		Compound D	Molecular formula	$\lambda_{\max}$	$[M+H]^+$	
Chlorogenic acid (5-CQA)	$C_{16}H_{18}O_9$	323.81	353.09		Caffeine	$C_8H_{10}N_4O_2$	272.81	195.09	

Figure 26. Name, structure,  $\lambda_{\max}$  (nm) and molecular ions ( $m/z$ ) of compounds identified in CP, CH, and CPH.

According to mass spectrum analysis (Figure 27a), compound B, extracted from CH, was identified as theobromine with  $[M+H]^+$  at  $m/z$  181.07, which corresponds to the protonated form. The fragmentation pattern ( $m/z$  67.03, 108.06, 110.07, 122.06, 137.08, 138.07, and 163.06) agree with that showed by  $m/z$  Cloud [320]. Four minor peaks at  $m/z$  163, 138, 135, and 110, from the fragmentation of  $m/z$  181.07 were detected, and correspond to the loss of:  $H_2O$  ( $m/z$  163),  $OCNH$  or isocyanic acid ( $m/z$  138),  $H_2O-CO$  ( $m/z$  135), and  $OCNH-CO$  ( $m/z$  110)[321]. Theobromine is the main alkaloid present in cocoa seeds [322], and its presence has been already described in CH at a concentration around 1.29 g/100 g (Table 3, cf. 2.3).

The peak of compound C, only present in CP, was assigned to chlorogenic acid, and gave the expected molecular ion  $[M-H]^-$  at  $m/z$  353 (Figure 27b), and the smaller mass  $[M-H]^-$  at  $m/z$  191, which corresponds to quinic acid (loss of the caffeoyl moiety from chlorogenic acid by fragmentation). A minor ion at  $m/z$   $[M-H]^-$  707 could correspond to a chlorogenic acid dimer.



**Figure 27. Full-scan ESI-MS spectrum of identified compounds on the ethanolic HSE extracts: a) (+) ESI-MS of theobromine. 272.81 Abs<sub>max</sub> at 273 nm; b) (-) ESI-MS of chlorogenic acid. 325.81 Abs<sub>max</sub> at 324 nm; c) (+) ESI-MS of caffeine. 272.81 Abs<sub>max</sub> at 273 nm**

Compound D, present in CP (major peak) and CH, was assigned to caffeine (Figure 27c and Figure 26). The ESI-MS analysis of compound D showed a base peak with  $[M+H]^+$  at  $m/z$  195. Fragment  $[M+H]^+$  at  $m/z$  138 arises from the parent ion by a neutral loss of methyl isocyanate ( $CH_3NCO$ )[323]. The content of caffeine in dry CP is reported about 1.5 g/100g dw (Table 2). In addition, this compound has been also identified in low concentration in cocoa seeds [37]. Commercial chlorogenic acid and caffeine were analyzed by UPLC-ESI-MS to confirm our results (not shown).

Interestingly, when CP, CH, and CPH were treated with CCLA for extraction times  $> 2$  h, the compound A ( $t_R = 9.5$  min) concentration was increased, and the extracts color has been changed. This phenomenon was observed also during a preliminary kinetic carried out with CCLA and CP. Indeed the increase of compound A was gradual until 9 h of extraction, after that the amount of compound A was constant until 22.5 h when it started to decrease.

The strong absorbance at 280 nm of compound A suggested the presence of at least one aromatic ring in its structure. An attempt to analyze compound A by RMN was made, since direct analysis of the filtered (0.45  $\mu$ m) diluted extract of CCLA-CP was not successful. Therefore, we tried to isolate them from the reaction mix using two methods:

- (i) Preparative-HPLC. The separation was carried out with Thermo Ultimate 3000, Thermo Fisher Scientific, France) equipped with a fraction collector (AFC-3000), and a Hypersil Gold C18 column (5  $\mu$ m, 175 Å, 21.2  $\times$  150 mm; Thermo Fisher Scientific, France) The elution solvents were (A) water (0.1 % acetic acid, v/v) and (B) methanol (0.1 % acetic acid, v/v). The elution gradient program was performed as follows (time (min), solvent B (%)): (3, 5), (13, 5), (23, 100), (33, 100), (34, 0), (40, 0), (45,5). The flow rate, injection volume, and detection wavelength were 10 mL/min, 2 mL, and 280 nm, respectively.
- (ii) Extract fractionation using a low pressure chromatography glass column, loaded with Amberlite XAD-4 polymeric resin. Two milliliters of an aqueous concentrated solution (2 mg/mL) were applied to the previously conditioned column with water (100 mL). The sample was eluted with  $H_2O$  and MeOH; obtaining three fractions ( $F_{1W}$ ,  $F_{2W}$  and  $F_{3W}$ ) from the first washing and four fractions ( $F_{1MeOH}$ ,  $F_{2MeOH}$  and  $F_{3MeOH}$ ) from the second one.

Sample from F<sub>2</sub> MeOH were diluted in MeOH (500 µL) for their characterization by HPLC and RMN.

Unfortunately, in the tested conditions, it was not possible to totally separate the DES components from compound A, and they interfered with the RMN analysis (results not shown). Therefore, in a first time, it was supposed that compound A was not a phenolic compound but probably a product of the lignocellulose degradation, as it was only extracted under high temperature, long-time, and acidic conditions ( $\text{pH}_{\text{CCLA}} = 0.78 \pm 0.18$ ). Moreover, a similar compound extracted from rice straw with ChCl:lactic acid (5:1 mole ratio), and strongly absorbing at 280 nm, was assigned (FTIR analysis) as lignin aromatic structural components [324]. To confirm our early hypothesis, the CPH extract obtained with CCLA was analyzed by UPLC-MS. The result showed that the molecule could be ionized, neither in positive nor in negative ESI. Afterward, different molecules formed by degradation of sugar (lignin and furfural) were analyzed in HPLC. The furfural appeared as a potential candidate, as on the one hand, it eluted at the same  $t_R$  as compound A, whatever the HPLC conditions (elution program, column, flow), and it presented the same UV spectrum than the commercial standard (not shown). On the other hand, this last did not ionize in UPLC-MS. Thus, the compound A was assigned as furfural (Figure 26). Furfural derives from the acid catalyzed dehydration of pentoses and its content in our extracts could be related to the hemicellulose content in each one of our samples: CP>CPH>CH (Table 12 *cf.* 8.1). According to the literature, furfural was detected after lignin extraction, when woody biomass was treated at 145 °C using ChCl-based DES in combination with lactic acid, acetic acid, and levulinic acid [325]. The furfural formation was also detected after the corncob was treated with ChCl-based DES at a concentration below of 0.2 % (w/w) [268]. Other studies have tried to use DES as reaction media for the conversion of C<sub>6</sub> and C<sub>5</sub> sugars for the production of second-generation biofuels [326]. In this context, Zhang and Yu [267] studied the conversion of xylose into furfural co-catalyzed by ChCl:citric acid:H<sub>2</sub>O from 90 °C to 140 °C, and they obtained yields ranging from 15 % to 59.3 %.

Although some studies have reported the apparition of furfural after biomass treatment with acidic DES, this is the first report on furfural production using DES from coffee and cocoa agro-residues, which may represent an economic and green strategy. In this study,

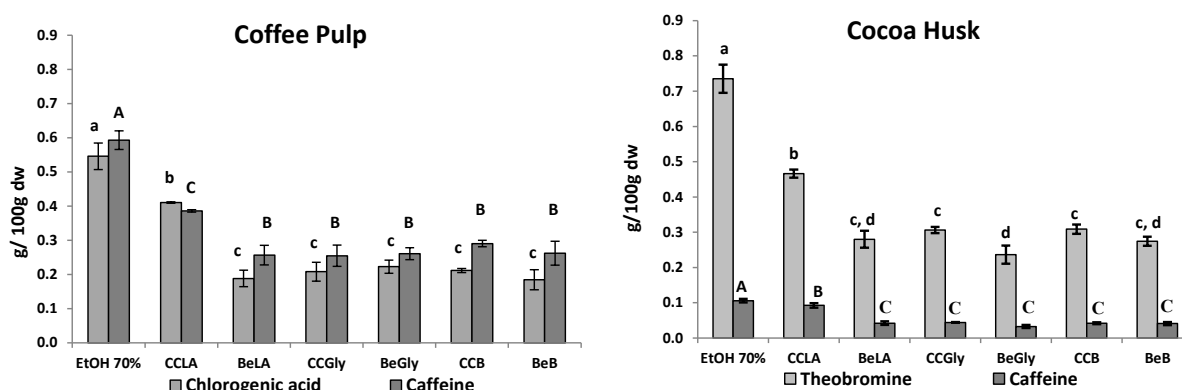


production rates of 181.13, 66.50 and 148.01 mg of furfural per 100 g of CP, CH, and CPH, respectively, was reached. The furfural has gained a renewed attention because of its interest in a wide range of applications. For example, it may be used as fungicides and nematicides in the agroindustry; as gasoline additives, lubricants, resins, decolorizing agents, jet fuel blend stocks in the fuel industry; as building block for biopolymers; or as flavor enhancers in the food industry [327]. Besides, with the more acidic DES (CCLA and BeLA) and whatever the sources (CP, CPH or CH), the color of the medium turned to purple during the extraction process.

In a first time, the presence of anthocyanins was suspected due to the extracts color change that become between pink and purple, but no absorbance was detected at 520 nm. This phenomenon has been already emphasized in studies dealing with the extraction of phenolic metabolites from safflower as well as from orange peel [220][328]. In the first example, the authors attributed this color change to the difference in the extractability of compounds [220]; while in the second study, this result was present when acids were used as HBD in the DES formation (namely, lactic or glycolic acids) [328]. It is possible that a reaction similar to that taking place in the Molisch's test for carbohydrates occurs during our acidic DES extraction. In the Molisch ring test, concentrated sulfuric acid is used both to produce the furfural derivatives and as the condensing agent [329]. In our case, CCLA would play the role of concentrated sulfuric acid. Additionally, pectin can be also released from CPH by DES extraction, and its hydrolysis in acidic medium releases uronic acid monomers that can further dehydrate in furfuryl derivatives [330]. It is also possible that color derives from furfural polymerization.

### **9.3 Quantitative analysis of the compounds A, B, C and D extracted from CP, CH and CPH**

As was aforementioned compounds A, B, C, and D were respectively identified as furfural, theobromine, chlorogenic acid, and caffeine, after a short-term extraction (HSE, 1 h, 60 °C) in the different biomass (CP, CH and CPH). From a quantitative standpoint, it was noticed that the DES nature has an influence on the amounts of each extracted compounds (Figure 28).



**Figure 28. Extraction yields of chlorogenic acid, caffeine, and theobromine with DES and EtOH, assisted by HSE in coffee pulp (CP) and cocoa husk (CH)**

Results are expressed as chlorogenic acid, caffeine, and theobromine equivalents (dry weight (dw)) and analyzed by HPLC. CC: choline chloride; LA: lactic acid; Be: betaine, Gly: glycerol; B: 1,4 butanediol.

EtOH 70 % (v/v) and CCLA were the most efficient solvents in extracting chlorogenic acid and caffeine from CP. The extracted amounts of chlorogenic acid in % w/w (dw) were  $0.55 \pm 0.04$  (EtOH 70 % (v/v)) >  $0.41 \pm 0.002$  (CCLA) >  $0.22 \pm 0.02$  (BeGly) ~  $0.21 \pm 0.01$  (CCB) ~  $0.21 \pm 0.03$  (CCGly) ~  $0.19 \pm 0.02$  (BeLA) ~  $0.18 \pm 0.03$  (BeB). According to the literature [145][44] chlorogenic acid is present in CP and its content varies from 0.014 g/100 g dw on dried CP, to 2.4 g/100 g dw on fresh CP, confirming that processing has an influence on the content of this molecule, that could be enzymatically hydrolyzed or oxidized on fresh CP (when not conditioned immediately) and on dried CP (if drying is too long for example) In our case, chlorogenic acid in CP presented an intermediate content compared to these reported values. For caffeine, the extracted amounts in % w/w (dw) were  $0.59 \pm 0.03$  (EtOH 70% (v/v)) >  $0.39 \pm 0.003$  (CCLA) >  $0.29 \pm 0.01$  (CCB) ~  $0.26 \pm 0.04$  (BeB) ~  $0.26 \pm 0.002$  (BeGly) ~  $0.26 \pm 0.03$  (BeLA) ~  $0.25 \pm 0.003$  (CCGly).

Regarding CH, the principal extracted compounds were theobromine and caffeine. For theobromine, the amounts were higher (but non-significantly different) when the ChCl-based DES were used in comparison to betaine-based DES, except for CCLA (ChCl:lactic acid). The last one extracted 1.7-fold more theobromine than the other tested DES. EtOH 70 % (v/v) was the most efficient solvent in extracting theobromine and caffeine. Non-significant differences in the theobromine extraction were observed when using BeLA, BeGly or BeB. The amount of theobromine extracted using CCB was slightly higher, but not significantly

different from that extracted using BeLA, CCGly or BeB. The extracted caffeine was similar when using BeLA, CCGly, DSB2, CCB or BeB.

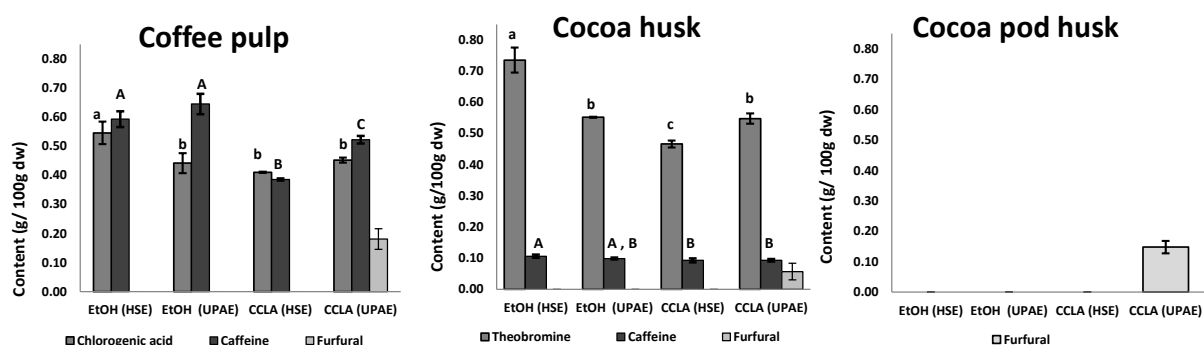
These results showed that the choice of HBA constituting the DES have a clear influence on the extracted compounds nature and on the phytochemical extraction efficiency. This result coincides with the reported by Qi *et al.* [273], who obtained a better extraction yields using relative strong polar compounds with ChCl-based DES than those containing betaine for the more polar target compounds.

#### **9.4 Effect of the assisted extraction method over the extraction yields: HSE versus UPAE**

After the extraction solvent selection, one of the most important critical steps is the choice of the extraction method. Thus, the study aimed at comparing two different and common assisted extraction methods, the HSE versus ultrasound (UPAE: 3 min constant duty cycle at 200 W). Similar to HSE extraction, EtOH 70 % (v/v in water) was used as a reference standard.

