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Water-Based Extraction of Bioactive Principles from Hawthorn, Blackcurrant Leaves and Chrysanthellum Americanum : from Experimental Laboratory Research to Homemade Preparations

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Phu Cao Ngoc. Water-Based Extraction of Bioactive Principles from Hawthorn, Blackcurrant Leaves and Chrysanthellum Americanum : from Experimental Laboratory Research to Homemade Preparations. Analytical chemistry. Université Montpellier, 2020. English. NNT : 2020MONT051 . tel-03173592

HAL Id: tel-03173592

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Submitted on 18 Mar 2021

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

En Chimie Analytique

École doctorale sciences Chimiques Balard ED 459

Unité de recherche Institut des Biomolécules Max Mousseron

Water-Based Extraction of Bioactive Principles from Hawthorn, Blackcurrant
Leaves and Chrysanthellum americanum: from Experimental Laboratory
Research to Homemade Preparations

Présentée par Phu Cao Ngoc

Le 27 Novembre 2020

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UNIVERSITÉ
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RESUME en Francais

De nos jours, la demande sociétale pour l'utilisation des plantes médicinales est forte et cela a été reconnu par la Présidente de l'Ordre des Pharmaciens en France qui déclarait en 2018, «Il y a une forte attente sociétale de produits naturels, sans effets indésirables, efficaces, et utilisés notamment en prévention ou contre les petits maux du quotidien». En médecine moderne, l'utilisation de plantes médicinales se fait généralement sous forme d'extraits secs standardisés de plantes (comprimés ou solutions) pour contrôler l'apport journalier et assurer l'efficacité du traitement. Leur consommation sous forme d'infusion (ou de macération / décoction selon les plantes) est rarement prescrite par le médecin, notamment en France. Néanmoins, la pratique de l'infusion est plus ou moins répandue en fonction des habitudes culturelles des personnes / familles. L'argument récurrent qui s'oppose à la consommation d'infusions de plantes médicinales est que les infusions ne sont pas standardisées, et donc que la quantité de composés bioactifs absorbée par l'organisme n'est pas connue. En effet, l'extraction de principes bioactifs à partir de plantes médicinales est souvent mal contrôlée à la 'maison', notamment lorsque les gens utilisent des infusions. En effet, ni le poids de la plante, ni sa granulométrie, la température d'extraction, ni le volume d'eau ne sont généralement bien contrôlés. La dose ingérée de composants biologiquement actifs extraits de la plante médicinale peut donc varier considérablement d'une infusion à l'autre. Il paraît donc important de tenter d'améliorer les modes d'extraction des plantes médicinales, et de considérer le passage de l'extraction à l'échelle du laboratoire vers une extraction réalisée à la 'maison'. Cela permet d'identifier les points clés expérimentaux permettant d'améliorer/optimiser les rendements, la répétabilité/reproductibilité, étapes importante pour aller vers une maîtrise de l'apport en composés bioactifs à l'échelle individuelle.

Dans cette thèse, la question de la standardisation, de la répétabilité et de l'optimisation de l'extraction des plantes médicinales dans l'eau a été étudiée. Trois plantes ont été sélectionnées, pour lesquelles les activités pharmacologiques sont basées sur différents flavonoïdes. Deux de ces plantes sont bien documentées (sommités fleuries d'aubépine et feuilles de cassis) aux propriétés bien connues, et la troisième a été peu étudiée (*Chrysanthellum americanum*). L'aubépine (*Crataegus*) est un arbuste à fleurs répandu qui pousse dans les climats tempérés d'Eurasie et d'Amérique du Nord, avec plus de 280 espèces différentes référencées, et est connu à la fois pour sa capacité à réguler le système sympathique et pour sa cardiotonicité (c'est-à-dire la diminution de la demande en oxygène des cellules cardiaques et leur meilleure contractibilité). Les feuilles de cassis (*Ribes nigrum*) sont surtout connues pour leur activité anti-

inflammatoire. *Chrysanthellum americanum* est une plante américaine et africaine utilisée pour sa capacité à stimuler et protéger la fonction hépatique et pour son activité hypocholestérolémiante.

Différents modes d'extraction dans l'eau (infusion, macération, percolation, ultrasons, micro-ondes) ont été testés et comparés pour l'extraction des principes bioactifs contenus dans les trois plantes en termes de rendement d'extraction, de quantité de composés phénoliques, de flavonoïdes et d'oligomères de proanthocyanidines, et de profils UHPLC des composés extraits. Les aspects quantitatifs et qualitatifs de l'extraction, ainsi que la cinétique d'extraction ont été étudiés. La spectrométrie de masse à résonance cyclotronique à transformée de Fourier à haute résolution (FT-ICR MS) a également été mise en œuvre pour obtenir des informations qualitatives supplémentaires sur les compositions chimiques spécifiques permettant de discriminer les trois plantes.

Le broyage de la plante s'est avéré être le meilleur moyen d'augmenter la cinétique et le rendement global d'extraction. Si la plante est broyée, l'infusion est le moyen le plus simple d'extraire les composants bioactifs des plantes médicinales, et les autres modes d'extraction n'ont pas amélioré de manière significative le rendement d'extraction. Les profils UHPLC sont également similaires d'un mode d'extraction à l'autre. Dans la mesure où la plante est broyée, l'agitation automatique de l'infusion n'est pas nécessaire et une simple agitation manuelle au début et à la fin de l'extraction suffit pour obtenir une extraction optimale. Une infusion de 10 minutes est nécessaire pour atteindre la température buvable (60°C), mais une durée plus courte est possible à condition qu'un glaçon (ou deux) soit ajouté à la préparation. L'utilisation d'un sachet filtre pour l'infusion n'est pas souhaitable car le filtre ralentit le processus d'extraction et diminue les rendements d'extraction tout en augmentant le coût global.

Un protocole simple, rapide et optimisé pouvant être utilisé par n'importe qui à la maison a été développé, en utilisant une cafetière à piston ('french-press coffee maker') et un broyeur à café électrique pour obtenir une granulométrie inférieure à 1 mm. Dans le cas de l'aubépine, ce protocole (infusion pendant 3 min de 2,5 g de sommités fleuries broyées dans au moins 250 mL d'eau bouillante) permet un apport journalier en polyphénols, flavonoïdes et oligomères de proanthocyanidines similaire à la dose recommandée d'extraits standardisés. Des rendements d'extraction d'environ 22% (en poids de la plante sèche d'aubépine initiale) sont atteints de façon répétable et contrôlée. Il est à noter que le rendement global d'extraction est resté inchangé pour les cinq lots d'aubépine testés, même si la répartition des composés actifs peut varier d'un lot à l'autre. Le coût global d'un mois de consommation quotidienne d'aubépine (à raison d'une infusion par jour) est d'environ 2,2 euros, soit environ 10 fois moins cher que

le coût de l'extrait de plante standardisé (sous forme de solution ou de comprimés). En utilisant le même protocole, les rendements d'extraction globaux atteignent environ 26% pour *Chrysanthellum americanum* et environ 28,5% pour les feuilles de cassis.

Concernant la composition chimique, les extraits de feuilles de cassis contiennent beaucoup plus de composés phénoliques que les deux autres plantes, tandis que les extraits d'aubépine contiennent beaucoup plus d'oligomères de proanthocyanidines que les deux autres plantes. Les extraits de *Chrysanthellum americanum* et de feuilles de cassis contiennent des quantités similaires de flavonoïdes. La technique UHPLC a révélé que les principaux composés détectés en UV sont les flavonols dans les feuilles de cassis, les dérivés d'acide hydrocinnamique, de flavone, de flavanone et d'aurone dans *Chrysanthellum americanum*, et les flavanols, flavonols et flavones dans l'aubépine. Dans les extraits de *Chrysanthellum americanum*, les profils d'UHPLC-ESI-MS révèlent la présence de dérivés de flavanomaréine et de martitiméine, tandis que la technique FT-ICR MS montre la présence spécifique d'acide oléanolique ou ursolique. Dans les extraits de feuilles de cassis, les dérivés de la quercétine et du kaempférol sont principalement identifiés. Le vitexin-2-O-rhamnoside, l'hyperoside et l'isoquercétine sont les principaux composants des extraits d'aubépine. Par FT-ICR MS, environ 2500 molécules ont été décelées pour chaque plante, parmi lesquelles environ 1100 sont communes aux 3 plantes, environ 350 sont communes à deux plantes, et environ 700 sont spécifiques à chacune des plantes.

Concernant leurs activités enzymatiques et antioxydantes, une inhibition intéressante de la hyaluronidase ($\geq 90\%$) a été rapportée pour les extraits d'aubépine (à une concentration de 1 g/L), similaire celle observée avec l'inhibiteur de référence de la hyaluronidase, et bien supérieure à celle des deux autres extraits de plante. Quant à l'activité anti-hypertensive, les extraits de *Chrysanthellum americanum* ont démontré une inhibition de l'ECA plus élevée ($\geq 90\%$ à une concentration de 1 g/L), que les deux autres extraits de plante. Concernant l'activité antioxydante, les extraits de feuilles de cassis ont montré la capacité antioxydante la plus élevée en corrélation avec les teneurs les plus importantes en composés phénoliques et en flavonoïdes, tandis que les extraits de *Chrysanthellum americanum* ont présenté les teneurs les plus faibles en composés actifs et la plus faible capacité antioxydante.

La formation de tea creaming (c'est-à-dire de nanoparticules d'une taille allant généralement de 10 à 400 nm) a finalement été étudiée dans ces infusions de plantes médicinales, à la fois d'un point de vue cinétique et de la distribution de taille. La formation de nanoparticules dans le thé lors de la diminution

en température est un phénomène rapporté dans la littérature avec des conséquences et/ou des applications parfois inattendues comme la stimulation du système immunitaire ou comme milieu de nucléation pour la synthèse de nanoparticules d'argent. Les processus de formation de ces nanoparticules ne sont pas complètement élucidés et il nous a paru important de les étudier sur les infusions de plantes médicinales avec des techniques de caractérisation adaptées. Nous avons montré que le tea creaming se forme dans le cas d'infusions de feuilles d'aubépine et de cassis, mais pas dans le cas de d'infusions de *Chrysanthellum americanum*. Cependant, cette quantité de particules est beaucoup plus faible que dans le thé vert, probablement en raison d'une faible teneur en caféine.

En parallèle de cette thèse, et en collaboration avec l'Université de Campinas (Brésil), une étude visant à déterminer les effets préventifs des extraits d'aubépine (issus de la thèse) dans un modèle de maladies inflammatoires de l'intestin appliqué à des rats, a été réalisée. Il a été constaté que les extraits d'aubépine produisaient une réponse anti-inflammatoire chez les rats atteints de colite induite par le TNBS (acide 2,4,6-trinitrobenzène sulfonique), en réduisant les médiateurs inflammatoires et en régulant les voies du stress oxydatif, induisant par conséquent moins de nécrose colique. L'aubépine présente des propriétés anti-inflammatoires en complément de son rôle protecteur et anti-hypertenseur cardiovasculaire déjà bien connu.