UPAE has been already described as a strategy that can promote the rates and the extraction yields of several biomolecules, such as oils, proteins, polyphenols, and anthocyanins, among others [331]. Extraction enhancement by ultrasound has been attributed to the propagation of ultrasound pressure waves, and resulting cavitation phenomena. The implosion of cavitation bubbles generates macro-turbulence, high-velocity inter-particle collisions and perturbation in micro-porous particles of the biomass which accelerates the eddy diffusion and internal diffusion [331]. These characteristics are especially effective when DES are used as extraction solvents, since the quickly increase of temperature during ultrasounds extraction can decrease the viscosity of DES, and therefore improve their extracting capacity. For example in the extraction of anthocyanins, (+)-catechin and quercetin-3-*O*-glucoside from grape skin, UPAE showed superior extraction efficiency in comparison with heating or microwave-assisted extraction [281]. The extraction of quercetin, kaempferol and isorhamnetin glycosides from *Flos sophorae*, using UPAE also showed satisfactory yields [284].

Regarding our experiments, CCLA exhibited the best results with respect to phytochemicals extraction. Results (Figure 29) showed that the highest chlorogenic acid extraction from CP was obtained with EtOH+HSE ( $0.55 \pm 0.04$  g/100 g dw). This amount is 1.2-, 1.3-, and 1.2-fold higher than EtOH+UPAE, CCLA+HSE, and CCLA+UPAE, respectively (no statistical difference observed among them). Regarding the caffeine, the highest quantity was obtained in CP with EtOH+UPAE ( $0.65 \pm 0.03$  g/100 g dw), but there was not a statistically significant difference with EtOH+HSE ( $0.59 \pm 0.03$  g/100 g dw), whereas CCLA+UPAE and CCLA+HSE were less effective. A similar trend was observed in the extraction of caffeine from CH, reaching  $0.11 \pm 0.005$  and  $0.10 \pm 0.003$  g/100 g dw of the extracted molecule with EtOH+HSE and EtOH+UPAE, respectively (Figure 29). Theobromine, which is very similar to caffeine in term of chemical structure, was the main compound extracted from CH. Once again, the best yield was reached with EtOH+HSE ( $0.74 \pm 0.04$  g/100g dw), following by EtOH+UPAE and CCLA+UPAE, whereas the combination CCLA+HSE ( $0.47 \pm 0.011$  g/100g dw) was the less efficient treatment (Figure 29).



**Figure 29. Comparison of phytochemical extraction using DES assisted by HSE or UPAE**

The most remarkable result was definitively obtained with the furfural production during UPAE extraction. It was previously emphasized that when longer extraction time (>2 h) was performed upon heating extraction, the peak assigned to furfural at 9.5 min with CCLA in CPH was substantially increased, whereas it remains missing or negligible in the other conditions (

Figure 25). As depicted in Figure 29, furfural was obtained from CPH ( $0.15 \pm 0.02$  g/100 g dw), CP ( $0.18 \pm 0.04$  g/100 g dw), and CH ( $0.06 \pm 0.03$  g/100 g dw) using CCLA and UPAE extraction. Once again, furfural was not detected when the assays were performed in classical solvent (EtOH 70 % v/v). This result confirmed our hypothesis about the lignocellulose degradation, promoted by long-term condition of extraction happening under acidic conditions (pH of CCLA = 0.78) and elevated temperature. Although the overall temperature in CCLA obtained with HSE ( $\sim 60$  °C) and UPAE ( $\sim 72$  °C) was not very dissimilar, one may say that UPAE treatments create a brief but a highest local temperature rise. This allows decreasing the high DES viscosity and consequently improves mass transfer. In addition, the energy provided may lead to superficial tissue disruption, and particle diffusion, therefore increasing the production and extraction yield of furfural precursors [331].

It is also important to point out the amounts of unknown molecules that have been detected in CP and CH, along elution time in HPLC. The highest quantities were observed with CCLA+UPAE ( $0.43 \pm 0.03$  g GAE/100 g dw) in CP, while for CH the most important amount was extracted using EtOH+HSE ( $0.05 \pm 0.004$  g GAE/100 g dw). In CPH, only traces of compounds were detected with EtOH+UPAE or CCLA+UPAE; whereas no compound was detected using HSE.

Interestingly, unlike the assays in EtOH, the UPAE seems to have a positive effect on the extraction capacity in DES. Indeed, regardless of the molecule and the raw material, UPAE did not promote the extraction in EtOH. Worse still, it had a negative impact, by decreasing the yields of chlorogenic acid in CP, and theobromine in CH. In opposite, UPAE could significantly help the extraction of chlorogenic acid, caffeine and theobromine, and furfural formation when applied in CCLA. UPAE, applied in organic solvent such as EtOH, could lead to degradation of dissolved molecule [332], whereas the strongest connections established in a DES could prevent such event [333]. Thus, UPAE could reinforce the positive effects of DES in the extraction processes.

## 9.5 Conclusions

During the phytomolecules extraction of CP, CH and CPH using different combinations of DES, it was demonstrated the versatile physicochemical effect of DES, and its capacity to extract a wide range of molecules of the treated samples.

Although the solvent CCLA (combination of ChCl and lactic acid) showed to be the most effective to extract the interest molecules in terms of quantity, a similar molecular profile was observed using all the DES formulations. However, it is clear that there is a relationship between the DES composition and yield extracted. The best CCLA ability to extract 5-CQA, caffeine, and theobromine from the samples, could be related to its characteristics. For example, it is possible that CCLA may present a great capacity to destructure the lignocellulosic material, thus improving its accessibility to the matrix and the extraction yield of the target molecules. In any case, the DES design is critical to obtain the maximal yields of the target compounds.

Moreover, the extraction method has an influence on both, the type of extracted biomolecules and the extraction yield. In this context, the use of UPAE had a positive effect on the yield extraction of chlorogenic acid, caffeine, and theobromine, identified as the main bioactive molecules in CP and CH.

On the other hand, the extraction of furfural by CCLA and UPAE (or long-term heating) was a remarkable result. The traditional production of furfural involves acid hydrolysis, therefore it is necessary largest volume of solvents (sulfuric acid generally), and high temperatures. Thus, these findings provide a new perspective for the use of CP, CH, and CPH to synthesize furfural from plant biomass by- and co-products using DES, since furfural has a wide spectrum of industrial applications. On the other hand, the use of DES as solvent extraction offers alternative molecule formation that could not be obtained with conventional organic solvent, therefore representing a green tool in adding-value to agro-residues.

## **X. Enriched DES formulation: evaluation of its effects in *in vivo* study**

According to results obtained in the last chapter and other researches [6], DES have been proved to be efficient for bioactive molecules extraction from agricultural resources. However as was already explained the recovery of target molecules is a challenge (*cf.* 4.4.4).

In this context, the goal of this chapter was to investigate the consequences of delivering an enriched-DES formulation in acute conditions, before conducting a long-term nutritional supplementation study, in order to evaluate if DES can be used for oral bioactive molecules or therapeutic agents delivery.

DES formulation was prepared from extracts of green coffee beans (GCB), for two main reasons: (i) because the TPC concentration found in DES after CP, CH and CPH extraction was too low ( $\approx 1$  % w/w dw) to achieve the objective to evaluate a rich phenolic DES formulation, whereas GCB are known to be richer in active phenolic compounds (6-10 % dw of CGA [334][20]; and (ii) as an alternative to take advantages of the low-grade GCB that cannot be used to the coffee fabrication because they not fulfil with quality standards (aroma and flavor, size, shape, color, hardness, and presence of defects), as a source for CGA extraction [335]. Besides, previous studies carried out in the Muscular Dynamics and Metabolism joint research unit (UMR 866 DMEM, INRA) , demonstrated the beneficial effects of a powdered polyphenolic extract (in classical ethanol extraction), on oxidative stress and lipid metabolism of rats fed with an obesogenic diet [336][337].

### **10.1 Extraction of phenolic compounds from GCB**

PC extraction from GCB was performed using the same six DES from (Table 9 *cf.* 7.2.1), since they proved to be efficient to extract PC from other samples (CP, CH and CPH).

Extraction rates were very similar with the six tested DES (in average 6.23 g CAE/100 g GCB dw). An extract using EtOH 70 % (v/v) as the reference was also obtained and contained  $7.29 \pm 0.71$  g CAE/100 g GCB dw (Table 16). Our choice was directed towards the use of the BeGly DES (betaine:glycerol:water combination), on one hand as extraction yield was slightly

superior to that of the others ( $7.37 \pm 1.09$  g CAE/100 g GCB dw). On the other hand, betaine is a methyl derivative of the amino acid glycine and it is present in microorganisms, plants, and animals and it is an essential component in many foods, as it is. Its LD<sub>50</sub>/oral in rats is about 11179 mg/kg, thus considered as practically non-toxic [21]. The physiologic function of betaine is either as an organic osmolyte to protect cells under stress or as a catabolic source of methyl groups via transmethylation for use in many biochemical pathways. It possesses a lipotropic activity and would reduce the risk for atherogenesis [338][339]. Hayes *et al.* [340] demonstrated in subacute and subchronic rat studies that betaine is nontoxic at all studied doses (0-5 % of the diet). Thus, BeGly was chosen in our current study to perform toxicity assessment on rats.

**Table 16. Extraction of PC from green coffee beans (GCB) using DES**

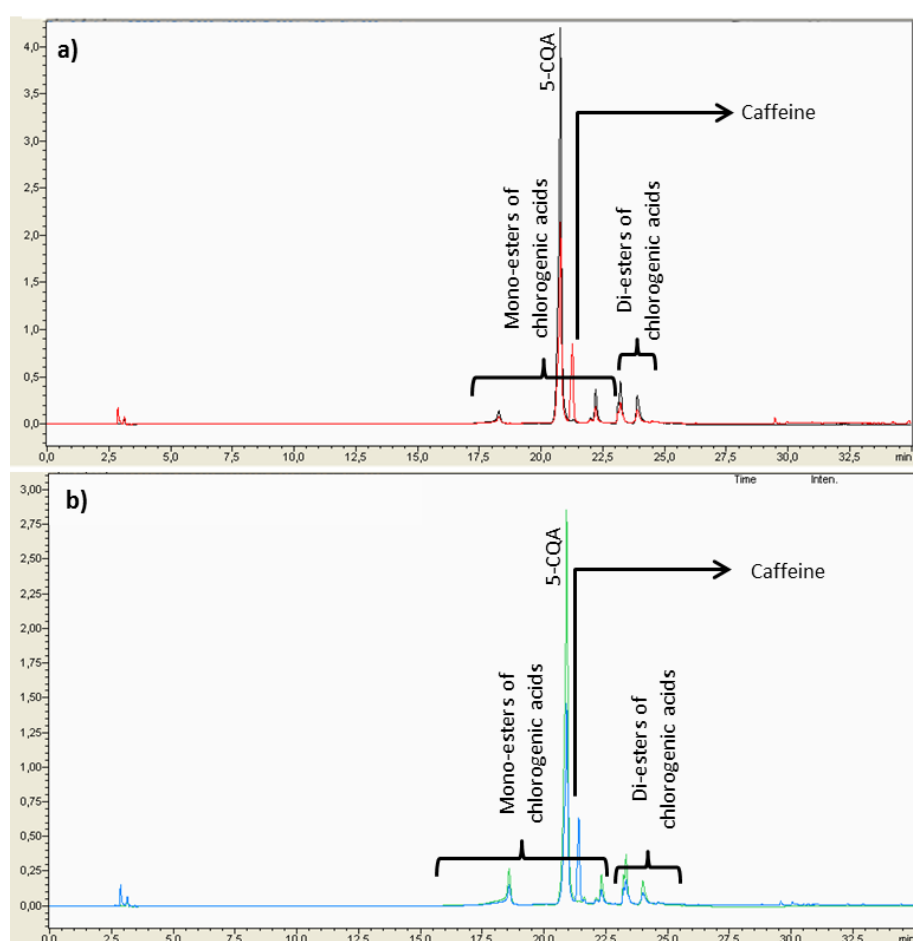
DES name	Extraction yield (% w/w) <sup>1</sup>
CCLA	$6.98 \pm 0.63$
BeLA	$5.85 \pm 1.44$
CCGly	$6.12 \pm 0.30$
BeGly	$7.37 \pm 1.09$
CCB	$5.43 \pm 0.04$
BeB	$5.67 \pm 0.11$

<sup>1</sup> Expressed at chlorogenic acid equivalents (CAE) on GCB dry weight. CC: choline chloride; LA: lactic acid; Be: betaine, Gly: glycerol; B: 1,4 butanediol.

BeGly GCB extract was analyzed and quantified by HPLC as described in section 7.2.3 and its final TPC concentration reached  $9.33 \pm 1.32$  mg/mL (expressed as CAE). Additionally, its PC profile was analyzed by HPLC according to spectra and retention times ( $T_R$ ) of standards. The results showed that BeGly GCB extract was composed of 83.9 % of mono-esters and 16.1 % of di-esters of CGA. Similar composition was observed when using ethanol 70 % (v/v) (86.1 % mono-esters and 13.9 % of di-esters of CGA). In both extracts, the main compound extracted was 5-CQA, which represented 75.2 % and 74.7 % of total chlorogenic acids extracted using EtOH 70 % (v/v) and BeGly, respectively (Figure 30). Results are not surprising, since 5-CQA and its related compounds are the main components found in the phenolic fraction of GCB. Among them, the most commons include caffeoylquinic acids, dicaffeoylquinic acids, feruloylquinic acids, p-coumaroylquinic acids and mixtures of 5-CQA diesters of caffeic and ferulic acids with quinic acid, each group with at least three isomers [55].



In addition, GCB BeGly and EtOH 70 % (v/v) extracts were analyzed by HPLC at 280 nm in order to quantify the possible caffeine content. The results showed that both extracts contained a non-negligible amount. Indeed,  $1.15 \pm 0.25$  % and  $0.96 \pm 0.05$  % (w/w) of caffeine was extracted from GCB using BeGly and EtOH 70 % (v/v), respectively (Figure 30). These results coincide with caffeine content reported in coffee Arabica from several origins (between 0.9-1.4 % dw) [341]. Accordingly, this means that the caffeine concentration in the GCB BeGly extract, used for the rat diet, was about  $1.96 \pm 0.32$  mg/mL. Thus, ingested caffeine in the rat's diet (56 mg/kg of rat by day) is below its lethal dose of 367 mg/kg determined in starved or fasted rats [342].



**Figure 30.** HPLC chromatograms of GCB extracted by a) EtOH 70 % (v/v) (red line at 280 nm, black line at 327 nm); and b) BeGly (blue line at 280 nm, green line at 327 nm). HSE (3 h, 60°C, 250 rpm)

## 10.2 Effects of enriched-DES extract from GCB *in vivo* study

After the extraction with BeGly, the enriched-DES formulation was diluted (in BeGly) to adjust the final concentration at 10 mg CAE/mL of DES. *In vivo* assay was performed on 6 rat groups called DES-group. The amount of enriched-polyphenolic DES extract, orally administrated, was set to give daily about 60 mg CAE to the rats, representing 6 mL of PC enriched DES extract per rat/day (3 mL morning and 3 mL evening). This represents a nutritional dose of PC, as the mean intake of PC is estimated about 1-1.5 g/day in European and Asian populations [343][344].

The gavage and sacrifice of rats, as well as the plasma and blood studies, were carried out in the laboratory of the DMEM team. The effects observed on the rats' health after enriched-DES extract administration, are described below.

### 10.2.1 PC enriched-DES GCB extract induced large belly and fatigue in rats

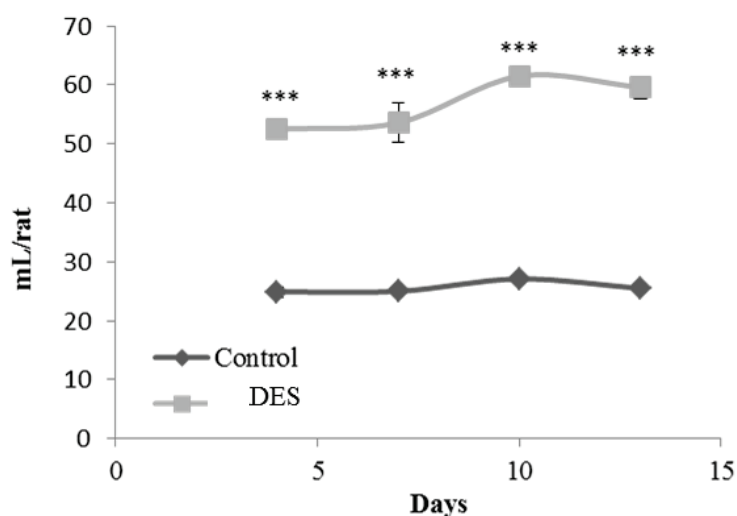
Although slightly hydrated at 10 % (v/v) in order to decrease its viscosity while maintaining its features, the extract previously prepared in BeGly was still too viscous for oral administration. Thus, before giving oral gavage, it was necessary to warm the mixture at about 37°C to adequately fluidize it, as the temperature is known to affect viscosity [207]. Rats were gavaged in the morning and at the end of the day. Rapidly, it was observed that the DES rats all have a large belly and a significant fatigue in the hours following the DES gavage. It is reported that glycerol oral administration to rats can provoke debility, loss of appetite and loss of weight [345]. Moreover, a first rat died within the DES group on the 5th days of gavage. After that, one rat was switched from the control group to the DES group to maintain balanced groups. On the 10<sup>th</sup> day of gavage, a second DES rat died. The aspect of its stomach was the same as for the first deceased rat. *i.e.* it was at least 10-fold the normal size and plenty of liquid (about 30 mL). Finally, the study was completed with 5 controlled rats and 5 DES rats. Indeed, acute toxicity tests on mice orally administrated with various ChCl:glycerol-based DES revealed mortality as well as a LD<sub>50</sub> lower with DES than with their individual components [346][347]. For example, working with ChCl: glycerol: (1:2 mole ratio), a single dose of 12 g of DES/kg of body weight, provoked behavioural disorders in mice and

death within 4 h and 7 days. For their study, Chen and co-workers reported a LD<sub>50</sub> of 7.7 g/kg [346], while in another study tested single doses of ChCl:glycerol (1:3 mole ratio) DES of 5, 10, and 20 g DES/kg of body weight on mice reported a LD<sub>50</sub> of 6.4 g/kg [347]. In our study, considering the BeGly density of 1.29 kg/L, the daily dose of DES was about 36 g/kg of initial rat body weight for an initial 210 g rat during 15 days. Thus, even if our doses are 4.5-fold higher, in our study only two rats died between 5 and 10 days.

In light of these results, it would have been interesting to compare the toxicity of PC enriched extracts in the same *in vivo* study, between betaine:glycerol and ChCl:glycerol. However, as mentioned above, this study was preliminary to verify the possible effect of a polyphenolic BeGly extract in rats, in order to not sacrifice animals unnecessarily, on the recommendation of the national ethics committee in France. Unfortunately, in the light of the preliminary results, the French national committee did not recommend to continue with the entire initially planned study.

### 10.2.2 PC enriched-DES GCB extract reduced dietary intake and weight gain, and increased water intake

From the beginning of and throughout the study, daily dietary intake was markedly reduced (-40 %) whereas water consumption was more than doubled in the DES group compared to controls (Figure 31, Table 17).



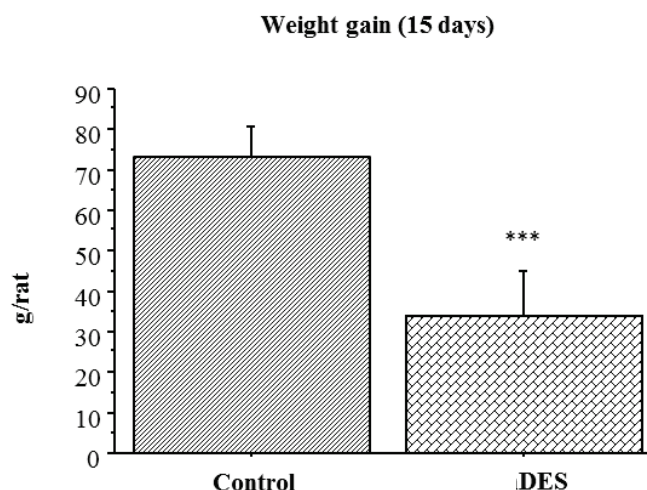
**Figure 31. Water rats consumption**  
Results are mean  $\pm$  SD, n=5. \*\*\*p<0.001

It is probably that BeGly, being viscous, contributed to a satiety sensation in rats, limiting their food consumption. However, according to literature, glycerol increases plasma osmolality and provokes diuresis [345], that may provoke thirst and so over drinking, as thirst is the body's defence mechanism to increase water consumption in response to perceived deficits of body fluids. Thirst can be stimulated in animals and man by intracellular dehydration caused by increases in the effective osmolality of the extracellular fluid [348]. It would have been interesting to follow the evolution of osmolality on the plasma. In accordance, reduced dietary intake after glycerol ingestion had been previously described in man in comparison to equicaloric glucose ingestion [349]. In addition to this reduced dietary intake and increased water intake, it was also observed a marked decreased in weight gain and in adipose tissue mass at the end of the study in the DES group compared to the control group (Figure 32, Table 17).

**Table 17. Rats parameters**

Parameter	Control group	DES group
Initial body weight (g/rat)	210 ± 3	210 ± 6
Final body weight (g/rat)	283 ± 5	244 ± 15***
Dietary intake (g/rat/d)	21.2 ± 0.3	12.5 ± 0.5***
Water consumption (mL/rat/d)	25.6 ± 0.4	56.8 ± 56.8***
Liver (relative weight %)	2.85 ± 0.17	4.44 ± 0.38***
Rate (relative weight %)	0.206 ± 0.012	0.195 ± 0.025
Heart (relative weight %)	0.286 ± 0.012	0.277 ± 0.017
Kidney (relative weight %)	0.382 ± 0.034	0.470 ± 0.053*
Gastro (relative weight %)	0.609 ± 0.044	0.591 ± 0.038
Soleus (relative weight %)	0.043 ± 0.04	0.043 ± 0.05
Adipose tissue (relative weight %)	2.66 ± 0.37	1.63 ± 0.10***

Results are mean ± SD, n= 5. A student t test was performed to detect statistical significance among groups. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001



**Figure 32. Rats weight gain at the end of the study**

Results are the mean  $\pm$  SD,  $n=5$ . A student t test was performed to detect statistical significance among groups. \*\*\* $p<0.001$ .

### 10.2.3 PC enriched-DES GCB extract increased plasma lipid levels

Plasma lipid levels were higher in the DES rats group than in the control group. Plasma cholesterol and plasma triglycerides levels were respectively increased by 90 % and 240 % compared to control rats (Table 18). The level of plasma glycerol was measured in order to avoid the interference that this parameter can have in plasma triglycerides assay. Although BeGly contains glycerol in a molar ratio 2:1, with respect to betaine, plasma glycerol level was similar in the group of DES and the control. Thus, this does not explain the high plasma triglycerides content in the DES rats. However, the increase in plasma lipid levels may results from an important alteration of lipid metabolism in particular a marked reduction in the adipose tissue mass with the BeGly DES and therefore the release of lipids into the circulation. Indeed, it has been reported that betaine, used as a dietary supplement in pigs, decreased the amount of fat tissue [350]. On the other hand, triglycerides can also derive from the glycerol-3-phosphate (G3P) pathway, also called the Kennedy pathway, where G3P is an important precursor for lipid biosynthesis [351].

**Table 18. Plasma and liver biochemical parameters**

Parameter	Control group	DES group
<b><i>Plasma parameters</i></b>		
Plasma ALAT (U/L)	38.5 ± 6.8	19.5 ± 6.8**
Plasma ASAT (U/L)	74.6 ± 16.0	39.9 ± 3.7**
Plasma cholesterol (g/L)	0.37 ± 0.06	0.70 ± 0.08***
Plasma triglycerides (g/L)	0.6 ± 0.3	2.04 ± 1.20*
Plasma free fatty acid (g/L)	0.458 ± 0.104	0.551 ± 0.127
Plasma glycerol (g/L)	0.125 ± 0.041	0.082 ± 0.040
Plasma glucose (g/L)	1.49 ± 0.06	1.47 ± 0.27
Plasma creatinine (mg/L)	6.57 ± 0.86	4.81 ± 1.75
Plasma uric acid (mg/L)	8.71 ± 0.86	11.9 ± 3.1
Plasma urea (mg/L)	328 ± 46	427 ± 39*
Plasma proteins (g/L)	61.1 ± 20.4	51.5 ± 8.9
<b><i>Liver parameters</i></b>		
Liver cholesterol (mg/g tissue)	3.34 ± 1.06	2.33 ± 0.56
Liver triglycerides (mg/g tissue)	15.9 ± 3.8	11.2 ± 2.2*
Liver free fatty acids (mg/g tissue)	7.41 ± 0.63	6.58 ± 0.64
Liver total lipids (mg/g tissue)	26.7 ± 3.7	20.1 ± 2.7*
Liver proteins (mg/g tissue)	138 ± 22	145 ± 23
Liver glycogen (mg/g tissue)	1.81 ± 0.14	3.30 ± 2.13

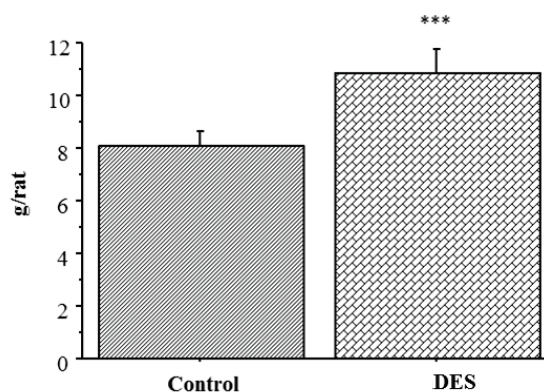
Results are the mean ± SD, n= 5. A student t test was performed to detect statistical significance among groups. \*p<0.05; \*\*p<0.001; \*\*\*p<0.001.

Betaine may be also involved in the increase in plasma cholesterol and triglycerides observed in the DES group. It has been reported that oral supplementation of betaine in humans increases plasma total cholesterol and low-density-lipoprotein cholesterol (LDL) [352]. A phosphatidylcholine moiety is needed to secrete very-low-density lipoprotein (VLDL, a precursor of LDL) from the liver. As betaine is formed from choline, it is possible that betaine supplementation spares this use of choline so that more choline is available for phosphatidylcholine biosynthesis in the liver, thereby making more available for VLDL formation.

#### **10.2.4 PC enriched-DES GCB extract induced hepatomegaly, but no liver, injury**

The enlarged liver (hepatomegaly) was observed in the DES group as liver weight was highly increased (Table 17, Figure 33), but without liver injury. Indeed the plasma activities of transaminases (ALAT and ASAT) were similar in both groups of rats and even lower in the

DES group than in the control group (Table 18). An effect of caffeine ingestion cannot be excluded, as Debry reported that large quantities of caffeine causes hepatic hypertrophy in animals [353]. It has been reported that betaine and CGA may decrease the activities of these enzymes [354]. This result differs from the results observed in others studies, where the plasma ASAT activity increased following acute oral administration of various DES in mice [347][355].



**Figure 33. Rats liver weight at the end of study**

Results are the mean  $\pm$  SD, n= 5. A student t test was performed to detect statistical significance among groups. \*\*\*p<0.001.

The observed hepatomegaly in the DES rats group was not associated with hepatic steatosis, defined when intrahepatic lipid content exceeds 5 % of hepatic tissue [356], as total lipid content was 2 % in the DES group. Total lipid content was even lower in the DES group compared to the control group (2.7 %). In particular, triglycerides content (1.1 % *versus* 1.6 %) (Table 18). Thanks to its lipotropic effect, betaine content in BeGly could prevent or eliminate an accumulation of excess fat in the liver [338][339]. It is important to highlight that CGA present in GCB BeGly extract possess several important and therapeutic roles as antioxidant activity, hepatoprotective, and can perform crucial roles in lipid and glucose metabolism regulation helping to treat hepatic steatosis [357]. Betaine has also been reported to help to prevent liver steatosis [358]. Thus, these two components in the GCB BeGly extract, betaine and CGA, could have contributed to preventing fat accumulation in rat liver.

Although glycerol supplementation was reported to increase liver glycogen content [359], it was hypothesized that hepatomegaly could be due to glycogen accumulation. However, liver glycogen content was not significantly modified in the DES rats compared to the control rats. Indeed, hepatic glycogen can be synthesized from glucose via the so called “direct pathway” or from gluconeogenic precursors (*e.g.* glycerol) by the indirect pathway [360].

According to Martins and co-workers [361], it is possible that in DES rats, a part of glycerol was converted into glucose by gluconeogenesis (due to reduced dietary intake in this group), and glucose in excess was used to synthesize glycogen[362], as liver plays a central role in maintaining a constant level of glucose in the bloodstream by balancing the uptake and storage of glucose via glycogenesis, glycogenolysis, and gluconeogenesis [363].

#### **10.2.5 PC enriched-DES GCB extract increased relative kidney weights and altered renal function**

Relative kidney weight was also increased in the DES group (Table 17), that can be related to the increased water consumption resulting in an increase in kidney fluid load, and kidney failure. In accordance, it was described in human that glycerol ingestion consistently resulted in higher values of fluid retention [364]. Nevertheless, the BeGly seems to impair renal function, because urea increased in the DES rats compared to the control rats, even though plasma levels of creatinine and uric acid remained unchanged (Table 17).

#### **10.2.6 PC enriched-DES GCB extract induced plasma but not liver oxidative stress**

Surprisingly, the results showed that oral administration of BeGly extract led to a marked oxidative stress in the plasma but not in the liver. In fact, lipid peroxidation products (TBARS), protein oxidation products (AOPP), and glucose oxidation products (AGE) were highly increased in the DES rats group compared to the control group (Table 18). This result is the most surprising as it occurs with a DES containing a large amount of PC, which are known to provide beneficial effects against oxidative stress [337]. However, a previous study demonstrated rather an *in vitro* opposite effect, as DES extract was shown to enhance the ROS inhibiting efficiency of phenolic compounds in fibroblast cells [286].



The BeGly extract contained two molecules possessing antioxidant and anti-inflammatory activities: CGA and betaine. In rats, CGA possesses a hepatoprotective activity [354]. Betaine administration decreases homocysteine levels and increases S-adenosyl methionine and GSH levels, which have strong antioxidant effects in organisms [358]. The antioxidant activity of betaine is suggested to be related to its effect on the metabolism of sulfur-containing substances in the liver [365]. Although betaine per se does not directly interact with oxidants, GSH has a direct antioxidant activity via ROS scavenging and chelating iron ions that inhibits hydroxyl radical generation. In addition, according to Singh and co-workers [366], glycerol provokes renal oxidative stress and derange renal functions. It increases renal lipid peroxidation and depletes the pools of antioxidant enzymes in rats provoking oxidative stress, which further release the free heme and destabilizes the intracellular heme containing proteins. It is possible that the observed results in this study derive from a balance between the deleterious effect of glycerol, and the antioxidant protective effect of phenolic compounds and betaine in a possible glycerol-induced renal failure in rats.