Enfin, ce travail de thèse met en évidence la complexité de la composition des trois extraits de plantes étudiées, à la fois en nombre de composants chimiques et en structures chimiques. Il confirme également l'intérêt potentiel de ces plantes médicinales pour un large éventail d'activités biologiques, y compris celles qui sont différentes de l'intention thérapeutique principale. Enfin, nous pensons que le protocole d'infusion optimisé pour une utilisation à la maison par le plus grand nombre est utile pour ceux qui s'intéressent aux plantes médicinales. Ce travail tente, à sa manière et de façon assez pragmatique, de contribuer à la promotion et l'intégration de la phytothérapie dans la médecine occidentale moderne, en réponse à une demande sociétale croissante dans ce domaine.

TITRE en Anglais

Water-Based Extraction of Bioactive Principles from Hawthorn, Blackcurrant Leaves and *Chrysanthellum Americanum*: from Experimental Laboratory Research to Homemade Preparations

RESUME en Anglais

This work deals with the question of standardization, repeatability and optimization of medicinal plant extraction in water. Three plants were selected, for which the complementary pharmacological activities are based on different flavonoids, two of which are well documented (hawthorn flowering tops and blackcurrant leaves) with well-known properties, and the third one has been little studied (*Chrysanthellum americanum*). We established a general extraction protocol in water for these three plants that can be used by each of us, based on infusion that can afford a reproducible daily uptake of bioactive components (phenols, flavonoids, proanthocyanidin oligomers) at drinkable temperature. Granulometry was the most important factor to get the best extraction yields (about 22% for hawthorn, 26% for *Chrysanthellum americanum* and 28.5% for blackcurrant). Chemical composition of these plants was investigated by colorimetric methods, and also using performant and complementary analytical instrumentations (UHPLC-ESI-MS and FT-ICR MS). Blackcurrant extracts contained much more phenolic compounds (the main UV-detected components detected in UHPLC being flavanols) than the two other plants. Hawthorn extracts contained much more proanthocyanidin oligomers (the main UV-detected components in UHPLC being flavanols, flavonols and flavones) than the two other plants. *Chrysanthellum americanum* and blackcurrant extracts contained similar amounts of flavonoids, the former one containing essentially hydrocinnamic acid derivatives, flavones, flavanones and aurones as UV-detected components. About 2500 hints were obtained for each plant, among which about 1100 are common to all 3 plants and about 700 are specific to each plant. Quercetin and kaempferol derivatives were identified in blackcurrant leaves extracts, while vitexin-2-O-rhamnoside, hyperoside and isoquercetin were identified in hawthorn flowering tops extracts and flavanomorein and martitimein derivatives, and Oleanolic or Ursolic acid were identified in *Chrysanthellum americanum* extracts. A significant inhibition of hyaluronidase ($\geq 90\%$) was reported for hawthorn extracts, much higher than that of the other two plant extracts. As for the anti-hypertensive activity, the *Chrysanthellum americanum* extracts demonstrated higher ACE inhibition than the other two plant extracts. Regarding antioxidant activity, blackcurrant leaf extracts showed the highest antioxidant capacity. Finally, the formation of nanoparticles in the herbal tea infusions (also known as tea creaming), was studied from a kinetic and size-distribution point of view as a function of temperature.

Keywords: hawthorn; blackcurrant; *chrysanthellum americanum*; standardization; extraction mode; infusion, water-based extraction; proanthocyanidin; polyphenol; flavonoid; granulometry; enzymatic activity, tea creaming

Intitulé et adresse de l'unité ou du laboratoire

IBMM, University of Montpellier, CNRS, ENSCM, Montpellier, France

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ACKNOWLEDGEMENT

I would like to express my deepest thankfulness to my supervisor, Dr. Laurent Leclercq and Prof. Herve Cottet from the Institut des Biomolécules Max Mousseron (IBMM), UMR CNRS 5247, Université de Montpellier, who accepted me as a Ph.D student and gave me all their important suggestions, valuable advices, comments, constant encouragement and kind help throughout my whole thesis in Montpellier, France. I am proud of being one of your student and I gratefully acknowledge this remarkable opportunity to work with you.

I would like to thank Prof. François Couderc, Dr. Sabrina Boutefnouchet and also thank other members of my board of examiners, Prof. Joseph Vercauteren and Dr. Celine Riviere for reviewing and giving me very useful comments in my thesis.

My specially gratefulness to Dr. Jean-Christophe Rossi and Isabelle Desvignes from the Institut des Biomolécules Max Mousseron (IBMM), UMR CNRS 5247, Université de Montpellier, France, who gave me valuable advices, and for teaching me the UHPLC methodology.

My sincere gratitude also conveys to Prof. Farid Chemat and Dr. Anne-Sylvie Tixier from the University of Avignon, INRA, UMR408, GREEN Extraction Team, Avignon, France for providing me valuable advices regarding extraction techniques and facilitating my study during the time I spent in their Lab and also my many thanks to all friends that I met there for their friendship and sharing knowledge.

I specially thank to Prof. Philippe Schmitt-Kopplin and Jasmine Hertzog from the Analytical BioGeoChemistry, Helmholtz Zentrum Muenchen, Neuherberg, Germany and Analytical Food Chemistry, Technische Universität Muenchen, Freising, Germany for their great collaborative work in High-resolution mass spectrometry. I would like also to thank to Prof. Reine Nehmé, Rouba Nasreddine and Ghassan Al Hamoui Dit Banni at ICOA, University of Orléans, CNRS, Orléans, France for their great colabolative work in enzymatic activity.

My special thanks to Laurent Boiteau for his support and explanations regarding technical aspects, and also to Marie-Sophie Monfort for her support in administrative work.

I would like to acknowledge the Ministry of Agriculture and Rural Development, Ministry of Education and Training, Vietnam and French Embassy in Ha Noi, Vietnam for awarding me a fellowship and to Campus France for additional support during my PhD studies.

I would like to thank to Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phu Tho, Vietnam for granting me the opportunity to enroll in a PhD. My great thank also conveys to my colleagues at NOMAFSI (Vietnam) for their kind assistance and encouragement.

Many thanks to my friends in Ph.D. Research Lab at Institut des Biomolécules Max Mousseron (IBMM), UMR CNRS 5247, Université de Montpellier, to Philippe for his technical supports, to Joseph for his straight advices and humor. I would also like to thank my colleagues: Nesrine, Charly, Mihai, Camille and Xiaoling for their friendship, encouragement and for sharing with me the interesting discussions during my PhD study.

Last and most of all, I would like to express my deepest gratitude to my family for all their love, encouragement, especially for my beloved wife for her great understanding, encouragement and constant support throughout my study.

Phu Cao Ngoc

Abbreviation list

aP2: activating protein 2
ACE: Angiotensin-converting enzyme
ABTS: 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid
API: atmospheric pressure ionization
APCI: atmospheric pressure chemical ionization
APPI: atmospheric pressure photo-ionization
BCC: black currant concentrate
BPR: blackcurrant press residue
BC: Blackcurrant leaves
BTH: hyaluronidase type I-S from bovine testes
BGE: background electrolyte
CE: Capillary Electrophoresis
CPA: corrected peak area
CY: cyanidin
C: -(-) catechin
CG: catechin gallate
COX-2: cycooxygenase-2
CF: Crataegii fructus
CCC: camphor *Crataegus* berry combination
CI: chemical ionization
CA: *Chrysanthellum Americanum*
DAD: diode array detector
DPPH: 2,2-diphenyl-1-picrylhydrazyl
DENA: diethylnitrosamine
EGCG: epigallocatechin gallate
ECG: epicatechin gallate
EGC: epigallocatechin
EC: epicatechin
EF: extraction factor

Eq: equation

El: electron impact

ESI-MS: Electrospray Ionization -Mass Spectrometry

ESI: electrospray ionization

E: extractor

epiCAT: epicatechin

eNOS: endothelial nitric oxide synthase

FT-ICR-MS: Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

FTICR: Fourier-transform ion cyclotron resonance

FRAP: ferric reducing antioxidant power

FAB: fast atom bombardment

GCG: gallic acid gallate

GC: gallic acid

GA: gallic acid

HAW: Hawthorn

HA: hyaluronic acid

HY: hyperoside

HCA: Hierarchical Cluster Analysis

HUVECs: human umbilical vein endothelial cells

UHPLC: ultra-high performance liquid chromatography

HPLC: high performance liquid chromatography

ICR: ion cyclotron resonance

IL-1 β : interleukin 1 β

IL-6: interleukin 6

IB: incubation buffer

iNOS: inducible nitric oxide synthase

LC-MS: Liquid Chromatography- Mass Spectrometry

LPS: lipopolysaccharide

LDL-C: low-density lipoprotein cholesterol

MPO: myeloperoxidase

MR: Myakuru
MS: Mass Spectrometry
MI: mixer
MW: microwave-assisted
NYHA: New York Heart Association
HDL-C: high density lipoprotein-cholesterol
NO: nitric oxide
OPC: total proanthocyanidins content
OPCs: Oligomeric proanthocyanidins
PAs: proanthocyanidins
PPAR γ : peroxisome proliferator-activated receptor-gamma
PGE₂: prostaglandin E₂
PCA: Principal Component Analysis
PLS-DA: Partial least squares-discriminant analysis
PVDF: polyvinylidenedifluoride
PI3: phosphatidylinositol-3
PDA: Photodiode Array Detection
p-Akt: phosphorylation of protein kinase B
Q: quercetin
ROS: reactive oxygen species
RG: red ginseng
SFE: supercritical fluid extraction
S: separator
SC: serum cholesterol
SREBP 1c: sterol regulatory element-binding protein 1c
TE: Trolox equivalent
TFs: theaflavins
TBs: thearubigins
TPC: total polyphenol content
TFC: total flavonoids content

TEAC: Trolox equivalent antioxidant capacity

TPTZ: 2,4,6-tripyridyl-s-triazine

TG: blood triglyceride

TC: total cholesterol

TG: triglyceride

TNF- α : tumor necrosis factor

US: ultrasound-assisted

WHO: World Health Organization

wt: weight

3HH-GGG: 3-Hydroxybutyl-Gly-Gly-Gly

GENERAL INTRODUCTION

We can classify the medicinal plants into two categories. The first one is composed of plants whose activities are based on a single molecule, such as alkaloids. Those plants are effective at low doses but can be very toxic at higher dosages, and their therapeutic concentration window is generally very short (see e.g. colchicum, digitalis, poppy). The second category is composed of plants whose activities are based on the *totum* of the plant, with a medicinal activity based on a (sometimes complex) mixture of components (e.g. mixture of flavonoids). Those plants generally present very few (or no) side effects and low toxicity (e.g. hawthorn, blackcurrant leaves). As it, there can be a good alternative to classical drugs targeting the same therapeutic indications, or they can be used in complement to modern medicine. Moreover, 70% of our pharmacopea is issued from the vegetal reign [1].

Traditional folk (Chinese and European) medicine is based on the use of medicinal plants [2]. Some of them have pharmaceutical properties that are recognized by clinical studies [3,4]. In medicine, they are generally delivered as standardized dry extracts of plants in the form of tablets or of hydro-alcoholic solutions to control the daily intake and to ensure the efficiency of the treatment. Their consumption in the form of infusion (or maceration, or decoction, depending on the plant) is rarely prescribed by the doctor (at least in France). Nevertheless, the practice of traditional infusion still exists with a strong variability in its use, depending on cultural habits of the people/families. The recurrent argument that is opposed to the consumption of medicinal plant infusions is that the infusions are not standardized, and therefore the amount of bioactive compounds absorbed by the organism is not known. Doctors are thus reluctant to prescribe these plants under that form. Still, in a decree published in 2008 in France [Decree No.2008-841 of August 22, 2008, relating to the sale to the public of medicinal plants listed in the Pharmacopoeia, see

<https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000019375944&categorieLien=id> for more details], the sale of 148 medicinal plants was liberalized because these plants were proved to be entirely safe. For unclear reasons, in the case of hawthorn, one of the 'best seller' of medicinal plants, only the fruit has been liberalized and not the flowering tops. The legislator

also prohibits the sale of plants in the form of mixtures as a precautionary principle [5]. The elaboration of mixtures of medicinal plants for infusion still remains the monopoly of pharmacists (official preparation), with clear guidelines given by the Agence Nationale de Sécurité du Médicament et des produits de santé (ANSM) monography.

Nowadays, the societal demand for the use of medicinal plants is strong; and this was recognized by the 'Présidente des Ordres des Pharmaciens' in France which declared in 2018: "There is a strong societal expectation of gentle natural products, without adverse effects, efficient, and used especially in prevention or against small daily ailments" [Compte-rendu de l'audition de représentants de l'ordre des médecins et de l'ordre des pharmaciens, 10-11 juillet 2018, see http://www.senat.fr/compte-rendu-commissions/20180709/mi_herbo.html]. In 2018, in France, about 21500 ha were devoted to the culture of medicinal plants, with a global French market for natural products in health and beauty of 3 billion of euros [1]. For the majority of the population, and in most of the situations, the use of medicinal plant is accepted and viewed as a complement of modern medicine. Sometimes, however, there is no satisfactory currently available alternatives in modern medicine, as for instance for venous return / heavy legs treatments for which red vine leaves (*Vitis vinifera*), butcher's broom (*Ruscus aculeatus*) and horse chestnut are known to be efficient. For more details on the plant pharmacological properties, the reader can refer to the European Medicine Agency (www.ema.europa.eu).

Clearly, there is still a huge work to perform through *in vivo* and clinical studies to investigate to pharmacological and medical properties of medicinal plants; even for well-known plants but for different therapeutic indications. Because medicinal plants are not patentable, only the academic scientists can launch such studies with the limitations that such research requires important funding that are generally not available in the academic world. Besides the clinical and *in vivo* studies, it seems important to standardize the preparation of medicinal plant infusion with an in-depth study of the impact of the different operating parameters (temperature, stirring speed, granulometry, filter use, extraction time, plant variability).

In this thesis, the question of standardization, repeatability and optimization of medicinal plant extraction in water was investigated. Three plants were selected, for which the pharmacological activities are based on different flavonoids, two of which are well documented (hawthorn flowering tops, see european monography EMA/HMPC/159075/2014) [6–15] and blackcurrant leaves, see european monography EMA/HMPC/745353/2016 [3,4,16,17]) with well-known properties, and the third has been only little studied (*Chrysanthellum americanum* [18–21]). Hawthorn (*Crataegus*) is a widespread flowering shrub growing in temperate climates of Eurasia and North America, with more of 280 different referenced species [22,23] and are known for their ability to regulate the sympathetic system and for their cardio-tonicity (i.e. less oxygen demand) [24]. Blackcurrant leaves are mostly known for their anti-inflammatory activity [3]. *Chrysanthellum americanum* is an American and African plant used for its capacity of stimulating the liver and for its cholesterol-lowering activity [18,20]. We will see if it is possible to establish a general extraction protocol in water for these three plants that can afford a standardized and reproducible daily uptake. The differences in chemical compositions between these plants will be also investigated using performant and complementary analytical instrumentations (UHPLC-ESI-MS and high resolution mass spectrometry). Enzymatic activities including antioxidant, anti-hypertensive and towards hyaluronidase will be also compared. Finally, the formation of nanoparticles in the herbal tea infusions (also known as tea creaming phenomenon), is studied from a kinetic and sizing point of view, and compared to those obtained in green tea as previously shown in the literature [25–28]. We will see whether tea creaming is a factor that limits the extraction yield by precipitation of active principles and how the temperature impacts the formation of tea creaming.

CHAPTER I. BIBLIOGRAPHY

CHAPTER 1: BIBLIOGRAPHY

This chapter sums up information on methodology of extraction, chemical composition and pharmacological properties of the three plants studied in this work: hawthorn (*Crataegus*), blackcurrant (*Ribes nigrum*), and *Chrysanthellum americanum*.

I.1. Phenolic compounds

I.1.1. Structure and classification

Phenolic compounds are one of the most numerous and widely distributed group of aromatic compounds in the plant kingdom, with over 8000 phenolic structures currently known, within which 6000 are flavonoids [29]. Phenolic compounds have one or several aromatic rings carrying hydroxyl groups. Generally, they can be divided into many different classes, depending on the basic skeleton (ranging from simple C₆ to polymerized forms), the degree of modification of this skeleton (degree of oxidation, hydroxylation, methylation ...), and finally the possible bonds with other molecules (carbohydrates, fats, proteins, other metabolites ...). Besides, according to the biological function, polyphenols can be classified into two main groups: the flavonoids and the non-flavonoids. Figure I.1 shows the major polyphenols found in plants [30].

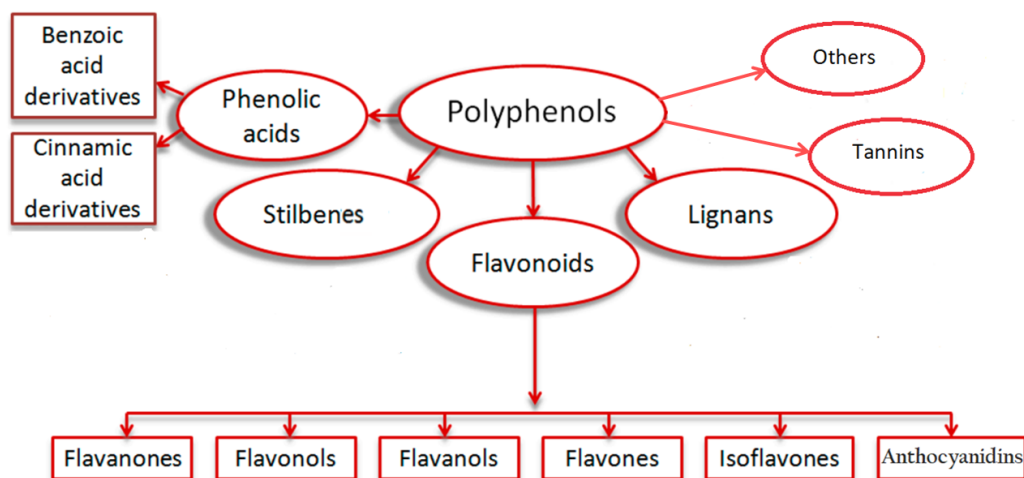


Figure I.1: The major polyphenolic compounds found in plants (taken and modified from [31]).

I.1.1.1. Non-flavonoid polyphenols.

The non-flavonoids can be classified into several classes of phenolic compounds: phenolic acids with subclasses derived from hydroxycinnamic acids and hydroxybenzoic acids, stilbenes, lignans, tannins and others [32].

Phenolic acids: Chemically, phenolic acids have one or more hydroxyl groups and a carboxylic acid function attached to the benzene ring. Based on the position of the hydroxyl group, phenolic acids can be divided into two main subclasses, namely benzoic acids and cinnamic acid derivatives [33] (Figure I.2).

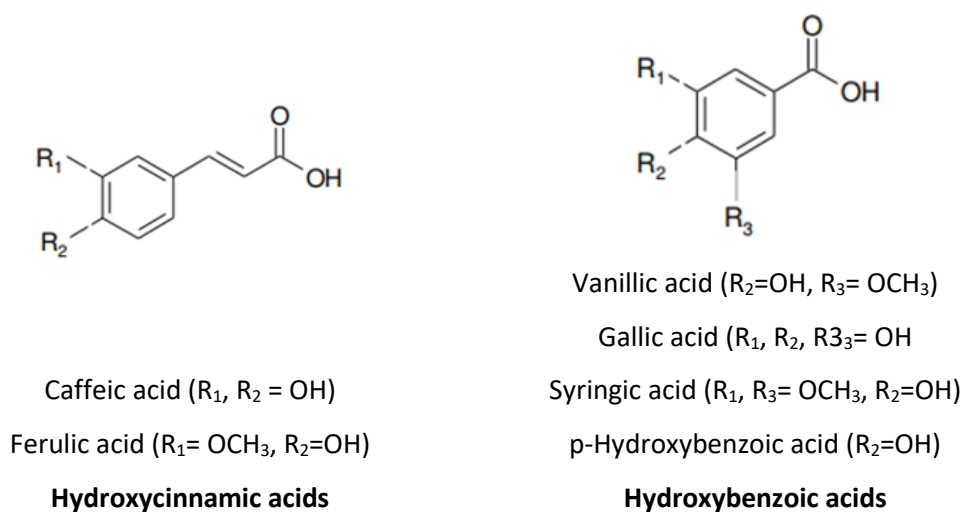


Figure I.2: Structure of phenolic acids found in plants.

Phenolic acids are found in all food groups and are abundant in cereals, legumes, oilseeds, fruits, and vegetables, beverages and especially herbal plants [34]. Caffeic acid and ferulic acid are the most abundant hydroxycinnamic acids, while gallic, vanillic and syringic acids are the most common hydroxybenzoic acids [35]. Phenolic acids have been reported as vital human dietary components and showed a tremendous antioxidant activity [36].