### **10.3 Conclusions**

The objective of this work was to investigate the preliminary consequences of delivering to rats, over a short period (14 days, 6 mL/day/rat), a GCB BeGly extract rich in phenolic compounds, before conducting a long-term nutritional supplementation study in rats. The results showed some adverse effects of oral administration of a high dose of GCB BeGly extract (betaine:glycerol,1:2 mole ratio + 10 % (v/v) of water) under these conditions. Indeed, the gavage of this extract induced mortality in two rats out of six, dietary restriction, excessive water consumption, weight loss, adipose tissue loss, hepatomegaly, plasma oxidative stress, and increased blood lipid levels. This occurs despite the fact that BeGly extract was rich in CGA, for which beneficial effects have been shown to be numerous. We think that these deleterious effects are possibly due to the large quantity of DES delivered to rats. In this case, the dose of DES have to be adapted in order to reduce its side effects and to find the adequate dose to be used for oral biomolecule delivering. Moreover, if the DES are partially or completely decomposed (suspected phenomenon) into its original constituents in the stomach or while being absorbed, the overall consequence results from the individual positive and/or negative effects of each constituent (glycerol, betaine, PC). In

both cases, a careful selection of DES components is needed, as well as the fine-tuning of molar ratios between components. Therefore, it is clear that a supplementary investigation is necessary to find out and to choose the right doses of DES to be delivered to rats, as well as DES formulation and concentration that are not only efficient and safe for the environment, but for animals or humans too.

## **XI. Isoprostanoids: first identification in coffee and cocoa by- and co-products**

The polyunsaturated fatty acids (PUFAs) are present in plant and animal membranes, where they are involved in the modulating of the membrane fluidity, among others function. Additionally, PUFAs can also be released from membranes and utilized for the enzymatic synthesis of oxidized fatty acids (oxylipins), some of which act as defense signals or antimicrobial lipids [165]. However, a non-enzymatic oxidation pathway of PUFAs, can form a series of mediators close to the prostaglandins [169]. These molecules have been recently described and called phytoprostanes (PhytoPs) and phytofurans (PhytoFs) when are produced in plants from  $\alpha$ -linolenic acid (C18:3 n-3, ALA). PhytoPs and PhytoFs study interest has grown in the latest years, since they could be involved in important biological activities (*cf.* 3.3).

Taking into account the lipid profiles of CP, CH and CPH (Table 14) into account, as well as the relevance of PhytoPs and PhytoFs, their elucidation (not described to date in CP, CH or/and CPH) was carried out. This can contribute to increase the knowledge about the phytomolecular profile of these agro-residues, and therefore increase their potential. In the next section, the results obtained will be described and discussed.

### **11.1 Isoprotanoids recover**

The recovery of PhytoPs and PhytoFs can be influenced by several factors, such as the efficiency of separation during liquid/liquid extraction step, the affinity of compounds for the selected phases or the selectivity of the used SPE cartridges [367]. In addition, the possible PhytoPs and PhytoFs co-elution with compound(s) present in the raw CP, CH, and CPH materials may affect the ionization during LC-MS/MS analysis, therefore creating an inaccuracy in measurements [368]. Thus, for taking into account these crucial factors, EY (extraction yield) and ME (matrix effect) parameters were determined. Those data, allow to better define the method and to deduce by calculation, a value closer to reality, of metabolites rates present in the raw material. In this study, we observed an excellent recovery rate for each matrix, notably for PhytoFs of CP with EY ranging from 95 to 99 % (Table 19). In this matrix, PhytoPs' EY varies between 71 and 96 %. In the case of CH, EYs

observed range from 72 to 97 % and 59 to 86 %, for PhytoPs and PhytoFs, respectively. Finally, for CPH, the values are between 95 and 111 % for PhytoPs and 83 and 96 % for PhytoFs. The EY of few metabolites reaching more than 100 % may correspond to the co-elution of a compound that presents the same MRM (multiple reaction monitoring) transition.

In this study, an important matrix effect (CP, CH, and CPH) on the isoprostanoids estimation was observed (Table 19), either by decreasing (for PhytoPs) or by increasing (for PhytoFs) the content of analyzed compounds. For instance, the matrix effect for the different PhytoPs was ranging from 30.4 % to 62.9 %, 71.7 % to 96.6 %, and 41.4 % to 66.5 %, in CP, CH, and CPH, respectively, whereas it was ranging from 49.7 % to 52.9 %, 46.2 % to 63.1 %, and 33.4 % to 102.2 %, in CP, CH, and CPH, respectively for PhytoFs (Table 19). Thus, one may say that the quantity in PhytoPs is actually underestimated with respect to the actual quantity in the starting materials, whereas it is likely overestimated for PhytoFs.

**Table 19. Percent of matrix effect (ME) and extraction yield (EY) of isoprostanoid derivatives.**

Compound	Concentration (pg/ $\mu$ L)	ME (%) <sup>a</sup>			EY (%) <sup>b</sup>		
		CP	CH	CPH	CP	CH	CPH
16-F <sub>1t</sub> -PhytoP	32	63	50.3	65.4	84.9	78.4	110.7
	256	55.7	49.8	42.8	95.7	79.6	102.2
16- <i>epi</i> -16-F <sub>1t</sub> -PhytoP	32	36.7	52.1	55.8	70.8	71.7	111.5
	256	30.4	50.2	41.4	92	74.1	102.4
9-F <sub>1t</sub> -PhytoP	32	52.9	64.5	65.5	78.2	73.6	103.2
	256	44.6	54	44.1	92.9	76.3	94.6
9- <i>epi</i> -9-F <sub>1t</sub> -PhytoP	32	57.8	64.7	66.5	77.8	77.9	105.9
	256	51.6	62	48.8	92.9	74.1	98.8
Ent-16-B <sub>1t</sub> -PhytoP	32	31.3	50	50	75.5	94.2	103.6
	256	38.6	52.8	42.4	92.2	80.6	98.6
Ent-9-L <sub>1t</sub> -PhytoP	32	39.9	52.1	49.8	74.9	96.6	101.4
	256	48.4	54.2	42.3	94.1	83.1	100.6
Ent-16(RS)-9- <i>epi</i> -ST- $\Delta^{14}$ -10-PhytoF	32	ND	ND	ND	ND	ND	ND
	256	52.9	46.7	33.4	97.2	84.3	87.6
Ent-9(RS)-12- <i>epi</i> -ST- $\Delta^{10}$ -13-PhytoF	32	ND	ND	ND	ND	ND	ND
	256	50.1	46.2	52.3	95.2	75.9	95.6
Ent-16(RS)-13- <i>epi</i> -ST- $\Delta^{14}$ -9-PhytoF	32	ND	ND	ND	ND	ND	ND
	256	49.7	63.1	103.2	97.3	86.1	93.6

<sup>a</sup>Matrix effect (%) and <sup>b</sup>extraction yield (%) measured by microLC-MS/MS for coffee pulp (CP), cacao husk (CH), and cacao pod husk (CPH). ND: not determined

## 11.2 PhytoPs: identification and quantification in CP, CH and CPH

CP, which is obtained after depulping in the coffee processing (

Figure 34), had the highest PhytoPs concentration (Table 20). In traditional primary green coffee wet processing, CP undergoes a natural fermentation, as it is left one night at ambient temperature before being treated or discarded. To avoid an uncontrolled fermentation, which could influence the PhytoPs concentration, the CP used in this study was immediately dried after the depulping step. Total PhytoPs content in CP was 654.45 ng/g dw, being 9-*epi*-9-F<sub>1t</sub>-PhytoP (171.37 ng/g dw) and 9-F<sub>1t</sub>-PhytoP (145.55 ng/g dw) the major-quantified PhytoPs (Table 20). These two compounds have already been reported as olive oil biomarkers of olive tree water stress, as their content in extra virgin olive oil correlated with a water deficit in olives tree plantations [369]. Similarly, an important variation of 9-F<sub>1t</sub>-PhytoP was identified in melons leaves when the plant was exposed to thermal stress [174]. In our case, the presence of these PhytoPs would not be dependent on the hydric stress in coffee tree, as coffee was harvested in a region located at 1300 m over the sea level, with average annual temperature and rainfall of 19.1 °C and 1825 mm [370], corresponding to good Arabica's growing conditions. Indeed, according to Chapagain and Hoekstra (2007), the coffee plant water requirement is about 1277 mm/year [371]. Thus, the PhytoPs amounts extracted from CP could be considered as base values on optimal climate conditions for growing Arabica coffee trees. Assessment of the PhytoPs profiles under different hydric and/or thermal stress growing conditions could allow establishing a predictive model to link these factors with the PhytoPs contents. Thus, PhytoPs could be used as biomarkers of coffee tree hydric and/or thermal stress, as previously demonstrated for olive trees and melon leaves [174].

On the other hand, it would be also interesting to investigate how the different edaphoclimatic conditions influence the qualitative and quantitative profile of PhytoPs and PhytoFs in the coffee plant and each by- and co-product.

Regarding cocoa products, the content in PhytoPs in CH and CPH were, respectively, 470.30 and 179.91 ng/g dw (Table 20). The post-harvest processing could partially explain this value since the CH was obtained after fermentation and roasting step (*cf.* 98). The main compound

found in CH was *ent*-9- $L_{1t}$ -PhytoP (138.82 ng/g dw), followed by *ent*-16- $B_{1t}$ -PhytoP (110.51 ng/g dw) and 9- $F_{1t}$ -PhytoP (76.52 ng/g dw). These compounds have already been identified in different rice flours [175]. The 9- $L_{1t}$ -PhytoP series has been reported in the melon leaves [174], extra virgin olive [369], walnut, macadamia, and pecan nuts as minor components [170]. In addition, it was observed that frying at 130 °C, increased 2.3-fold the concentration of these compounds in macadamia nut [170].

**Table 20. Qualitative and quantitative assessment of Isoprotanoids in coffee pulp (CP), cocoa husk (CH), and pod husk (CPH).**

Compound	Isoprotanoids concentration (ng/g dw) <sup>1</sup>		
	CP	CH	CPH
<i>Ent</i> -16- $F_{1t}$ -PhytoP	123.4 ± 3.1 <sup>a</sup>	41.2 ± 2.7 <sup>a</sup>	19.0 ± 1.6 <sup>a</sup>
16- <i>epi</i> -16- $F_{1t}$ -PhytoP	86.8 ± 2.5 <sup>b</sup>	39.6 ± 2.6 <sup>a</sup>	20.2 ± 1.6 <sup>a</sup>
9- $F_{1t}$ -PhytoP	145.6 ± 6.2 <sup>c</sup>	76.5 ± 3.0 <sup>b,c</sup>	37.0 ± 0.8 <sup>b</sup>
9- <i>epi</i> -9- $F_{1t}$ -PhytoP	171.4 ± 4.8 <sup>d</sup>	67.7 ± 1.4 <sup>c</sup>	37.1 ± 1.1 <sup>b</sup>
<i>Ent</i> -16- $B_{1t}$ -PhytoP	60.7 ± 2.2 <sup>e</sup>	110.5 ± 9.5 <sup>b</sup>	34.0 ± 4.0 <sup>b</sup>
<i>Ent</i> -9- $L_{1t}$ -PhytoP	66.7 ± 3.0 <sup>e</sup>	138.8 ± 12.5 <sup>d</sup>	32.6 ± 3.3 <sup>b</sup>
<i>Ent</i> -16( <i>RS</i> )-9- <i>epi</i> -ST- $\Delta^{14}$ -10-PhytoF	163.2 ± 5.8 <sup>f</sup>	62.8 ± 5.6 <sup>f</sup>	43.5 ± 1.2 <sup>f</sup>
<i>Ent</i> -9( <i>RS</i> )-12- <i>epi</i> -ST- $\Delta^{10}$ -13-PhytoF	183.4 ± 7.1 <sup>g</sup>	89 ± 0.7 <sup>g</sup>	142.7 ± 8.9 <sup>g</sup>
<i>Ent</i> -16( <i>RS</i> )-13- <i>epi</i> -ST- $\Delta^{14}$ -9-PhytoF	196.6 ± 0.6 <sup>g</sup>	126.2 ± 6.6 <sup>h</sup>	207.6 ± 11.7 <sup>h</sup>

<sup>1</sup>Expressed on dry weight and quantified by micro LC-MS/MS. Results are the mean of triplicates (mean values ± standard deviations measured according to Pearson (divided by *n*), values followed by the same letter are not significantly different in Tukey's test using 95 % confidence level). Coffee pulp (CP), cacao husk (CH), cacao pod husk (CPH).

9-*epi*-9- $F_{1t}$ -PhytoP (37.1 ng/g dw), *ent*-16- $B_{1t}$ -PhytoP (34.0 ng/g dw), and *ent*-9- $L_{1t}$ -PhytoP (32.7 ng/g d.b) were the main compounds extracted from CPH. Stereoisomer 9- $F_{1t}$ -PhytoP was found in CP (145.6 ng/g dw), and CPH (37.0 ng/g dw). The series of 9- $F_{1t}$ -PhytoP was predominant in the kernel nuts, up to 7.83 ng/g [170]. In the olive trees, hydric stress has been shown to increase the content of certain  $F_{1t}$ - and  $B_{1t}$ -PhytoP. Accordingly, the authors proposed that 9- $F_{1t}$ -PhytoP, 9-*epi*-9- $F_{1t}$ -PhytoP, and 16- $B_{1t}$ -PhytoP + *ent*-16- $B_{1t}$ -PhytoP could be considered as early biomarkers of water stress [369]. On the other hand, the series  $B_{1t}$ -PhytoP have been identified in high levels in linseed oil [372], and likely involved in the regulation of gene expression in *Arabidopsis thaliana* and tobacco [373].

Several vegetable matrices have been characterized by their content in PhytoPs, *e.g.* rice bran (22.20–118.00 ng/g), and white and brown rice flours (2.55–32.89 ng/g) [175]; gulupa shell (1.3 to 67.6 ng/g dw) [374]; raw green table olive fruits (5.81–9.99 ng/g) [375]; almonds (40.25–238.09 ng/g) [376]; fresh birch pollen (1380.00 ng/g) [24]; and dry leaves of melon plants (1600.00 ng/g dw) [174], the ones with the highest concentration of total PhytoPs.

In our study, CP was the material with the highest content of PhytoPs (659.8 ng/g dw). This result could be ascribed to ALA concentration since CP has the highest content (160.7 mg/g dw), in comparison with CPH and CH (82.8 and 52.1 mg/g dw, respectively) (Figure 35). Moreover, CPH showed the lowest PhytoPs concentration (181.2 ng/g dw), likely because this by-product did not undergo further processing steps, except a moderate drying before being stored.

In conclusion, considering the metabolites' interests (biological activities among other), the total amounts of PhytoPs in CP, CH, and CPH (ranging from 180.0.91 to 654.5 ng/g) are very interesting and could confer new applications for these unexploited agro-residues.

### 11.3 PhytoFs: identification and quantification in CP, CH and CPH

Three different PhytoFs were characterized in our samples (Table 20,

Figure 34). Similar to PhytoPs, those molecules are produced by non-enzymatic oxidation of PUFAs via a parallel pathway after the initial endoperoxide formation and requires further oxygenation [377] (

Figure 34).