Stilbenes: Stilbenes are another class of non-flavonoid polyphenols with 1,2-diphenylethylene as basic structure.

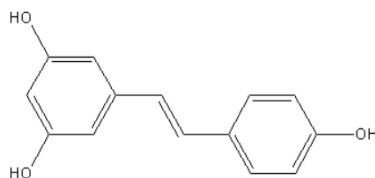


Figure I.3: Basic structure of stilbenes (structure of resveratrol) (Source: Pubchem).

Resveratrol (Figure I.3) is the main representative compound of this group and has been found at high concentrations in red grapes that have been proposed as a treatment for hyperlipidemia and to prevent fatty liver, diabetes, atherosclerosis and aging [37,38].

Lignans: Lignans are also a subgroup of non-flavonoid polyphenols and are defined as phenylpropanoid dimers, where two phenylpropane units (C6-C3) are linked by their carbon 8 (β - β' link) as represented in Figure I.4 [10].

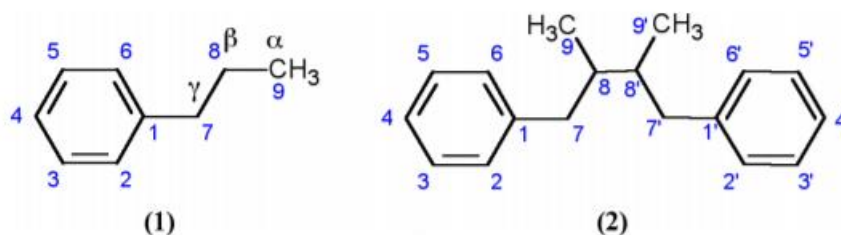


Figure I.4: Phenylpropane unit (1) and lignan structure (2) (taken from [39]).

Lignans are widely distributed in the plant kingdom, being present in more than 55 plant families [41]. They have showed many biological activities such as antiviral, anticancer, anti-inflammatory, antimicrobial, antioxidant, immunosuppressive properties, hepato-protective effect and osteoporosis prevention [39,40,42].

Tannins: Tannins are a group of polyphenols in which some of them found in wine are non-flavonoids like hydrolysable tannins (gallic acid, ellagic acid) while the others are polymers of flavonoids (named proanthocyanidins or condensed tannins) [43]. The basic structure of tannins has two or three phenolic hydroxyl groups on a phenyl ring. There are two main groups of tannins, which are different both in their chemical reactivity and in their compositions:

hydrolysable tannins and condensed tannins are presented in Figure I.5. Hydrolyzable tannins are abundant in broadleaf weeds and some trees, which are industrial sources such as oak tannins, chestnut, etc. They are first characterized by the fact that they can be degraded by chemical (alkaline or acid) or enzymatic hydrolysis. Then, they release a non-phenolic part (often glucose or quinic acid) and a phenolic part, which can be gallic acid or a dimer of the same acid, such as ellagic acid. Condensed tannins are oligomers or polymers of flavane-3-ols (optionally flavane-3,4-diols) derived from (+)-catechin or its isomers. Unlike hydrolysable tannins, they are resistant to hydrolysis and only strong chemical attacks can degrade them. Thus, by hot acid treatment, they are transformed into red pigments and, for this reason, the dimeric or oligomeric forms are called proanthocyanidins. Several studies also indicated that tannins possess potent antioxidant activity as well as exhibit radical scavenging activity that may reduce the risk of many diseases [44,45].

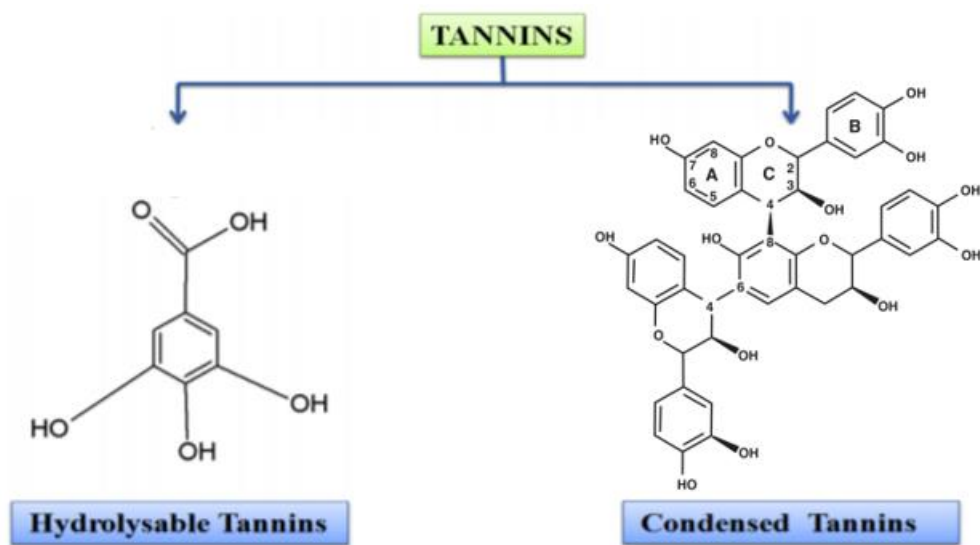


Figure I.5: Types of tannins and their basic structure (taken and modified from [45]).

1.1.1.2. Flavonoids

The general structure of flavonoids is a 15-carbon skeleton, consisting of two phenyl rings (A and B) and a heterocyclic ring C. In plants, flavonoids often exist as *O*- or *C*-glycosides, but *O*-bonding appears more frequently than *C*-bonding [46]. Flavonoids are categorized into various

subclasses including flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins with the same basic structure (Figure I.6) [47].

Flavonoids play a variety of significant roles in plants. They are responsible for the pigments of flowers as well as aroma that attracts pollinating insects [48]. They play a role in plant defense mechanism and especially in plant growth control by inhibiting and activating enzymes [49]. They also play a role in plant heat acclimation and freezing tolerance [49].

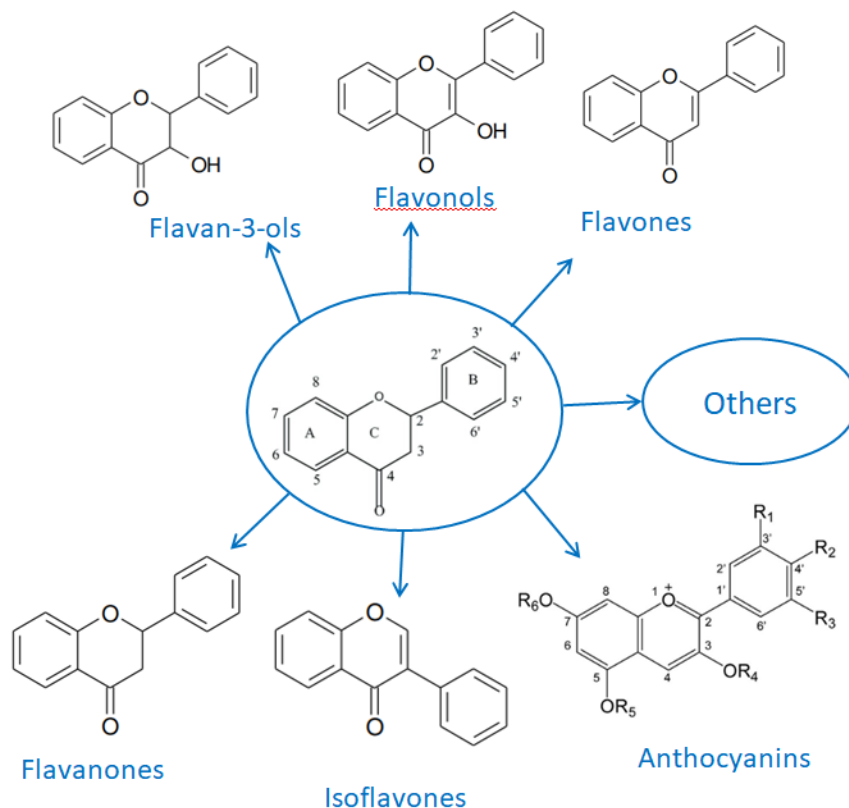


Figure I.6: Basic structure of flavonoid subclasses.

Flavones and flavonols: Flavones are characterized by the presence of a double bond between C2 and C3 in the heterocycle of the flavan skeleton [50], while flavonols are hydroxylated flavone derivatives and distinguished by the presence of hydroxyl functional group in C3 position [51] (Figure I.6).

Flavanones and flavan-3-ols: Compared to flavonols and flavones, these two groups are characterized by the absence of the double bond between C2 and C3 and have the precursor 2-

phenyl-benzopyrone [52,53] (Figure I.6). Flavanols and flavan-3-ols exist as simple monomers such as (+)-catechin and (-)-epicatechin, but oligomers or polymers are called condensed tannins or proanthocyanidins because they release anthocyanidins when they are treated by hot acidic solutions [53].

Anthocyanins: Anthocyanins are the main class of flavonoids and their basic structure is presented in Figure I.6. They are responsible for the colors of most of the petals, fruits and vegetables, and of some varieties of cereals such as black rice. Actually, they appear from red, pink and purple to blue colors to berries, red apples, red grapes, cherries and of many other fruits, red lettuce, red cabbage, onions and eggplants, and also red wine [54]. From a chemical point of view, anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts, the only differences between individual anthocyanins being the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and number of sugars attached to the molecule and the position of this attachment, and the nature and number of aliphatic or aromatic acids bound to the sugars in the molecule [55]. Aglycones of anthocyanins (i.e. the free-sugar molecule) called anthocyanidins, have been described, and the most common six ones found in vegetables and fruits are pelargonidin, cyanidin, delphinidin, petunidin, peonidin and malvidin, depending on the number and position of hydroxyl and methoxyl groups [56]. Anthocyanins are present in plants and plant foods in the form of glycosides that are linked to one or more sugar units. The most common sugars present in these natural pigments are glucose, fructose, galactose, xylose, arabinose and rhamnose [57].

Isoflavones: isoflavones are a group of compounds derived from flavanones (Figure I.6). The main factor that differentiates them from other flavanones is given by the orientation of the C3 position of the benzene ring C [58]. Isoflavones possess both estrogen-agonist and estrogen-antagonist properties, thus, they are classified as phytoestrogens [59]. Isoflavones are the major flavonoids found in legumes, particularly soybeans [60]. The most representative compounds of this class are daidzein and genistein [61].