*Ent-16(RS)-9-epi-ST- $\Delta^{14}$ -10-PhytoF*, *ent-9(RS)-12-epi-ST- $\Delta^{10}$ -13-PhytoF*, and *ent-16(RS)-13-epi-ST- $\Delta^{14}$ -9-PhytoF* were identified in CP, CH, and CPH. As shown in Table 20, the total PhytoFs content in ng/g dw was: 543.1 (CP), 393.7 (CPH), and 278.0 (CH). These values are higher than the ones found in brown rice flours (1.59–27.74 ng/g dw) [175], but similar concentration range was measured in the melon leaves (130–4400 ng/g dw). The epimeric mixture of *ent-16(RS)-13-epi-ST- $\Delta^{14}$ -9-PhytoF* predominates in the three samples. These compounds were identified and quantified for the first time in different nuts and seeds in

concentrations up to 10 and 16.06 ng/g in the walnut [167] and brown rice flour [175], respectively. The second main compound extracted from CP (183.35 ng/g dw), CH (89 ng/g dw), and CPH (142.66 ng/g dw) was *ent*-9(*RS*)-12-*epi*-ST- $\Delta^{10}$ -13-PhytoF, already detected in the melon leaves [174] and in brown rice bran (4.62 ng/g dw) [175]. The less predominant PhytoF in CP, CH, and CPH was *ent*-16(*RS*)-9-*epi*-ST- $\Delta^{14}$ -10-PhytoF, present at 163.2, 62.8, and 43.5 ng/g dw, respectively. This compound was identified in different rice cultivars and the highest concentrations detected were 1.25, 4.21, and 12.88 ng/g dw for white flour, brown flour, and bran respectively [175].

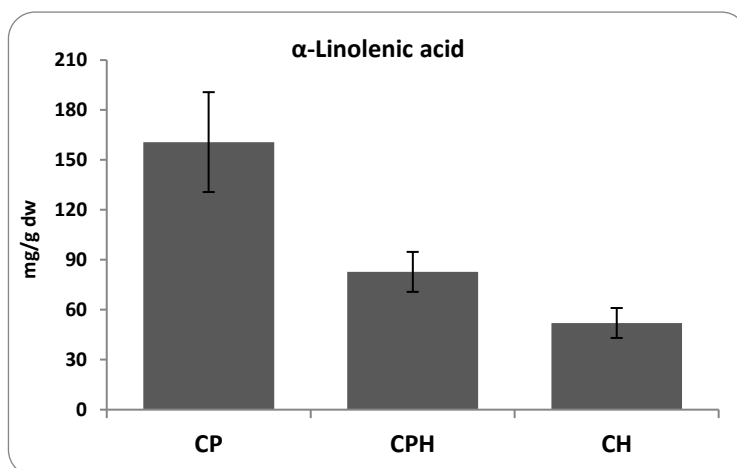
The highest concentration in PhytoFs was detected in CP, followed by CPH, and CH. Interestingly, the same order in the concentration of ALA was observed in our investigated samples (160.73, 82.76, and 52.07 mg/g dw) in CP, CPH, and CH, respectively) (Figure 35). It would be interesting to quantify these compounds on immediately fresh harvested samples of CP, CH, and CPH, and to follow the influence of each primary and/or secondary processing step on the content of ALA, PhytoFs, and PhytoPs, in order to demonstrate if a correlation exists. Some authors working on nuts, seeds, and an animal tissue demonstrated that the PhytoFs concentration was not ALA-dependent, but probably oxidative stress condition-dependent [376]. Anyhow, the quantification of PhytoPs and PhytoFs could provide a unique sensitive indicator of oxidant injury (crop conditions as well as postharvest factors).



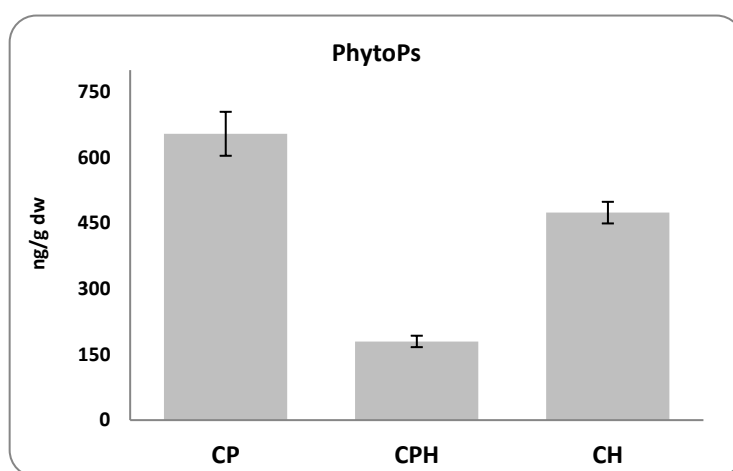
Chemical structure	Name
<b>Phytosteranes</b>	
	16-F <sub>1t</sub> -PhytoP
	16- <i>epi</i> -16-F <sub>1t</sub> -PhytoP
	9-F <sub>1t</sub> -PhytoP
	9- <i>epi</i> -9-F <sub>1t</sub> -PhytoP
	<i>Ent</i> -16-B <sub>1t</sub> -PhytoP
	<i>Ent</i> -9-L <sub>1t</sub> -PhytoP
<b>Phytofurans</b>	
	<i>Ent</i> -16( <i>RS</i> )-9- <i>epi</i> -ST-Δ <sup>14</sup> -10-PhytoF
	<i>Ent</i> -9( <i>RS</i> )-12- <i>epi</i> -ST-Δ <sup>10</sup> -13-PhytoF
	<i>Ent</i> -16( <i>RS</i> )-13- <i>epi</i> -ST-Δ <sup>14</sup> -9-PhytoF

Figure 34. Chemical structures of phytosteranes and phytofurans present in coffee pulp, cocoa husk, and cocoa pod husk

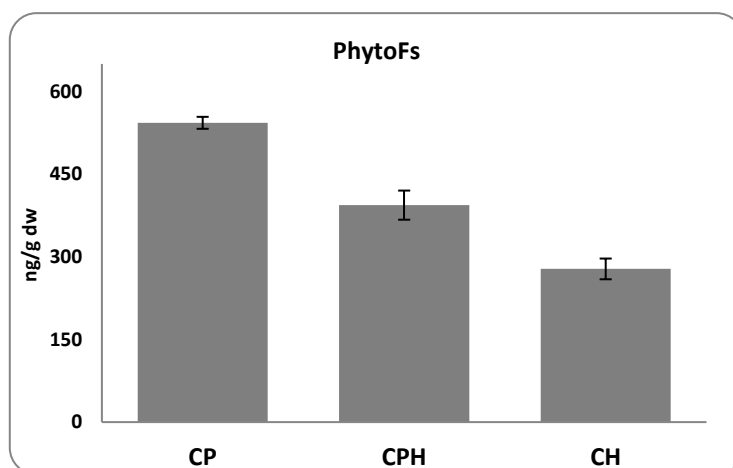
a)



b)



c)



**Figure 35. Comparative assessment between the concentrations of α-linolenic acid, phytosterols (PhytoPs) and, phytofuran (PhytoFs) in coffee pulp (CP), cocoa husk (CH) and pod husk (CPH)**

The values of each column are the mean of triplicates (mean values  $\pm$  standard deviations).

## 11.4 Conclusions

The coffee and cocoa co- and by-products represent a major problem for waste disposal, leading diseases propagation or environmental issues, both in the producer countries as well as in the processing companies. To the best of our knowledge, this is the first time that a qualitative and quantitative evaluation of PhytoPs and PhytoFs in CP, CH, and CPH was performed. The concentrations found in coffee and cocoa by- and co-products suggest that these residues are a valuable source of PhytoPs and PhytoFs. Thus, this research contributed to broaden the current portfolio of interesting biomolecules derived from the coffee and cocoa by- and co-products. The use of such residues as a source of bioactive compounds could provide an economical alternative to the farmers and smallholders in the producing countries. Indeed, taking into account the wide range of already demonstrated biological activities (anti-inflammatory, apoptosis, immune function regulation, neuroprotection [164]) and the still remaining prospects, such compounds may represent an interesting source of development. PhytoPs and PhytoFs rich extracts could be obtained from these residues and used in the functional food industry by enriching food or to be used as nutritional supplements. In addition, the quantification of PhytoPs and PhytoFs in coffee and cocoa could represent a unique sensitive indicator of oxidant injury of plants (crop conditions as well as postharvest factors).

Although additional studies are still necessary, this work open new insights and prospects, not only to the add-value to these agro-wastes but also it paves the way on the interest of enlarging this study to green coffee bean and cocoa nibs and their derived products.

## **XII. First identification and quantification of thiol precursors in coffee and cocoa by- and co-products**

The aroma is one of the most important characteristics related with the food quality. This feature is generally related to the volatile compounds. Among them, those containing sulfur are some of the most significant in the development of flavor and odor in a large number of food products [378]. In particular the volatile thiols are keys to ensure the coffee and chocolate quality. In many cases, these compounds are not present in raw material, but they are released during their fermentation from odorless precursors [25], as it happens in the wine production [26]. Indeed, varietal thiols (*cf.* 3.4) confer olfactive notes that are particularly appreciated by the consumers of products such as wine and beer, where they have been identified [25] [379]. Thus, their identification could help to improve the final quality of the products.

In an attempt to valorize other interesting and potential added value biomolecules present in CP, CH, and CPH, we went to investigate the presence of thiol precursors in our samples. This work was carried out in collaboration with Nyséos (Montpellier), a company specialized in the analysis and consulting on wine and beverage aroma.

### **12.1 Thiol precursor identification**

The 3-mercapto(hexan-1-ol)-L-cysteine (3MH-Cys), 3-mercapto(hexan-1-ol)-glutathione (3MH-Glu), 4-mercapto-4-methylpentan-2-one-L-cysteine (4MMP-Cys), and 4-mercapto-4-methylpentan-2-one-glutathione (4MMP-Glu) were synthesized according to published method [301], and fully characterized by <sup>1</sup>H NMR using trimethylsilylpropionic acid as internal standard. Additionally, the reference solutions of the four thiol precursors were infused in the UPLC–MS/MS, in order to optimize their fragmentation.

The analytical parameters, including sensitivity, accuracy, repeatability, and precision were determined to validate the methodology for thiol precursor's identification in our samples. Limits of detection (LOD) and quantification (LOQ) were measured according to the International Conference on Harmonization recommendations, using a signal to noise ratio

equal to 3 and 10 respectively. The LOD was between 0.01 and 0.18 µg/L (0.04 and 0.61 µg/L concerning LOQ) for the four precursors (Table 21).

**Table 21. Validation of the methodology**

Parameters	3MH-Cys	3MH-Glu
Repeatability (%)	3	4
Accuracy (%)	100	97
LOD <sup>1</sup> (µg/L)	0.16	0.24
LOQ <sup>2</sup> (µg/L)	0.53	0.79

<sup>1</sup> Limit of detection. <sup>2</sup>Limit of quantification

The identification of these compounds was carried out by taking into consideration their HPLC chromatogram profiles and retention time ( $T_R$ ), with those obtained from ethanolic solutions of CP, CH, and CPH. The standards of each analyzed molecule were added in each extract to confirm their presence. The observed  $T_R$  of each identified compound in CP and CPH matched with the respectively added standards. The UPLC–MS/MS spectra of standards were recorded using the molecular ions of the different target compounds.

The fragmentation of 3MH-Cys showed typical peaks at  $m/z$  222, 205 and 83  $[M+H]^+$  corresponding to the loss of ammonia  $[M+H-NH_3]^+$ , the loss of cysteine ( $[M+H-Cys]^+$ ) and the loss of water from the fragment at  $m/z$  101 ( $[M+H-Cys-H_2O]^+$ ). The fragmentation of 3MH-Glu consisted in typical ions at  $m/z$  408  $[M+H]^+$ ,  $m/z$  279 (loss of the dehydro-c-glutamyl moiety (glu)  $[M+H-glu]^+$ ) and  $m/z$  262  $[M+H-glu-NH_3]^+$  (Table 22,

Figure 36). The observed fragmentation of 3MH-Cys and 3MH-Glu have been reported previously in commercial tannins, grape must, hops and wine [301][380][27][379]. Accordingly, 3MH-Cys and 3MH-Glu thiol precursors were clearly identified.

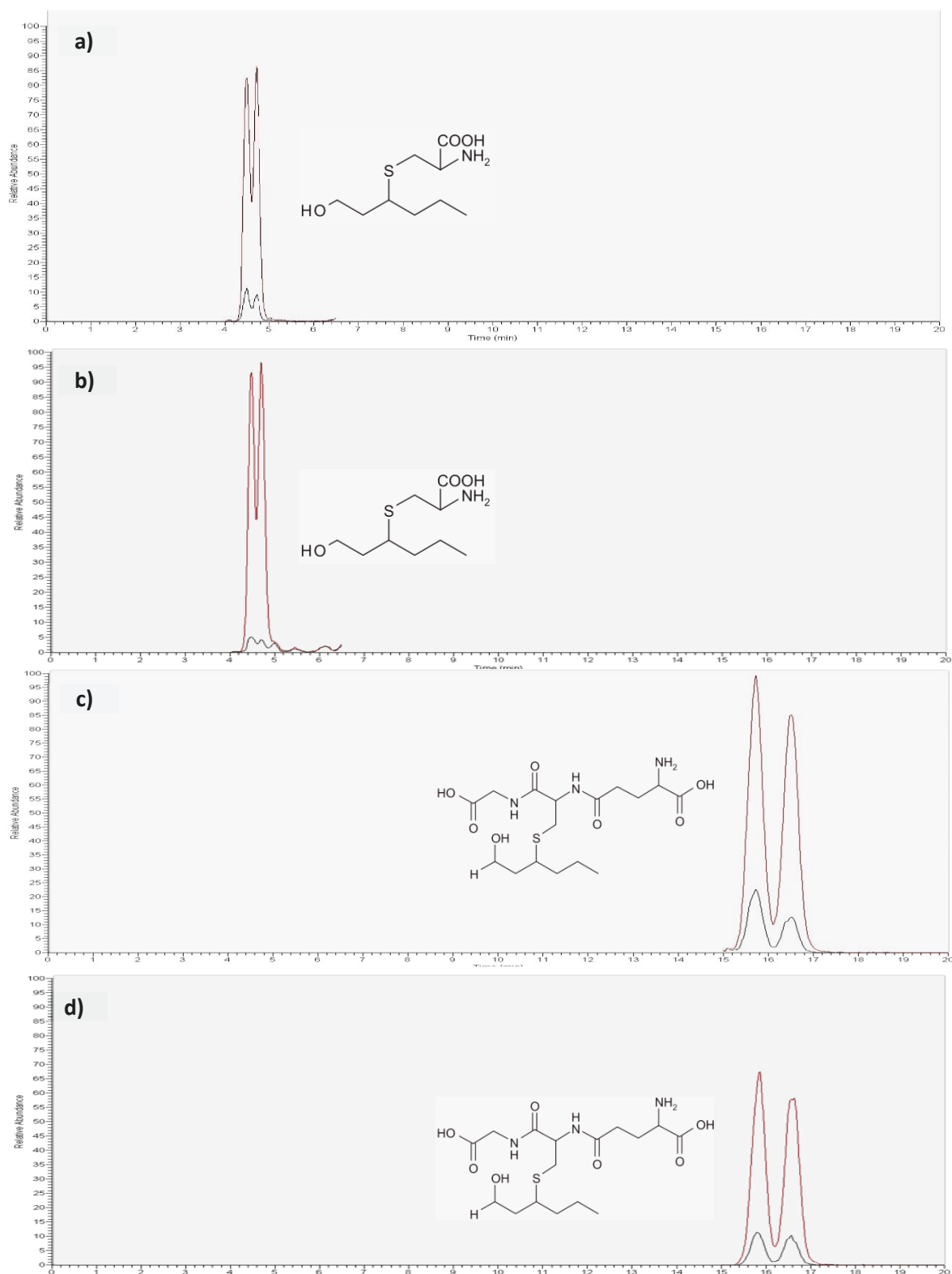
Table 22. MS/MS parameters<sup>1</sup>

Compound	Retention time (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)
3MH-Cys	4.5	222.1	83.1	17
			205.0	9
			162.0	22
3MH-Glu	15.8	408.2	262.1	16
			279.1	12

<sup>1</sup>Quantifier ion in blond (relative intensities) obtained by infusing solution of thiol precursor standards at 5 mg/L (mixture composed by 50 % ACN and 50 % water) into mass spectrometer in manual tune mode (triple quadrupole, Thermo TSQ Quantiva).