Other flavonoids: Chalcones and aurones are classified as minor flavonoids. These compounds possess a special structure compared to other subclasses of flavonoids. From the chemical view, chalcones consist of two aromatic rings (A and B) that are interconnected by a three-carbon α,β -unsaturated carbonyl system (Figure I.7). Chalcones showed various biological activities such as antioxidative, antibacterial, antiulcer, antiviral, insecticidal, antiprotozoal, anticancer, cytotoxic and immunosuppressive activity [62,63]. Chemically, aurones consist of three-ring C₆-C₃-C₆ system (A, B and C) with the heterocyclic C-ring being a five-membered ring (Figure I.7). In some flowers and fruits, aurones contribute in pigmentation and color formation and generally produce a yellow color. Aurones possess therapeutic potential including anticancer, antidiabetic, antiviral, anti-malarial, anti-hepatitis, anti-hormonal, anti-diabetic, anti-obesity, anti-cholinesterase inhibitor and anti-inflammatory activity [62,64].

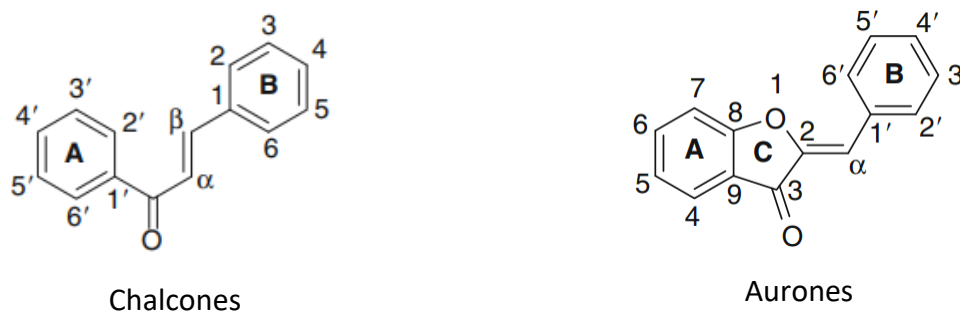


Figure I.7: Basic structure of chalcones and aurones (taken from [62]).

1.1.2. Methods generally used for extracting phenolic compounds from plants

Nowadays, there has been an increasing interest in investigating polyphenols from herbal plants due to their health benefits, either as food supplementation or for cosmetic/pharmaceutical uses. Polyphenols have been reported as the main group of bioactive principles [65]. Extraction is the first step of any medicinal plant study, and the extraction mode plays a significant role on the outcome.

Numerous extraction methods have been investigated in order to extract the bioactive compounds from herbal plants. Traditional methods such as infusion, maceration, percolation and Soxhlet extraction are generally used, while the more advanced ones, such as ultrasound-

assisted extraction, microwave-assisted extraction, or super-critical fluid extraction, are selected with the aim to increase the yield and kinetics of extraction. This part will review the most common used methods based on their principle. Table I.1 shows the difference between all extraction modes including their advantages and disadvantages.

- **Infusion:** Infusion is a very simple, low cost, rapid and green (no organic solvent) technique, in which the active compounds are released in boiled water (Table I.1). Boiled water is poured over the plant materials that are placed in a container. The mixture is allowed to stand for a certain period, while the temperature decreased. Depending on the purpose for which the infusion is being prepared, the infusion time can range from seconds to hours. Finally, the solution may be then filtrated or the plant materials are removed from the liquid [103,104].

- **Maceration:** This method is also simple, depending on the solvent used, the extraction efficiency in organic solvent is better than in water (Table I.1). The plant materials are placed in a double jacket reactor of 1 L volume (or a container) with the solvent, and allowed to stand for a certain period at a fixed temperature and under stirring. In this case, temperature is maintained constant by a cooling system using thermo-regulator machine connected to the double-jacket reactor. The mixture is extracted, then filtrated, the marc is also pressed and the combined liquids are clarified by filtration or decantation after standing [68,103,104].

- **Percolation:** This method generally uses a percolator. The plant material is put in a percolation tube plugged with cotton or fitted with a filter and a stopcock [71]. Water or organic solvent such as methanol or ethanol is added to the plant material that is then allowed to stand for a certain period in a well-closed container, after which the mass is packed and the top of the percolator is closed. The mixed liquid is clarified by filtration or by standing followed by decanting. Percolation is a typical example, where the solvent is pressurized boiling water [69,103] (Table I.1).

Table I.1: Comparison between the different extraction modes (summarized from [66–71]).

Extraction mode	Solvent	Temperature	Pressure	Power	Advantages	Disadvantages	Remarks	Ref
Infusion	Water	80-100°C	-	-	Simple, rapid, low cost, green (no organic solvent), usable by everyone	The extraction yield is generally lower than when using US or MW	Conventional extraction techniques	[72–74]
Maceration	Water	20-80°C	-	-	Simple, low cost, green, usable by everyone	Long process (generally > 1 day), the extraction yield strongly depends on the solvent, temperature and time		[75]
	Methanol (70-100%)	4-60°C			Simple, better extraction yield than in water			[76–79]
	Ethanol (45-95%)	4-60°C			[80–82]			
	Acetone (70-80%)	4-20°C			[83,84]			
Percolation	Ethanol 40-95%	20-100°C	10-20 bar	-	Green technology using water as extractant, suitable for thermally-labile substances, can be scaled up for industrial production, ability to perform extraction at lower operating pressure, very safe to use	High cost for the high-pressure equipment needed for operation at higher pressure		[85]
	Methanol 40-100%							[86]
	Water							[86]
Soxhlet	Ethanol 50-96%	Boiling solvent	-	-	Often used for seeds and fruits, can be scaled up for industrial production	Not usable by everyone, hazardous and flammable organic solvents		[87–89]
	Methanol 40-80%							[74]
	Water						[90,91]	
	Non-polar solvent						[92]	
Ultrasonic	Methanol 25-100%	0-20°C	-	-	Can be performed at atmospheric pressure and room temperature,	Not usable by everyone, may cause undesirable changes in the structure	Preferred advanced extraction technique for alkaloids,	[93,94]
	Ethanol 45-100%	20-50°C					[95]	

	Methanol/Acetone /Water (3.5/3.5/3)	20°C			high extraction yield, rapid, low solvent consumption, very safe to use	of active molecules (due to free radicals formation generated by ultrasound energy), scaling up limited	flavonoids, polyphenols, anthraquinones, saponins, polysaccharides and fatty acids	[96]
	Water	20°C						[97,98]
Super-critical CO ₂	CO ₂ (sometimes with methanol or ethanol as co-solvent)	30-90°C	100-400 bar	-	Green technology with CO ₂ as extractant, can be scaled up for industrial production, suitable for thermally-labile substances, rapid, high extraction yield, very safe to use	High cost for the high-pressure equipment needed, may be difficult to extract polar components, not usable by everyone	Preferred advanced extraction technique for alkaloids, polyphenols, anthocyanins, flavonoids, carotenoids, saponins and essential oils	[99,100]
Microwave	Ethanol 50-96%	60-120°C		80-140W	High throughput with the commercially-available system, suitable for thermally-labile substances, organic solvents and water can be used as an extractant, rapid, high extraction yield	(Amount of sample / volume of extractant) ratio is important, scaling up is challenging, potential explosion risks as a result of pressurization with closed vessel, may cause degradation of polyphenolics due to excessive heat (>100 C)	Preferred advanced extraction technique for alkaloids, polyphenols, polysaccharides, anthocyanins, flavonoids, saponins and essential oils	[101]
	Water	80-100°C		600W				[102]

- **Soxhlet:** Soxhlet extraction is used when the extracted compounds have low solubility in a solvent. With this extraction mode, small amount of solvent is able to dissolve a large amount of chemicals (Table I.1). A Soxhlet extractor has three main parts: a percolator (boiler and reflux) which circulates the solvent, a thimble which retains the solid to be extracted, and a siphon mechanism, which periodically empties the thimble [69,103]. In this process, as presented in Figure I.8, the solvent (1) in flask (2) is heated to reflux. Then, the solvent vaporizes and travels up an arm (3) and floods into the chamber (4) housing the thimble of plant material (5), condenses in the condenser (8) and drips back down into the chamber containing the solid material. The chamber containing the solid material is slowly filled with hot solvent. Some of the desired compound dissolves in the hot solvent. When the liquid in the chamber (4) is almost full and reaches the top of siphon (6), it is emptied into the flask (2) using siphon exit (7). The solvent is returned to the distillation flask and circulated for the next cycle. The cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the extracted compound is concentrated in the distillation flask, the solvent is removed, and the non-soluble portion of the extracted solid remaining in the thimble is also discarded [69,71,103].

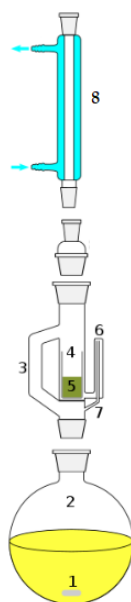


Figure I.8: Extraction by Soxhlet (Source: Wikipedia). 1: Solvent; 2: Still flask; 3: Distillation path; 4: Chamber housing the thimble; 5: Solid or material; 6: Siphon top; 7: Siphon exit; 8: Condenser.

- **Ultrasound-assisted extraction:** This technique (US) is rapid, very safe to use, requires low solvent consumption, and extraction efficiency is generally high (Table I.1). The difference of US with other methods is the impact of high frequency sound (more than 20 kHz) on the plant tissues that can damage the cell wall, facilitating the release of the natural compounds [105]. One disadvantage of this process is that the heat labile compounds may decompose because of the deleterious effect of ultrasound energy on the active constituents of medicinal plants through formation of free radicals, and consequently, undesirable changes in the drug molecules [106]. The plant materials are placed in a double jacket reactor of 1 L volume which is connected to a ultrasonic generator (or a ultrasonic bath), a temperature sensor and a mechanical stirrer. Temperature is maintained constant by a cooling system using thermo-regulator machine connected to the double-jacket reactor. After extraction, the solvent is removed by filtration [104].

- **Microwave-assisted extraction:** This method (MW) is rapid and the extraction efficiency is generally high (Table I.1). It is considered as a novel method for extracting bioactive components using microwave energy with the frequency ranging from 300 MHz to 300 GHz [107]. With controlled pressure and temperature, the usage of microwave heating the solvents and plant tissue can help to increase the kinetics of extraction, reduce the time needed for extraction and decrease the amount of solvent to be used, resulting in increasing the yield of extracted bioactive compounds [108].

An example of MW equipment is presented in Figure I.9. MW is performed on a microwave apparatus using a closed-vessel system. The plant materials are placed in a 500 mL flask containing solvent, for example water. The flask is then placed in the microwave oven with a controlled power. If the solvent is water, the extraction process is often performed at boiling point. After extraction, the solvent is removed by filtration [104].