The 3-mercapto-hexan-1-ol (3MH) is a powerful odoriferous thiol that was detected for the first time in Sauvignon Blanc grape juice. Nowadays, it is one of the most relevant varietal thiol, which is responsible for grapefruit and passion fruit aroma [25]. The 3MH-Cys and 3MH-Glu have been already identified in the must, skin, leaves, and seeds of some grape varieties such as Sauvignon Blanc, Chardonnay, Merlot, and Koschu, among others [26]. More recently, 3MH-Cys and 3MH-Glu were also identified in eight hop varieties, a key ingredient in the beer fabrication [379].

Unlike CP and CPH ethanolic extracts, no thiol precursors were identified in the CH extract. It is possible that, if present in the raw CH, thiol precursors were released during fermentation via the action of a yeast  $\beta$ -lyase [28]. CH are separated from cocoa beans after their fermentation and drying, therefore it is possible that thiol precursors degrade during these processes (Figure 4 *cf.* 2.2).



**Figure 36. NanoLC-MS/MS chromatograms of 3MH-Cys in a) CP and b) CPH extracts, and of 3MH-Glu in c) CP and d) CPH. Black line: 3MH-Cys present in samples; red line: addition of 3MH-Cys or 3MH-Glu standards to samples**

In this study the free thiol (3MH) was not examined, however Tominaga and co-workers [381] proved that its release was proportional to the concentration of its precursor (3MH-Cys) after alcoholic fermentation of Sauvignon blanc must. Yet, some Oenology studies have shown that the final concentration of the polyfunctional mercaptans in wine depends on many factors, because the results were not only related to the precursor levels in the must and yeast, but also to the prefermentative process steps. Additionally it has been suggested that the final concentration of 3MH or 3MHA in Sauvignon Blanc may be derived from pathways that do not use glutathionylated or cysteinylated precursors [382].

## 12.2 Thiol precursor quantification

The quantification of glutathionylated and cysteinylated precursors was carried out in CP, CH, and CPH (Table 23).

The concentration of 3MH-Cys was lower (8.7 and 0.9  $\mu\text{g}/100\text{ g dw}$ ) than 3MH-Glu (30.43 and 25.32  $\mu\text{g}/100\text{ g dw}$ ) both in CP and CPH, respectively, whereas these compounds were not detected in CH.

**Table 23. Concentration of cysteinylated and glutathionylated precursors in CP and CPH**

Sample	Thiols precursors ( $\mu\text{g}/100\text{g dw}$ ) <sup>1</sup>	
	3MH-Cys <sup>2</sup>	3MH-Glu <sup>3</sup>
CP	8.7	30.43
CH	n.d.	n.d.
CPH	0.9	25.32

<sup>1</sup>Results expressed in dry weight (dw).; n.d.= no detected; <sup>2</sup> 3MH-Cys: 3-mercapto(hexan-1-ol)-L-cysteine. <sup>3</sup> 3MH-Glu: 3-mercapto (hexan-1-ol)-glutathione

The 3MH-Cys and 3MH-Glu quantification reported in the literature is showed in Table 24. The quantity of these compounds ranged according to the vegetal material. For example, Barbe Rouge hops showed high concentration of 3MH-Glu of until 1.9 mg/ 100 g; whereas in Koshi grape seed was reported only a few  $\mu\text{g}/100\text{ g}$  (Table 24).



In our samples, we found higher concentration of 3MH-Glu than 3MH-Cys, similar to what Roland and co-workers observed when they evaluated these thiol precursors in several hop varieties [379]. According to another study made by Larcher and co-workers [380], this variation can be related to the biological origin of the samples, as well as the different formation and thiol precursors degradation pathways.

The 3MH-Cys concentrations found in CP (8.7  $\mu\text{g}/100\text{ g dw}$ ) and CPH (0.9  $\mu\text{g}/100\text{ g dw}$ ) were within the range of those observed in samples of extracted tannins from tree fruits (0.1 – 13.5  $\mu\text{g}/100\text{ g}$ ) [380]. Regarding 3MH-Glu, the concentration found in CP and CPH was within those reported in Koshu grape leaves (0.4 – 122  $\mu\text{g}/100\text{ g}$ ), but superior to Koshu grape skin or seeds [383].

However, despite the low concentration found in CP and CPH, these precursors could have a large sensorial importance due to the low perception threshold of molecules that they release. For example, the 3MH-Cys perception threshold reported in the juice of grapefruit was between 0.004 and 0.06  $\mu\text{g}/\text{L}$  [384].

**Table 24. Concentration of cysteinylated and glutathionylated precursors reported in different samples**

Sample	3MH-Cys ( $\mu\text{g}/100\text{ g}$ )	3MH-Glu ( $\mu\text{g}/100\text{ g}$ )	Reference
Chinook hops (pellets)	53.5	447.9	
Saaz hops (cones)	17.9	511.2	
Barbe Rouge hops (cones)	114	128.3	
Cascade hops (pellets)	42.9	1905.6	[379]
Mistral hops (cones)	22.5	283.8	
Aramis Alsace (cones)	56.2	236.3	
Strisselspat hops (pellets)	47.9	914.1	
Mandarina hops (cones)	12.9	211.9	
Cabernet Sauvignon grape	0.22	ND <sup>1</sup>	[183]
Merlot grape skin	2.5	ND	
Merlot grape must	0.16 – 0.35	ND	
Koshu grape must	0.22 – 1.1	0.4 – 4	
Koshu grape skin	0.33 – 1.68	0.4 – 6.11	
Koshu grape leaves	60.78	0.4 – 122	[383]
Koshu grape stem	0.33	0.4 – 0.8	
Koshu grape seeds	0.22	0.4 – 0.6	
Acacia (tannins)	0.7	5.3	
Tree fruits (tannins)	0.1 - 13.5	0.6 – 10.5	
Tara tree (tannins)	n.d.	17	[380]
Grape skin (tannins)	20.4 – 42.5	59 – 64	
Grape seed (tannins)	0.6 – 617	0.8 – 3360	
French Oak (tanins)	0.1 – 0.3	0.4 – 0.5	

<sup>1</sup> ND: Not detected

### 12.3 Interest and potential of thiol precursors in CP and CPH

Nowadays, the food-processing operations, from premature harvesting to extended storage and physical treatments, may cause a loss of aroma (volatile flavor), so in many cases the subsequent supplementation is necessary. Nearly 80 % of the flavors and fragrances used worldwide are produced chemically. However, since 1990 there is a trend growing forward to increase the use of natural and healthy products [385]. In this context, the identification of 3MH-Cys and 3MH-Glu in agroresidues from coffee and cocoa opens a new perspective in the potential of these sources. These thiols precursors are involved in the formation of the called varietal thiols, which have a great importance in the wine field. However, they cannot

be used to enhance of wine aroma because the specifications speculated in the European wine regulation [386]. Fermented food and beverages provide some 20-40 % of the food supply in the world, especially distributed in dairy food products, beverages, and cereals [387]. As already mentioned, desirable organoleptic features can be lost during fermentation processes, whereas this is in this step that the thiol precursors are released. Thus, this process could offer an opportunity to use the thiol precursors identified in CP and CPH for supplementation of certain food products, *e.g.* some beverages. Indeed, Larcher and co-workers hypothesized that about 3 % of the precursor's molar content could be converted to the free forms after fermentation [380].

Before reaching that stage, it would be interesting to analyze the effect of the different factors such as the biological origins, the crop conditions, and the pretreatment steps over the thiol precursor contents. Besides, it is necessary to keep in mind that other factors are involved in the release of 3MH, such as the fermentation conditions, formation pathways, and the involved yeasts, among others [388][26].

## 12.4 Conclusions

To the best of our knowledge, this is the first time that 3MH-Cys and 3MH-Glu are identified and quantified in coffee and cocoa residues. This study, carried out using analytical methods developed for the thiol precursors in grapes, allowed to highlight thiol precursors in CP and CPH.

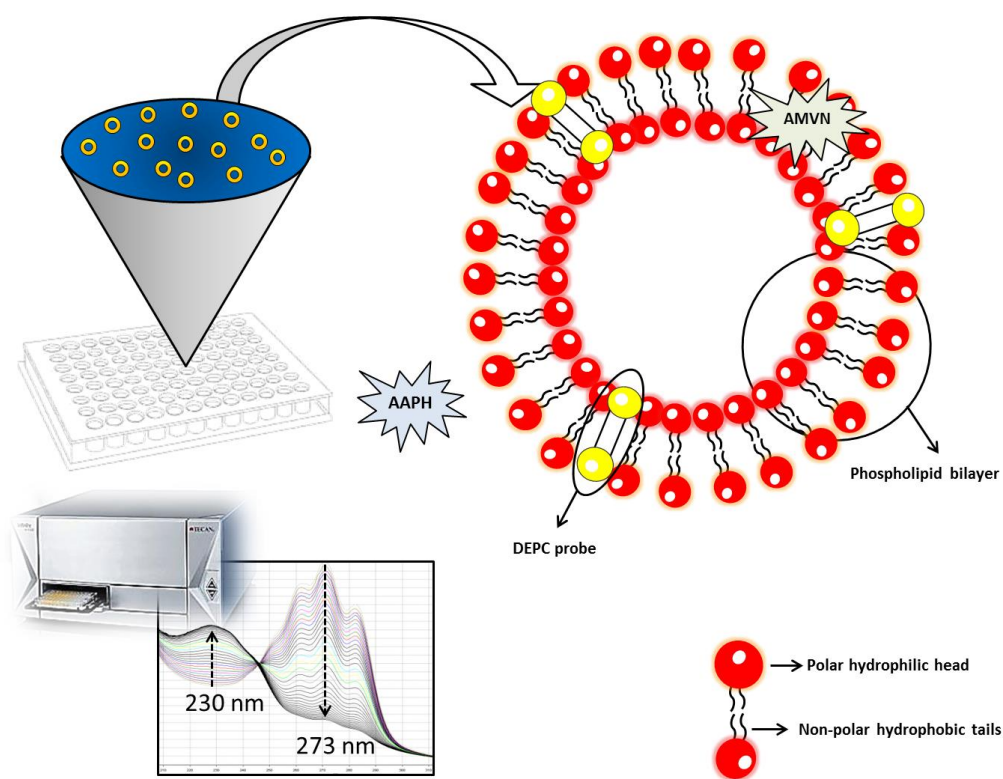
These findings open interesting perspectives for the potential applications of CP and CPH, in particular on fermented foods and beverages, where they can be used to improve certain fruit aromas. A deeper and wider characterization of thiol precursors in our samples would be of a great interest. For example, assessment and comparison of thiol precursors according to different varieties, origins, culture conditions, or postharvest processes, among others, would be interesting approach. Anyhow, this preliminary study provides a new option in diversifying the uses of CP and CPH as sources of interesting biomolecules.

### **XIII. Evaluation of antioxidant capacity of coffee and cocoa co- and by-products by the Vesicle Conjugated Autoxidizable Triene (VesiCAT) assay**

Liposomes are sphere-shaped phospholipids vesicles which stabilize an aqueous core from the external medium [389]. They consist of one (or more) lipid bilayers surrounding by aqueous environment, where the polar head groups of phospholipids are oriented toward aqueous phases. Accordingly, the liposomes can be classified as multilamellar vesicles (MLV), and unilamellar vesicles (ULV). MLV contain several bilayers surrounding each other, whereas unilamellar liposomes are made of a single bilayer. These latter can be distinguished as small unilamellar vesicles (SUV; diameter<100 nm) or large unilamellar vesicles (LUV; diameter> 100 nm) [11].

Liposomes have been widely used as bioactive delivery vehicles or membrane models to mimic complex biological systems. More recently the food and pharmaceutical industries have extensively studied the use of liposome encapsulation to produce delivery systems that can entrap unstable compounds (for example, antimicrobials, antioxidants, flavors and bioactive elements) and shield their functionality [390]. Despite their relative simplicity, liposomes exhibit drawbacks owing to their relatively poor physical or chemical stability [391], as well as the chemical degradation mainly via oxidation of their fatty acid constitutive moieties. Generally, the strategies to protect the liposomes involve the use of exogenous antioxidants [390], that can act by various mechanisms depending of their chemical reactivity and the interactions and localization in liposome systems. In this context, the liposomes represent an interesting tool to investigate the antioxidant potential of a compound as well as drug-membrane interaction. Although many probes are widely used to assess antioxidant capacity in liposomal systems, the obtained results are questionable since these probes are not naturally present in living systems. In addition, most of the synthetic fluorescent probes used to evaluate the antioxidant capacity (*e.g.*, BODIPY [392] and its derivatives HDAF (hexadecanoylamino fluorescein) [393], or DPH-PA (diphenylhexatriene propionic acid) [394] have different polarities and charge properties, as a result, different membrane affinity, mobility, location, and penetration depth that can lead to misinterpretation of obtained results.

Recently, the Vesicle Conjugated Autoxidizable Triene (VesiCAT) assay was developed in our team to address an alternative and more efficient strategy to measure membrane oxidation of liposomes, and therefore antioxidant capacity of molecules. This method uses a new synthesized, di-eleostearic phospholipid probe (DEPC), as new marker to assess oxidation once incorporated into model membrane systems (liposomes). Indeed, the conjugated trienes presented on the fatty acid tails of the phospholipid (eleostearic acid) exhibit very high oxidative sensitivity and strong absorption UV, therefore allowing a direct oxidation assessment at 273 nm through UV-Vis spectrum (Figure 37). The method was implemented with micro-plate reader to allow several experiments in a row. The oxidation is induced by a radical generator, since the natural oxidation of the DEPC in a liposomal system is much slower (~days) than artificially induced oxidation (~hours). In this study two different radical generators, namely AAPH (hydrophilic) and AMVN (lipophilic)) were used with the aim at comparing water-soluble and interface membrane radical initiation [11]. ULV was targeted, since they maintain their structural integrity when exposed to reactive oxygen species (ROS) generated outside the bilayer (in aqueous media) [395].



**Figure 37. VesiCAT assay**

### 13.1 VesiCAT with water radical initiation (AAPH)

To express the results, we used trolox as reference and equivalent, since it has been already used in several antioxidant tests, especially in the one (CAT assay [396]) that inspired the development of the VesiCAT assay. The trolox concentrations used in the suspension were 0.5, 1 and 2  $\mu\text{g/mL}$ . The antioxidant capacity of the molecules, extracts, as well as the reference (trolox) was assessed by observing the oxidation delay of the DEPC probe, by means of the in the absorbance decay at 273 nm (Figure 37). The calculation of the area under the curve (AUC) was used to represent the antioxidant capacity, since this method exploits both inhibition time and degree of oxidation, thus provides global information about the oxidation [11]. For AAPH the net AUC protection of the reference ( $\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{Control}}$ ), as well as the samples ( $\text{AUC}_{\text{samples}} - \text{AUC}_{\text{Control}}$ ) versus concentration, had a good linear relationship ( $R^2 = 0.99$ ). All calculation was carried out in mass concentration ( $\mu\text{g/mL}$ ), due to the molecular complexity of the vegetal extracts.

As it was showed in section 9.2, CP, CH, and CPH contain PC, and 5-CQA was extracted and identified from CP extracts. The antioxidant capacity of 5-CQA has been largely known, and it has been evaluated by several methods in monophasic (DPPH, ORAC, and ABTS)[397][398] and biphasic (CAT) systems [396]. Very recently, the antioxidant capacity of 5-CQA as was evaluated in a liposomal system, thought VesiCAT assay [11].

In this context, it is interesting to assess the antioxidant capacity of our extracts using the VesiCAT assay, in order to study their behavior in a liposomal system.

The VesiCAT assay in aqueous generated oxidant (AAPH) exposed the following antioxidant ranking  $\text{CGA} > \text{CH} > \text{CP} > \text{CPH}$  (Table 25). As caffeine and theobromine, present in our extracts, have their maximum absorbance at 273 nm (as it was shown in Figure 27 *cf.* 9.2). Anyhow, the standard molecules were tested in VesiCAT assay in order to assess their activity or any perturbation in assay. As expected, they do not exhibit antioxidant activities. According to the literature, studies have reported that these alkaloids have low or negligible antioxidant activities [399]. However, other works showed that caffeine and theobromine have moderate antioxidant performances, measured with ABTS and ORAC assays [400], but their effect seems to be concentration dependent [401]. On the other hand, the curves obtained

from caffeine and theobromine analyses, especially the baseline levels, were slightly different to that of the control (Figure 38). This is due to the use of relatively high concentrations for such assays (because of their low antioxidant capacities) in addition to absorbance at 273 nm (wavelength of measurement).

CGA showed the best antioxidant activity, which is not a surprise since this compound was documented to have antioxidant activity [400][402]. Recently, Durand and co-workers [11] found a VesiCAT value of  $2.41 \pm 0.01$  for CGA, which is similar to the found value in this study ( $2.41 \pm 0.07$ ) if taking the molar concentration for calculation.

Regarding the extracts, surprisingly, CH showed the best antioxidant activity; although (*cf.* 9.2) caffeine and theobromine (no detected antioxidant capacity) were described as the main compounds. Thus, the presence of unknown molecules ( $0.05 \pm 0.004$  g GAE/100 g dw) in CH extract (*cf.* 9.4) is strongly suspected to contribute to the antioxidant activity. When natural products are isolated from natural material, they retain some of the original metabolomic complexity of their biogenetic source. This concept known as *residual complexity* can exert a significant influence on the biological assessments [403][404]. For example, a study compared the antioxidant activity of crude caffeine (produced in the decaffeination of coffee) and pure caffeine. The results showed that the antioxidant activity of crude caffeine was 24 fold higher than the pure caffeine. A biochemical study revealed the presence of minor compounds (e.g. 1 % of PC) in crude caffeine, which according the authors could explain the observed activity in crude caffeine. Additionally, the same study showed that antioxidant activity of caffeine crude was better in a hydrophilic environmental [399].

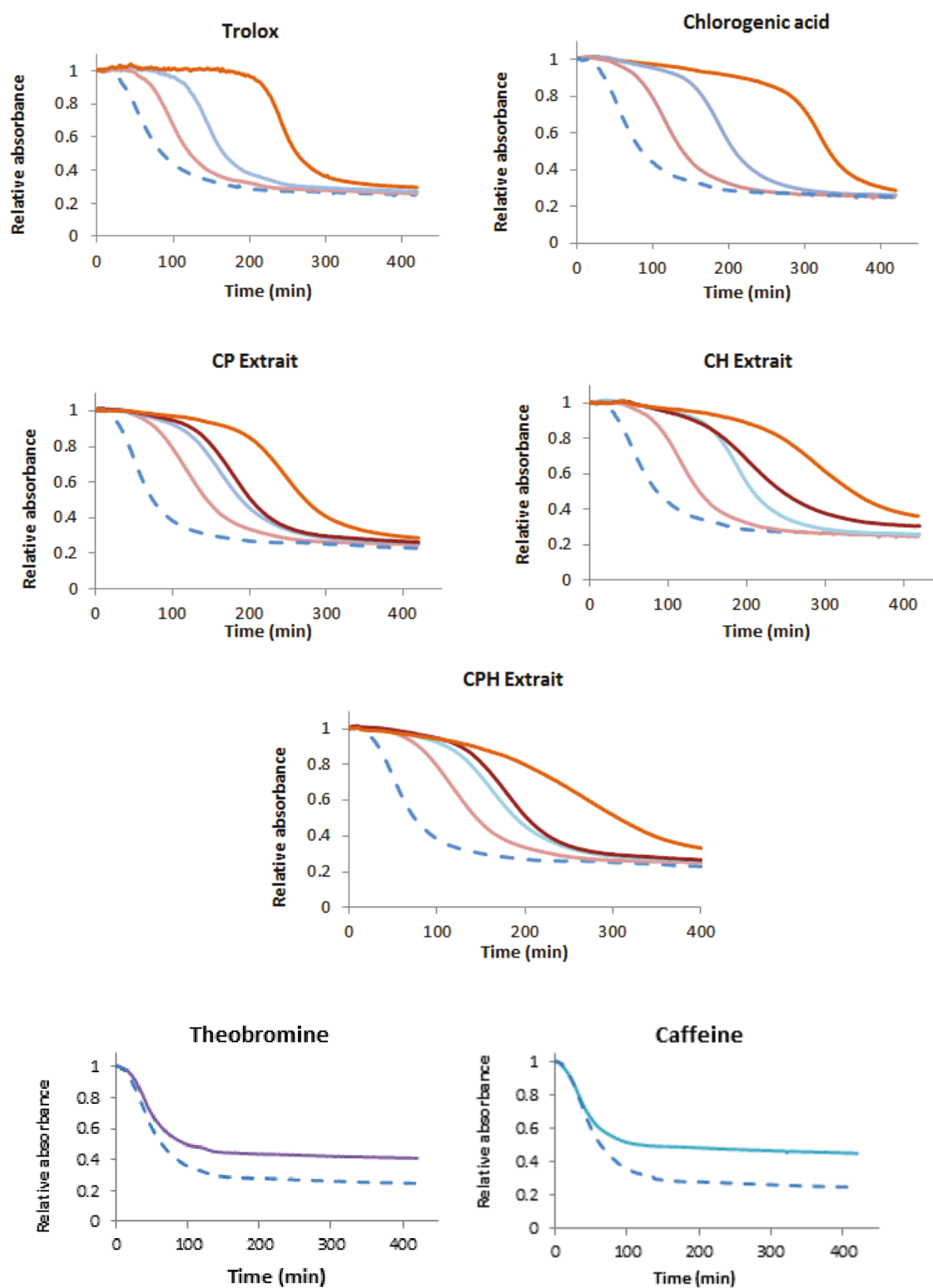


Figure 38. Kinetics of relative absorbance decay in the absence and presence of trolox (0.5 - 2  $\mu\text{g/mL}$ ), 5-CQA (0.35-1.4  $\mu\text{g/mL}$ ), CP and CH (10-30  $\mu\text{g/mL}$ ), CPH (20-100  $\mu\text{g/mL}$ ), theobromine (9  $\mu\text{g/mL}$ ) and caffeine (19.41  $\mu\text{g/mL}$ ). Without (---) and with (—) the molecule or extract. The liposome suspension was made with DEPC/DLPC (1:10, w:w ratio) in PBS, pH 7.2, at  $34.5 \pm 0.5^\circ\text{C}$



Regarding the antioxidant activity observed with CP ( $0.065 \pm 0.001$  TE), it can be attributed to the presence of CGA. This compound is well-known for its properties to reduce free radicals, and its hydrophilic nature gives it the ability to react in aqueous phase [405][354][406].

**Table 25. VesiCAT value (TE <sup>1</sup>)**

Sample	AAPH	AMVN
Caffeine	ND	ND
Theobromine	ND	ND
CGA	$1.711 \pm 0.068$	$0.057 \pm 0.004$
CP	$0.065 \pm 0.001$	$0.005 \pm 0.000$
CH	$0.079 \pm 0.001$	$0.013 \pm 0.001$
CPH	$0.019 \pm 0.001$	$0.005 \pm 0.000$

<sup>1</sup> Results expressed as mean (n=3) of Trolox equivalents (TE)  $\pm$  standard deviation. ND not detected.

### 13.2 VesiCAT with membrane radical initiation (AMVN)

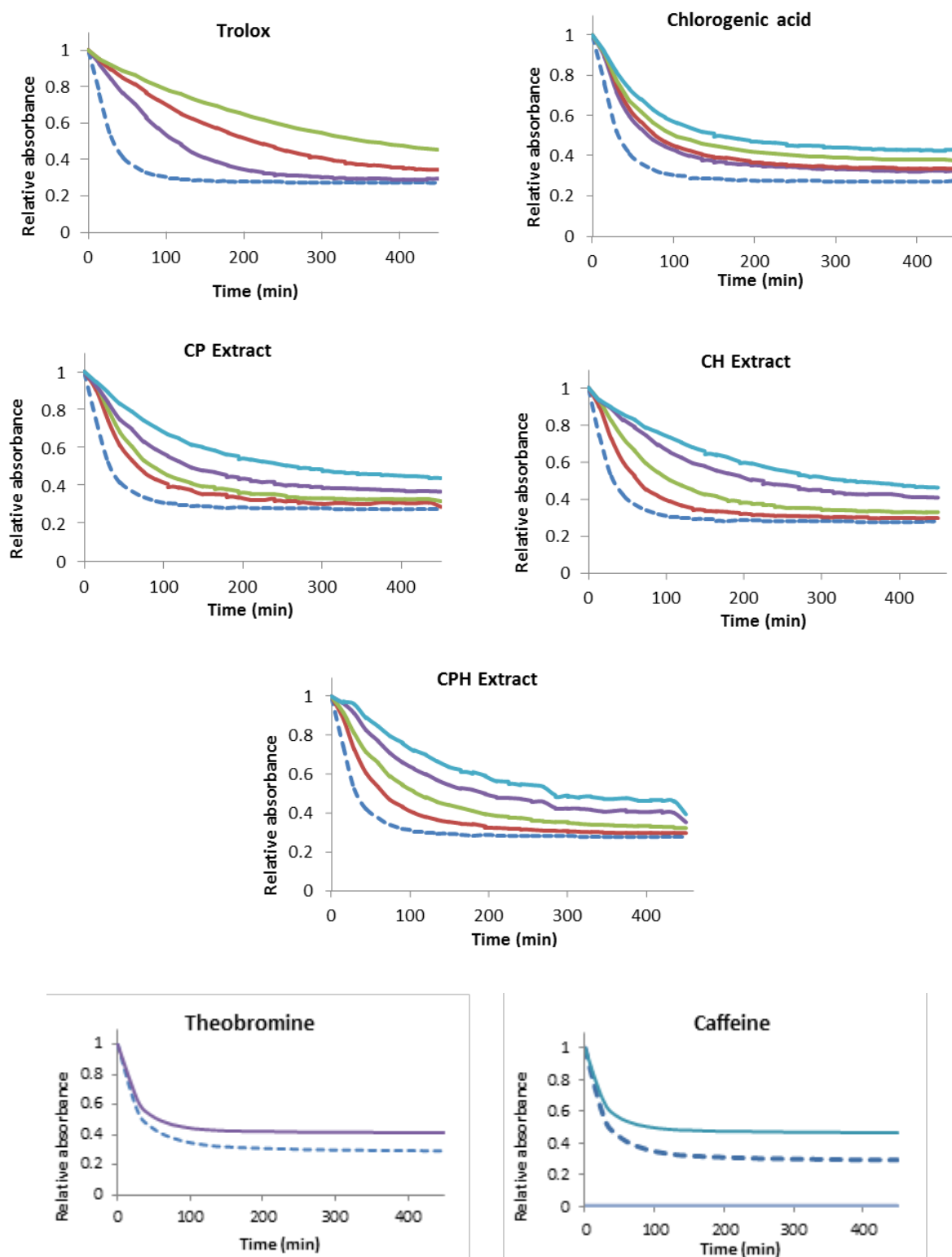
VesiCAT using liposoluble AMVN was carried out using the same methodology, except that the membrane-based initiators must be added before vesicles formation. Thus, the test was developed with DLPC membranes (lower liquid-crystalline phase transition temperature,  $\sim 20^\circ\text{C}$ ), thus preventing the radical production during liposome preparation. In this assay the kinetic behavior was slower; possibly due to more complex reactivity between antioxidants and oxidant species, that is why the return to baseline was different and slower [11]. Thus, as it was aforementioned in section 7.6.4 the  $\text{AUC}_{80}$  corresponding to the area under the curve obtained after 80 % of DEPC oxidation was calculated. The net protection  $\text{AUC}_{80}$  of the reference ( $\text{AUC}_{80\text{Trolox}} - \text{AUC}_{80\text{Control}}$ ) versus concentration, allowed for a good linear relationship ( $R^2 = 0.98$ ).

As previously described, trolox was also used as reference at concentrations ranging from 0.37 to 1.25  $\mu\text{g/mL}$  (1.5 to 5  $\mu\text{M}$ ). Additionally, theobromine and caffeine standards also were tested in this system, and once again they did not show antioxidant activity. Nevertheless, as already mentioned with AAPH assay, these molecules slightly increased the baselines, mostly due to their absorbance at the wavelength at which the assay was performed, in combination with their high concentrations (Figure 38). CP, CH, CPH and CGA

were able to delay the liposome oxidation (Figure 39). According to the results; CGA was the most effective followed by CH, while CP and CPH showed the same performance (Table 25).

Although CGA showed the best value, it revealed a poor antioxidant efficacy. This coincide with the resulted found by Durand and co-workers [11], where compared CGA in molar concentration ( $0.084 \pm 0.033$ ) to other phenolic compounds, such as rosmarinic acid ( $0.549 \pm 0.01$ ) or gallic acid ( $0.107 \pm 0.02$ ). By changing the region where oxidant are generated and spread, led to distinct CGA antioxidant performance. Indeed, CGA is an efficient antioxidant with aqueous generated oxidants, but poor antioxidant while oxidant species are generated in membrane domain. One reason could be a slow mobility combined, or not, with a low distribution in the interfacial domain [11].