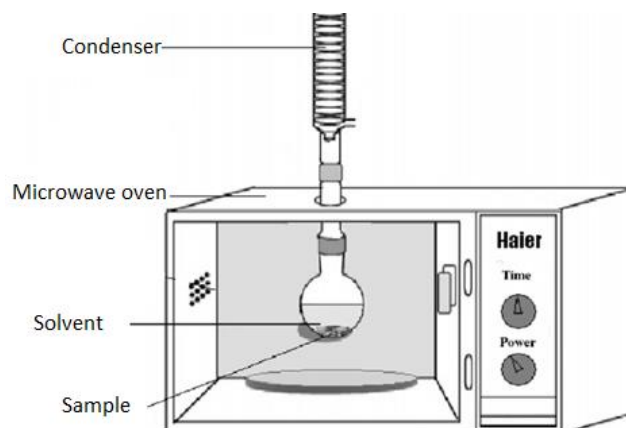


Figure I.9: Microwave-assisted extraction.

- **Supercritical fluid extraction:** This method (SFE) is the most technologically advanced extraction system (green technology). It is very safe to use and extraction efficiency is generally high (Table I.1). In general, SFE is the process of separating one component (the extractant) from another one (the matrix) using supercritical fluids as the extracting solvent. Extraction is usually from a solid matrix, but can be also from liquids. SFE can be used as a sample preparation step for analytical purposes, or on larger scale to remove some unwanted constituents from material (e.g. decaffeination), or to collect the desired components (e.g. essential oils) [109]. The most used supercritical fluid is carbon dioxide CO_2 , which is considered as an excellent solvent for non-polar analytes and it has low toxicity. The extracting conditions for supercritical carbon dioxide are above the critical temperature of $31\text{ }^\circ\text{C}$ that is close to room temperature and critical pressure of 74 bar [68,110]. SFE process is divided into two steps, the first one is extraction and the second one is separation of the extract from the solvent. Figure I.10 shows a schematic diagram of the SFE process. R1 is the CO_2 reservoir, R2 is the buffer tank. The solvent is pumped by B1 and through the extractor filled with the plant materials (E). The co-solvent is stored in R3 (if it is necessary to add). It is pumped by B2 then mixed with the supercritical CO_2 in the mixer (MI) before the inlet of the extractor (E). Based on the convection and diffusion principles, the solutes are extracted. The extraction step (C2) is ended, followed by a separation step and in this process, the reduction of pressure leads to the decrease of the solvation power of the fluid, and the precipitation of the solutes in the separator (S). When the separation step ends, the solvent can be recovered for re-use (C4) in the system [111,112].

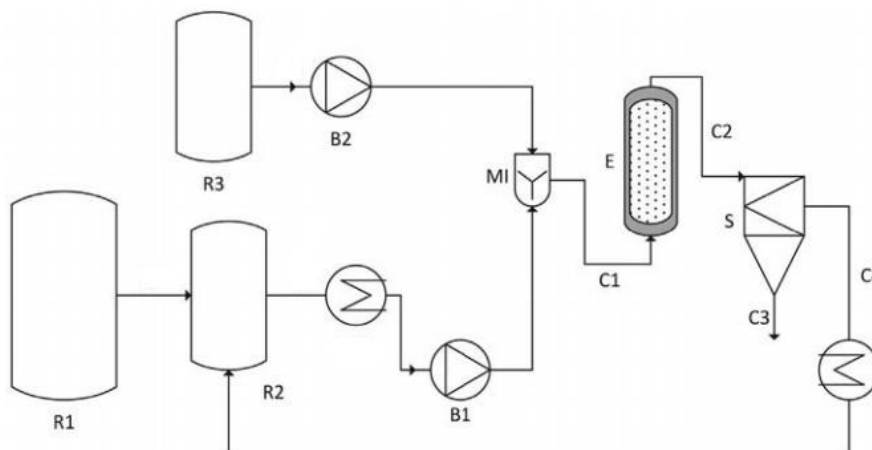


Figure I.10: Schematic diagram of SFE process (taken from [111]). *R*₁, CO₂ reservoir; *R*₂, CO₂ buffer tank; *R*₃, co-solvent reservoir; *B*₁, CO₂ pump; *B*₂, co-solvent pump; *M*_I, mixer; *E*, extractor; *S*, separator; *C*₁, solvent inlet stream in the extractor; *C*₂, solvent + extract outlet stream from the extractor; *C*₃, extract (+co-solvent) outlet stream; *C*₄, CO₂, recycling stream.

1.1.3. Methods for the quantification of plant-extracted phenolic compounds

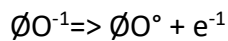
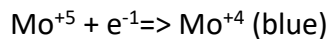
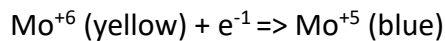
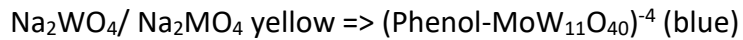
To quantify and characterize the phenolic compounds from plant extracts, various spectrophotometric and chromatographic methods have been investigated. Among spectrophotometric methods, colorimetric methods are widely used for the estimation of total polyphenol, total flavonoids, and total proanthocyanidin content. In addition, different chromatographic techniques have been developed for the separation, identification and quantification of individual phenolic compounds, particularly, ultra-high performance liquid chromatography (UHPLC) equipped with UV-visible spectrophotometry [113]. For the identification and analysis of phenolic compounds, the combination of UHPLC equipped with Electrospray Ionization -Mass Spectrometry (ESI-MS) or High-Resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS) is very useful, accurate and suitable method to determine, and identify the phenolic compounds of plant extracts [114].

1.1.3.1. Colorimetric methods

1.1.3.1.1. Folin-Ciocalteu reagent assay for total phenolic content

Folin-Ciocalteu assay, described by Singleton & Rossi [115] is the most common method used for determining the total polyphenol content (TPC) in plant extracts using gallic acid as a

standard. The Folin-Ciocalteu reagent consists of a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) in which the molybdenum and the tungsten are in the 6^+ oxidation state. This method bases on a single electron transfer mechanism. The mixture is reduced during the oxidation of phenols into a mixture of blue tungsten oxides (W_8O_{23}) and molybdenum oxides (Mo_8O_{23}) according to the following reactions:



where \emptyset refers to phenyl moiety and the exponent refers to the global charge of the species.

TPC is estimated after reaction of the sample with diluted Folin-Ciocalteu reagent and sodium carbonate. The reaction with phenolic compounds produces a blue color which has a maximum absorbance at around 725-765 nm. TPC is calculated from calibration curve using gallic acid as a reference and is expressed as milligram gallic acid equivalent per gram of dry extract (mg GAE/g extract) as following:

$$TPC = C_g \times \frac{V}{m} \quad (1)$$

where C_g is the concentration of gallic acid in mg/mL giving the same absorbance as the plant extract, V is the volume of plant extract in mL, and m is the mass of pure dry plant extract in g.

I.1.3.1.2. Aluminium chloride colorimetric method for total flavonoids content

Aluminium chloride colorimetric method, described by Lamaison & Carnet [116], is widely used for determining the total flavonoids content (TFC) in plant extracts using quercetin (for example) as a standard. The principle of this method bases on the formation of stable complexes of aluminium chloride with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Furthermore, aluminium chloride also forms acid labile complexes with

the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. Rutin, hyperoside, or catechin can also be chosen as suitable standard for building the calibration curve. TFC is calculated from calibration curve of standard solution and is expressed as milligram quercetin (Q) equivalent per gram of dry extract (mg Q/g extract) as following:

$$\text{TFC} = C_q \times \frac{V}{m} \quad (2)$$

where C_q is the concentration of quercetin in mg/mL giving the same absorbance as the plant extract, V is the volume of plant extract in mL, and m is the mass of pure plant extract in g.

I.1.3.1.3. Acid butanol assay for total proanthocyanidins content

Acid butanol assay, described by Porter *et al.* [117], is widely chosen for determining the proanthocyanidin oligomers (or condensed tannins) content (OPC) in plant extracts. Proanthocyanidins become colored when exposed to strongly acidic conditions such as hydrochloric or sulfuric acid, and under these conditions, are hydrolyzed at the inter-flavan bonds and converted to anthocyanidins and flavanols as shown in Figure I.11. The red-colored anthocyanidins absorb UV light at a wavelength around 450- 540 nm and can be used for OPC quantification.

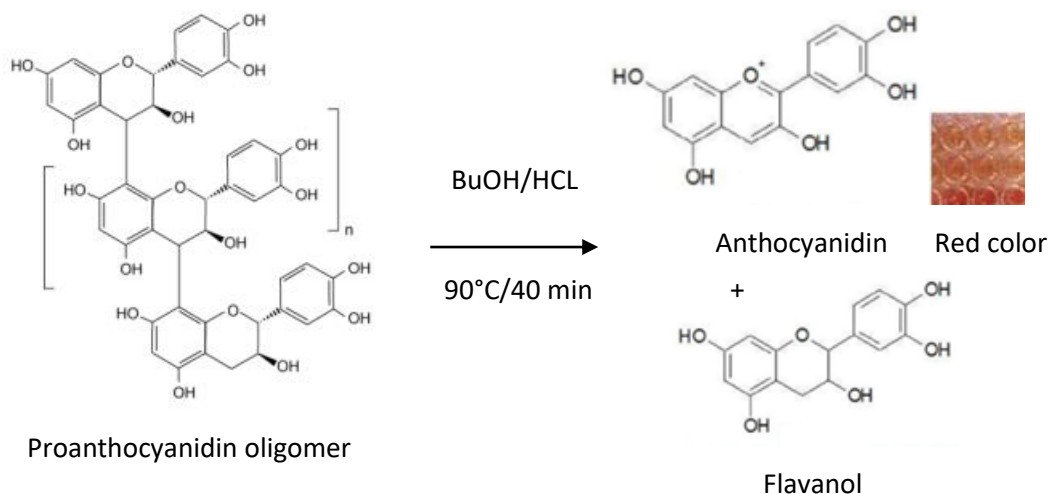


Figure I.11: Hydrolysis reaction on proanthocyanidin oligomers (summarized from [118]).

OPC is calculated from calibration curve using cyanidin as standard and is expressed as milligram of cyanidin equivalent per gram of dry extract (mg cyanidin /g extract) as following:

$$\text{OPC} = C_c \times \frac{V}{m} \quad (3)$$

where C_c is the concentration of cyanidin in mg/mL giving the same absorbance as the plant extract, V is the volume of plant extract in mL, and m is the mass of pure plant extract in g.

1.1.3.2. Separation, identification and analysis methods of polyphenols

1.1.3.2.1. Ultra-High Performance Liquid Chromatography (UHPLC)

UHPLC is an advanced technique of liquid chromatography, combining various innovative technologies to obtain higher resolution, speed and sensitivity. It performs at higher pressure than that used in HPLC and uses fine particles (less than 2.5 μm size) and mobile phases at high linear velocities [119]. UHPLC is a method able to separate compounds from complex mixtures based on polarity, solubility, and size of each compound. UHPLC equipped with UV-visible spectrophotometry (diode array detector or DAD) provides extensive information on the structure of phenolic compounds [120].

For the identification of phenolic compounds, the most frequent way is to compare the retention time of a particular compound with standard molecules. Based on the UV/visible spectral data of the separated compounds, a peak can be identified if it matches with the retention time and the UV/Vis spectrum of a standard molecule [121]. Finally, the analytes are quantified based on calibration curves for each standard, which are plotted for different concentrations. Due to the lack of phenolic compound standards and the existence of isomers, this method has limitations and may lead to wrong identifications. Thus, Liquid Chromatography coupled to Mass Spectrometry (LC-MS) is a better tool to identify the structure of phenolic compounds [122].

I.1.3.2.2. Mass Spectrometry (MS)

Mass Spectrometry (MS) is an analytical technique that gives access to the molar mass of (unknown) compounds based on the molecular ions and characteristic fragment ions. Its high sensitivity and the possibility of combination with chromatography techniques indicate that MS is the most suitable physicochemical method to study and identify natural products like phenolic compounds [123].

The principle of mass spectrometry bases on the specific mass-to-charge ratio (m/z) of ionized molecules. The first step in MS analysis of compounds is to produce ions issued from the compounds in gas phase, often by loss or addition of protons. Then, these ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. The MS instrument is composed of three major components: an ion source, an analyzer and a detector as presented in Figure I.12 [124,125].

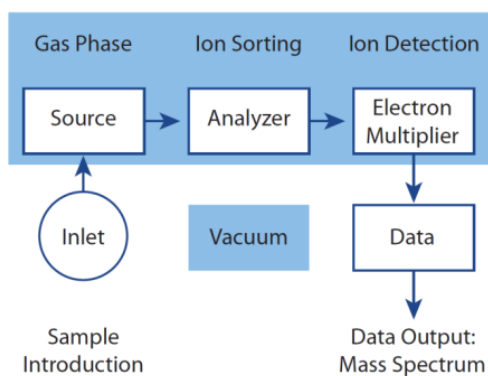


Figure I.12: Principle of mass spectrometry (taken from [125]).

Various instruments have been investigated depending on the availability of different sources and analyzers and the specificity of the source and analyzer to provide a large range of analytical possibilities. For the analysis of phenolic compounds, several sources such as electron impact (EI), chemical ionization (CI), fast atom bombardment (FAB), atmospheric pressure ionization (API) including atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI), and electrospray ionization (ESI) are used. APCI and ESI are the most commonly used ionization methods for the identification and characterization of phenolic

compounds, and can be used to establish polyphenol fingerprints of complex plant extracts [124,127].

Tandem MS (or MS/MS) is a technique using two or more (MS^n) mass analyzers to break down selected ions (precursor ions) into fragments (product ions). The fragments then reveal some aspects of the chemical structure of the precursor ion. Figure I.13 shows the basic principle of MS-MS. The samples are ionized by ESI, MALDI, EI, etc...to generate a mixture of ions, named precursor ions, of specific mass-to-charge ratio (m/z), that are selected (MS_1) and then analysed (MS_2). This technique can increase the sensitivity and selectivity of detection and is useful for identification of phenolic compounds [128].

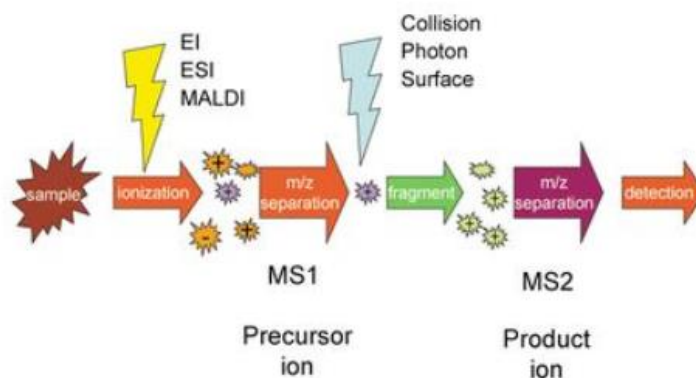


Figure I.13: Schematic diagram of tandem mass spectrometry (MS/MS) (taken from [129]).

Fourier-transform ion cyclotron resonance (FTICR) mass spectrometry is a high-resolution technique that can be used to determine masses with very high accuracy. FT-ICR-MS provides the highest level of mass accuracy compared to other forms of mass spectrometer [130]. This technique is based on the principle of ion cyclotron resonance (ICR). In general, the externally formed ions are trapped in a FT-MS analyzer cell, which is at the center of a strong magnetic field. A cyclotron motion results when the ions spiral rapidly around the magnetic field lines. The cyclotron frequencies of the different ions depend on their mass/charge ratios and the uniform magnetic field strength. Detection of the ions is performed by recording image current signals, which are induced by the ions passing a pair of receiver plates. Frequencies are recorded more

precisely than any other physical parameter leading to mass spectra with very high resolution and mass accuracies [124,128,130].

1.1.4. Methods used for the assessment of the antioxidant activity

There are several methods developed to determine the antioxidant activity. The principle of those assays bases on the measurement of the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. Each method uses different chromogenic redox reagents with different standard potentials.

1.1.4.1. DPPH assay

The DPPH (common abbreviation for 2,2-diphenyl-1-picrylhydrazyl) method is the most commonly used to determine the antioxidant activity of herbal plant extracts. The use of DPPH assay provides a simple and rapid way for the evaluation of antioxidant activity by spectrophotometry. The principle of this method bases on the electron-transfer from a phenolic compound to DPPH radical to produce purple-colored solution as shown in Figure I.14. Purple color appears due to the delocalization of DPPH molecule with an absorption wavelength at around 515 nm. The DPPH radical scavenging ability is calculated by the following equation [131,132]:

$$\text{DPPH Scavenged (\%)} = \frac{AB-AA}{AB} \times 100 \quad (4)$$

where AB is the absorbance of blank at $t= 0$ min and AA is the absorbance of the antioxidant at $t= 30$ min. A calibration curve using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, its structure presented in Figure I.14) as a reference standard is plotted with % DPPH scavenged versus concentration of standard antioxidant and is expressed as Trolox equivalent antioxidant capacity (TEAC) values [133].

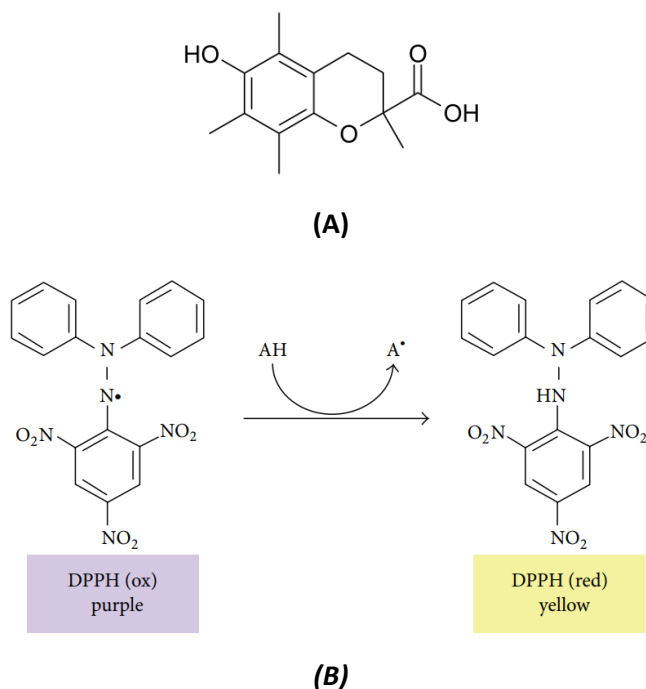


Figure I.14: Structure of Trolox (A) and reduction of DPPH radical (B) (taken from [134]).

1.1.4.2. ABTS assay

The ABTS (common abbreviation for 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) assay is another important spectrophotometric technique widely used for measuring the antioxidant activity of herbal plant extracts. The principle of this assay bases on the discoloration of $ABTS^+$ during its oxidation by antioxidant compounds. The ABTS radical scavenging ability is evaluated within a fixed period. Firstly, the reaction between ABTS solution with potassium persulfate in water generates directly the radical $ABTS^+$ which is stored in the dark at room temperature for 12 hours before use. $ABTS^+$ solution is then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. After adding the plant extract solution or antioxidant compounds to the diluted $ABTS^+$ solution, the absorbance of the resulting mixture can be followed by spectrophotometry at 734 nm. Percent inhibition of absorbance at 734 nm is calculated using the following equation [135–137]:

$$ABTS^+ \text{ scavenging effect (\%)} = \frac{AB-AA}{AB} \times 100 \quad (5)$$

where AB is the absorbance of ABTS radical in methanol and AA is the absorbance of ABTS radical in methanol in the presence of the sample extract/standard. Trolox is used as a reference standard.

1.1.4.3. FRAP assay

The FRAP (common abbreviation for ferric reducing antioxidant power) assay is another spectrophotometric technique often used for measuring the antioxidant activity of herbal plant extracts. This assay is different from ABTS and DPPH assays, because the mechanism of this assay is based on electron transfer instead of hydrogen atom transfer. The principle is based on the reduction of Fe³⁺-TPTZ (2,4,6-tripyridyl-s-triazine) complex to Fe²⁺-tripyridyltriazine and the color changing from colorless to blue colored complex formed by the action of electron donation from antioxidants under low acidic condition. The color change is monitored by spectrophotometry at a wavelength of 593 nm. By comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe³⁺, the FRAP value is obtained and expressed as mg of Trolox equivalent per gram of sample. A calibration curve using Trolox as reference standard is plotted with different concentration of FeSO₄ versus concentration of standard antioxidant [138,139].

1.1.5. General medicinal and pharmacological properties

Phenolic compounds as well as flavonoids are the most common natural compounds of medicinal plants [140]. They are interesting because of their benefits for human health, curing and preventing many diseases. A large number of medicinal and pharmacological properties such as antioxidant, anti-inflammatory, antihypertensive, cardioprotective, anti-hyperlipidemic, anti-arthritic, anti-allergic and antimicrobial activities [6–15] have been attributed to bioactive compounds contained in plants. Table I.2 summarizes the pharmacological properties of some major phenolic compounds. The specific pharmacological properties of hawthorn, *Chrysanthellum americanum* and blackcurrant leaves are given in more detailed in the next sections.

The next step of this bibliographical part aims at presenting a detailed overview of the chemical composition, as well as the medicinal and pharmacological properties of three medicinal plants studied in this thesis: hawthorn, blackcurrant leaves and *Chrysanthellum americanum* that have complementary pharmacological effects.