Just like AAPH-induced assays, CH was the best antioxidant among the tested extracts. Usually, the antioxidant activity of cocoa co-products has been attributed to the presence of PC. Indeed, Martínez and co-workers [121], found a strong correlation between TPC and CH antioxidant capacity assessed by DPPH and ABTS assays. In this study, the main identified compounds in CH extract were theobromine and caffeine, which did not show antioxidant protection in VesiCAT assay. However, it has been shown that synergistic and antagonistic interactions between PC may explain unpredictable results when measuring the antioxidant effect of complex vegetal extracts [407]. Thus, that could explain the superior antioxidant protection showed by CH extract. In comparison, the antioxidant activity of CP and CPH were in the same order of magnitude than those observed with pure tocopherols ( $0.005 \pm 0.002$  TE).



**Figure 39.** Kinetics of relative absorbance decay in the absence and presence of trolox (1.5-5  $\mu\text{M}$ ), 5-CQA (10-60  $\mu\text{M}$ ), CP (20-150  $\mu\text{g/mL}$ ), CH (10-75  $\mu\text{g/mL}$ ), CPH (50-200  $\mu\text{g/mL}$ ), theobromine (5-100  $\mu\text{M}$ ) and caffeine (5-50  $\mu\text{M}$ ). Without (---) and with (—) molecule or extract. The liposome suspension was made with DEPC/DLPC (1:20, w/w) in PBS, pH 7.2, at  $43 \pm 0.5$  °C and contained AMVN (0.3 mM)

### 13.3 VesiCAT: AAPH versus AMVN

VesiCAT assay was not only helpful to determinate the antioxidant activity of molecules or extracts, but also to evaluate the influence of other aspects that can affect the antioxidant capacity. Among them, the antioxidant location, concentration, distribution, or even penetration depth in membrane could have a key effect [408]. Indeed, It was considered that the AAPH experiments are good indicators of the scavenging/reducing free radicals ability in water environment, whereas the AMVN assays provide more informative about the reaction happening in the lipid bilayer, and therefore the membrane affinity [11].

Regarding AAPH assay, CH extract value (TE) showed to be 1.2 and 4.15 fold higher than CP and CPH, respectively. This could indicate that certain compounds in CH could have better radical scavenger/reducer activity. Likewise, in AMVN-induced oxidation assays, CH extract value (TE) was 2.6 fold higher than those obtained for CP and CPH. Thus, CH seems to retain a better overall antioxidant action. Although the antioxidant ranking obtained in both assays followed the same trend, the values observed using AMVN were inferior. This could be explained by a predominant activity with aqueous generated oxidants, combined with the stronger observed trolox antioxidant activity (NB: results are expressed in TE).

Anyhow, these results confirm that the effectiveness of antioxidants could be related to the type of inducer and the region where oxidant species are initiated and spread [11]. In fact, in the AAPH-induced peroxidation, free radicals are formed in the aqueous dispersive phase and attack the liposome membrane from the outside. By contrast, when the assay is performed with AMVN, the oxidant species are generated within the liposomal bilayer [395]. Additionally, the antioxidant nature, can affect its mobility in liposome systems. Thus, when different phases coexist in a system, the combination between mobility and equilibrium constant distribution is essential.

The results showed that in general the CP, CH, and CPH extracts seem to express a better antioxidant activity in the aqueous phase. Nevertheless, the complexity of vegetal matrices of our samples could influence many aspects of their antioxidant behavior, and therefore increase the complexity of their antioxidant interpretation.

## **13.4 Conclusions**

Recently developed VesiCAT assay for measuring the liposome oxidation have the advantage to use a natural probe. In addition, the antioxidant responses obtained are more precise, since DEPC probe avoid imprecisions such as probe location and alteration of the membrane physical properties. Additionally, VesiCAT assay allows performing experiments with two oxidation inducers having opposite solubility, leading to complementary knowledge regarding antioxidant reactivity and distribution.

The assessment of CP, CH, and CPH antioxidant capacity using VesiCAT, highlighted its interest and showed that this method could represent a useful tool to examine antioxidant performance of natural extracts.

# *Concluding remarks and perspectives*

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## **Concluding remarks and perspectives**

The world human population is growing faster. To face this challenge, the food industry has increased its production with the goal to feed about 10 billion people in the year 2050 [29]. However, this has led to the increase of waste derivate from food industrialization, with unfavorable environmental, economic and social consequences. Indeed, it has been estimated that  $2 \times 10^{11}$  ton are generated each year derivate from (i) the agro-residues which are left in the field directly after crops harvest and (ii) those separated from the product as it is processed [31].

The processing of coffee and cocoa generates abundant by- and co-products that, despite their rich composition, do not focus interest. Thus, they do not have real economical uses; expect maybe to be used as fertilizers. Moreover, they usually represent an important pollution problem both in producer and consumer countries, which is still a challenge.

In this context, the main objective of this research work was to offer new perspectives to the valorization of coffee pulp (CP), cocoa husk (CH), cocoa pod husk (CPH), and GCB: (i) by proposing an innovative environmental friendly approach to obtain extracts enriched in bioactive molecules using different DES as the extraction solvent, (ii) by evaluating the effect of the administration by oral administration in rats of a GCB extract in DES, in order to evaluate if DES can be used for oral bioactive molecules or therapeutic agents delivery, and (iii) by completing the actual portfolio of molecules possessing interesting physicochemical and biological properties by the characterization of isoprostanooids and thiol precursors in coffee and cocoa by- and co-products, (iv) by assessing the antioxidant capacity of CP, CH, and CPH ethanolic (70 % v/v) extracts through their ability to preserve liposome membranes from oxidation.

First, a chemical characterization of CP, CH, and CPH, using reported methods in the literature, was performed. For the monosaccharide profile description, a modification of the reported method [293] was necessary with the addition of a pre-hydrolysis step, in order to achieve the complete hydrolysis of cellulose. It would be interesting to characterize the physicochemical properties of the cellulose microfibrils from CP and CPH, to evaluate their

potential as dyes adsorbents in wastewater treatment, as it was made for CH in collaboration between our laboratory and Pr. El Achaby team [112]. CP, CPH, and CH presented an interesting lipid profile that is why we looked for the characterization and description of isoprostanooids. This line of research proved to be a worthwhile study, as it is the first time that these compounds were reported on coffee and cocoa by- and co-products. The obtained results confirmed that CP, CH, and CPH represent an economic source of several interesting biomolecules. It is known that the qualitative and quantitative molecular profile of vegetal sources (CP, CH, and CPH) can be affected by their growth conditions and geographic origins. Thus, although the results obtained in this work can be used as a reference, it is important to carry out this characterization study for each particular case.

Several proposed PC and alkaloids extraction technologies from plant biomass involve the use of conventional solvents (*e.g.* MeOH, EtOH, acetone, and propanol or their mixtures [253]). These kinds of solvents have several intrinsic drawbacks, among them their flammability, high toxicity, cost, and non-biodegradability [254]. Besides, the current environmental challenges and the growing trend for green Chemistry are pushing the scientific community to develop safer and alternative strategies. In this context, DES offer several environmental and economic advantages, besides their wide chemistry diversity. Additionally and according to previous researches, they have shown an interesting extraction capacity from vegetal sources. On this perspective, the second part of this work was dedicated to implement an innovative environmental friendly extraction methodology using DES in order to obtain enriched biomolecules extracts from CP, CH, and CPH. Six different ChCl- or betaine-based DES were investigated as extraction media and by HSE and UPAE assisted extraction. EtOH 70 % (v/v) was used as a control. Results showed that the DES composition and the type of extraction method have an influence on both, the type of extracted biomolecules and the extraction yield. On the one hand, ChCl-based DES were the most effective, specifically CCLA (ChCl:lactic acid:water, 1:2:1.4 mole ratio). On the other hand, DES extraction assisted by UPAE was the most efficient as it had a positive effect on the yield extraction of 5-CQA, caffeine, and theobromine, identified as the main bioactive molecules in CP (5-CQA, caffeine), and CH (caffeine and theobromine). UPAE allows decreasing the relatively high DES viscosity and consequently improves mass transfer. Under these extraction conditions (CCLA-UPAE), furfural was formed during extraction and



identified in CP, CH, and CPH. This formation is related to the extraction conditions, especially time and temperature, as well as the pH. These findings provide a new perspective for the use of CP, CH, and CPH to synthesize furfural from plant biomass by- and co-products using DES, since furfural has a wide spectrum of industrial applications.

In conclusion, this study showed that DES can be used as sustainable solvents to extract and/or synthesize biomolecules from coffee and cacao by- (CP and CPH) and co- (CH) products.

Regarding the extraction process with DES, it is important to consider not only the target molecule but also other compounds that may be extracted and/or hydrolyzed and/or chemically modified (proteins, polar lipids, sugar residues, cellulose, etc.). Indeed, considering the abundance, richness and complexity of plant biomass, and the strong and unusual solvation properties of DES, thinking with a systemic approach could offer interesting prospect. For example, the DES properties have already been used for the treatment/fractionation of lignocellulosic material [261] to valorize lignin streams produced from papermaking and biomass refinery processes, as it was shown that the ChCl-based DES could be a lignin selective solvent [ref 307], or to produce ethanol from cellulosic materials [ref 246]. Thus, it would be interesting to develop a biorefinery flowchart (inspired on those reported in the literature, *e.g.* Figure 20 *cf.* section 4.4.4) including successive extraction and isolation steps to fractionate the different classes of molecules present in coffee and cocoa by- and co-products. Both, polar and non-polar DES, with successive precipitation (with adding water and/or EtOH) and filtration steps, could promote an integral biorafinery of these residues in a sustainable way.

Although some studies have proposed strategies to separate the target molecules from DES, they involve usually specific equipment, organic solvents, production of waste and additional fees. Bearing this in mind, one attractive strategy is to use DES formulations directly in food, pharmaceutical and/or cosmetic applications. Indeed, depending on the nature of the starting components, DES may be fully compatible with any targeted application. They offer all characteristics to design atom economy, efficient, low cost and sustainable development, for “ready-to-use” extraction. Consequently, another option would be to use DES as, first,

extracting media, and, second, formulation system, thus bypassing the problem of solute recovery. That is why, a preliminary in vivo study using a GCB PC enriched DES extract by HSE was performed in order to evaluate its effects in acute conditions [22], knowing that the final objective was to evaluate if DES could be used for oral bioactive molecules or therapeutic agents delivery. The extract was obtained from GCB, since they are known as an abundant and natural source of phenolic compounds, especially 5-CQA. BeGly (betaine:glycerol, 1:2 mole ratio) was chosen as extraction solvent since it showed the better extraction yield from the six tested DES. The oral administration of DES extract led to several negative effects on the health of the rats, and even the death for two of them. Despite this, it was noted that although the rats in DES group showed hepatomegaly symptoms, it was not associated with excess lipid content. Hence, betaine and 5-CQA contained in DES formulation could have contributed to preventing fat accumulation in rats' liver. Yet, the conditions that we used in this study are not enough to support the safety of DES formulation, neither to deny it, since several factors could be related to DES toxicity. Among them, the molar ratio, the HBA and HBD nature, the dose and the way for sample administration [228]. Unfortunately, in the light of the preliminary results, the French National Committee did not recommend to continue with the entire initially planned study. Although there is still a long way to go regarding DES toxicity, this research about the effects of a phenolic DES extract supplementation is pioneer, and would contribute to understand the factors and risks associated with in vivo DES application.

In conclusion, although still complementary studies are necessary DES showed an enormous potential not only as extraction solvent, but also as a formulation media that could be used in a wide range of applications from the green Chemistry standpoint.

As aforementioned, CP, CH, and CPH are rich sources of a wide spectrum of biomolecules, beyond PC and alkaloids. The innovation of the last part of this project was to enrich the biomolecules' portfolio by extracting and characterizing for the first time PhytoPs, PhytoFs, and thiol precursors from coffee and cocoa by- and co-products. We found this perspective interesting for two main reasons, first, these compounds are raising recent consideration and curiosity, as they possess interesting physicochemical and biological activities, and

second, these compounds have never been described in CP, CH, and CPH and therefore could confer specificity.

Since PhytoPs and PhytoFs show some similarity to IsoPs and prostaglandins in humans, there is growing interest in studying these compounds' profile and levels in plant foods and their response to cultural and postharvest transformation practices and/or environmental conditions. The results showed that these agro-residues are an interesting source of PhytoPs and PhytoFs. CP, CH, and CPH contained respectively 654.45, 474.30 and 179.91 and 543.10, 278.02 and 393.70 ng/g dry weight (dw), for PhytoPs and PhytoFs. Indeed, six and three different kind of PhytoPs and PhytoFs, respectively, were characterized with respect to the molecular profile and concentration [374]. As perspectives of this part, it would be interesting to increase knowledge on the influence of the edaphoclimatic conditions, crops variety and the postharvest process (primary and second transformation process) on the qualitative and quantitative PhytoPs and PhytoFs profile in CP, CPH, CH, and low-grade green coffee. Besides, it has been suggested the possibility to "*functionalize*" plant products through the manipulations of certain agronomic and industrial procedures in order to enhance PhytoPs and PhytoFs content [367]. From this perspective, it would be very interesting to study these factors in coffee and cocoa crops, in order to establish a relationship between them and PhytoPs and PhytoFs concentration in coffee and cocoa co- and by-products.

The identification and quantification of thiol precursors in CP, CPH, and CH, not only widened their biomolecular profile, but also paved the way to new valorization perspectives. For example, a serious and suspected consequence of CPH accumulation in cocoa plantation is the propagation of diseases, such as black pod rot (*Phytophthora spp.*). This disease causes pod losses until 30 %, and kills up to 10 % of trees annually [409]. However, even the CPH affected by black pod rot could be concentrated sources of thiol precursors, as it was demonstrated for wine obtained from grapes affected by Noble rot (*Botrytis cinerea*) that contained more 3MH than those obtained from healthy grapes, indicating that these grapes probably contain higher concentrations of its precursor [395]. Obviously, further studies are necessary in order (i) to better characterize the CP, CPH, and CH thiol precursors profile associated with the influence of the variety, the culture conditions, and the post-harvest

processes, (ii) to determine the best conditions able to exploit this potential, and finally to (iii) examine their possible application in fermented food and/or drinks.

Additionally, since CP, CPH, and CH are sources of antioxidant such as PC, we conducted an evaluation of their antioxidant capacity using a new method that was developed in our laboratory, called “Vesicle Conjugated Autoxidizable Triene” (VesiCAT). This method allows monitoring the membrane oxidation of liposomes, with the means of a new synthesized di-eleostearic phospholipid probe (DEPC). Overall, the CP, CPH, and CH extracts, showed poor antioxidant activity, especially when oxidation was initiated and spread in the membrane region. Anyhow, this studied showed that VesiCAT assay could represent a useful tool to examine the antioxidant performance of natural extracts.

As mentioned in the literature review, some DES have been suggested to possess antimicrobial activity. Such properties may be advantageous, especially if DES could be associated after extraction step with compounds such as PC, which are natural antimicrobial agents. Indeed, it is possible that an additive or synergistic antimicrobial effect take place between PC and DES. Moreover, caffeine and CGA are known to have an inhibiting effect on *Aspergillus* mycotoxin production [23][410]. To complete this work, it would be valuable to study the antimicrobial (antibacterial and antifungal) and anti-mycotoxigenic activity of the obtained CP, CPH, and CH extracts in DES, so they could be used as preservatives in food or non-food products.

To conclude, these thesis works demonstrated that CP, CPH, and CH, are still an unexploited (under evaluated) sources of biomolecules. In these researches, we tried to offer new possibilities to valorize these vegetal materials, with an environmental and friendly approach. Moreover, the identification for the very first time of new kinds of molecules could reinforce their interest and promote the research for food and not food applications.

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# *Appendix*

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