Table I.2: Plant sources, bioactive compounds and pharmacological properties of some major phenolic compounds (taken and modified from [141]).

Phenolics	Some source(s)	Active components	Pharmacological properties	References
Phenolic acids	<i>Barringtonia racemosa</i> , <i>Cornu officinalis</i> , <i>Cassia auriculata</i> , <i>Polygonum aviculare</i> , <i>Punica granatum</i> , <i>Rheum officinale</i> , <i>Sanguisorba officinalis</i> , <i>Terminalia chebula</i> , <i>Cinnamomum cassia</i> , <i>Lawsonia nermis</i> , <i>Foeniculum vulgare</i> , <i>Ipomoea turpethum</i> , <i>Lavandula officinalis</i> , <i>Rosmarinus officinalis</i> , <i>Crataegus</i> , <i>Ribesnigrum</i>	Gallic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, syringic acid, ferulic acid, caffeic acid, p-coumaric acid, chlorogenic acid, sinapic acid, salicylic acid, protocatechuic acid, vanillic acid, syringic acid	Cancer-preventive, cardio-protective, anti-ulcer, antioxidant, antiseptic, antimicrobial, anti-inflammatory, anti-spasmodic, anti-depressant, treatment of dyspepsia	[17,142–147]
Stilbenes	<i>Reynoutri ajaponica</i> , <i>Polygonum cuspidatum</i> , <i>Rhodomyrtus tomentosa</i> , <i>Passiflora edulis</i>	Resveratrol, oxyresveratrol, piceatannol, petrostilbene, pinosylvin	Chemoprotective, Cancer-preventive, antioxidant, anti-aging, anti-angiogenic, neuroprotective, anti-fungal, immune modulation	[148–150]
Lignans	<i>Podophyllum peltatum</i> , <i>Podophyllum emodi</i> , <i>Phyllanthus amarus</i> , <i>Linum usitatissimum</i> , <i>Schisandra chinensis</i>	Matairesinol, enterolactone, podophyllotoxin	Antimicrobial, antioxidant, anti-inflammatory, Cancer-preventive, antiviral, anti-pyretic, diuretic, analgesic, anti-rheumatic, antineoplastic, phytoestrogenic, cathartic, immunosuppressive, hepato-protective, cardioprotective, treatment of osteoporosis, rheumatoid arthritis, gastric and duodenal ulcers	[40,151,152]
Condensed tannins	<i>Rhus javanica</i> , <i>Quercus lusitanica</i> , <i>Geranium thunbergii</i> , <i>Tellima grandiflora</i> , <i>Mimosa tenuiflora</i> , <i>Caesalpinia pyramidalis</i> , <i>Agrimonia pilosa</i> , <i>Crataegus</i>	Procyanidin, prodelphinidins	Antioxidant, Cancer-preventive, anti-diarrhoea, anti-inflammatory, antibacterial	[146,153–155]
Hydrolysable tannins	<i>Neoregelia laevis</i> , <i>Diospyros crassiflora</i> , <i>Embelia schimperi</i> , <i>Polygonatum falcatum</i>	Gallotannins, ellagitannins	Anti-diarrhoea, antidote empoisoning by heavy metals	[151,154]
Flavonoids			Anti-inflammatory, Antioxidant, Cancer-preventive, anti-viral, anti-allergic, anti-stress, estrogenic antibiotic, antidiarrheal, antiulcer	[156,157]

Flavones	<i>Scutellaria baicalensis</i> , <i>Biophytum umbraculum</i> , <i>Gentiana triflora</i> , <i>Torenia hybrid</i> , <i>Crataegus</i> , <i>Ribes nigrum</i> ,	Luteolin, apigenin, baicalein, chrysin, diosmin, rutin, sibelin and their glycosides	Anti-tuberculosis, antimicrobial, antioxidant, anti-carcinogenic, anti-inflammatory, anti-proliferative, anti-angiogenic, anti-estrogenic, improvement of blood circulation	[145–147,154,158]
Flavonols	<i>Aconitum tanguticum</i> , <i>Sutherlandia frutescens</i> , <i>Acacia nilotica</i> , <i>Terminalia arjuna</i> , <i>Aloe barbedensis</i> , <i>Moringa oleifera</i> , <i>Ficus religiosa</i> , <i>Crataegus</i> , <i>Ribesnigrum</i>	Quercetin, kaempferol, myricetin, morin, galangin, and theirglycosides	Anti-mutagenic, antioxidant, antimicrobial, anti-carcinogenic, anti-hypertensive, anti-allergic, anti-depressant, anti-diabetic, enzyme-inhibitors, neuroprotective, cardioprotective, chemopreventive	[142,145–147,159,160]
Flavanones and flavanonols	<i>Harungana madagascariensis</i> , <i>Arachis hypogaea</i> , <i>Hemizonia increscens</i> , <i>Eriodictyon glutinosum</i> , <i>Thymus vulgaris</i> , <i>Sophora flavescens</i>	Naringenin, hesperetin, eriodictyol, fisetin, taxifolin, and theirglycosides (e.g. naringin, hesperidin, andliquiritin)	Antioxidant, antiproliferative, estrogenic, radio-protective, anti-inflammatory, analgesic, anti-hypercholesterolemic, anti-carcinogenic, anti-microbial, hepatoprotective	[161,162]
Flavan-3-ols, Flavan-3,4-diols	<i>Humulus lupuhts</i> , <i>Sambucus nigra</i> , <i>Camellia sinensis</i> , <i>Onobrychis viciifolia</i> , <i>Pseudotsuga menziesii</i> , <i>Mammea longifolia</i> , <i>Crataegus</i>	(+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, (-)-teracacidin, proapigeninidins, propelargonidins, proluteolinidins, procyanidins, prodelphinidins, andprotricitinidin	Antioxidant, antimicrobial, antiviral, anticarcinogen, anti-inflammatory, neuroprotective	[146,147,163–165]
Isoflavones	<i>Psoralea corylifolia</i> , <i>Trifolium pratense</i> , <i>Erythrina variegata</i> , <i>Pueraria lobata</i>	Genistein, daidzein, glycitein, fromononetin, and theirglycosides	Cancer-preventive, neuroprotective, cardioprotective	[142,166,167]
Anthocyanins and Anthocyanidins	<i>Hibiscus rosasinensis</i> , <i>Vacciniumspp.</i> , <i>Saussureamedusa</i> , <i>Crataegus</i> , <i>Ribesnigrum</i>	Cyanidin, delphinidin, malvidin, peonidin, pelargonidin, petunidin	Antioxidant, anti-platelet, chemopreventive, antimicrobial, anticarcinogenic, proapoptotic, neuroprotective, cardioprotective, antihepatotoxic, anti-lipolytic, vasodilatory, enhance memory	[3,145–147,168–170]
Aurones	<i>Antirrhinum majus</i> , <i>Coreopsis spp.</i> , <i>Cosmos spp.</i> , <i>Dahlia spp.</i> ,	Hispidol, sulfuretin, aureusidin, auresin, maritimetin, letopsin, bracetin	Cancer-preventive, antimicrobial, antioxidant, anti-hormonal, anti-inflammatory, anti-leishmanial, enzyme inducing/ inhibitoryactivities, potential for treating malaria, diabetes, viral infections, skin diseases, Alzheimer's disease	[171–173]

I.2. Hawthorn

Hawthorn, the common name for *Crataegus* species in *Rosaceae* family, is a part of a genus of spiny shrubs trees, mostly growing to 5 to 15 m tall, with small pome fruit and usually thorny branches native to temperate regions in the Northern Hemisphere in Europe, Asia and North America [174]. It consists of bright green leaves, white flowers and bright red berries (Figure I.15). Depending on the classified interpretation, from 200 to 1000 species of *Crataegus* have been listed [175].

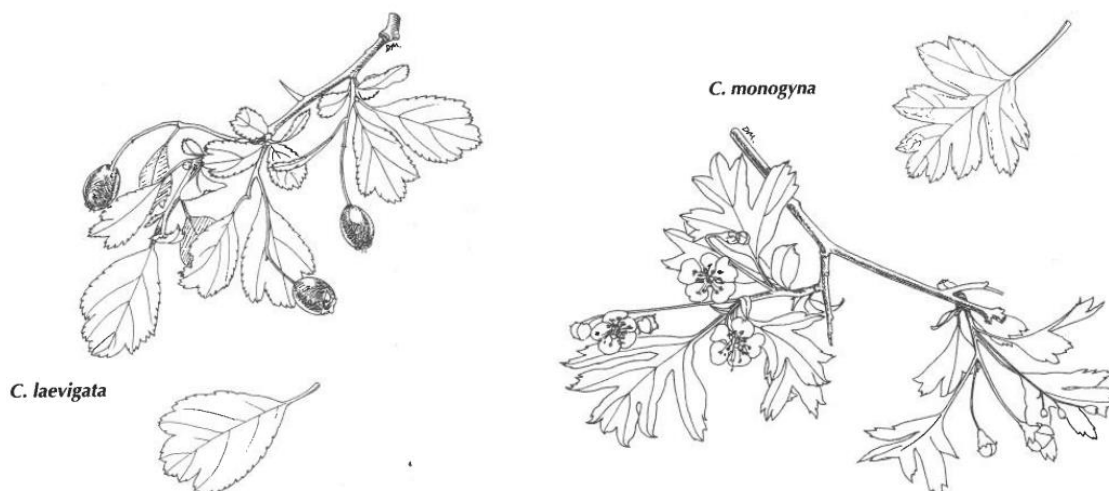


Figure I.15: Hawthorn leaves and flowers (*Crataegus laevigata* = *Crataegus oxyacantha*, and *Crataegus monogyna*) (taken from [176]).

Hawthorn has been used in Asia and Europe as food and folk medicine for a long period. In the framework of traditional medicine used in China, India and some European countries, the properties and effects on health of hawthorn extracts have been well documented. Hawthorn is generally used for its stimulating properties of digestion and gastric function and for the improvement of blood circulation [85,145–147,177–179]. For example, dried fruits are often consumed fresh or processed into jams, jellies, soft drinks, candies and canned fruits, especially in Asia [155]. In Europe and North America, flowering tops (leaves and flowers) of hawthorn are used for their astringent, antispasmodic, cardiotonic, diuretic, hypotensive, vasodilative, sedative, antiatherosclerotic and antihyperlipidemic properties [6–15]. In addition, in the recent

clinical trials, it is demonstrated that hawthorn could be used as complimentary therapy for class I through III heart failure according to the classification of the New York Heart Association (NYHA) [11,12,13].

1.2.1. Chemical composition

Generally, various studies indicated that *Crataegus spp* showed high phenolic content which is considered as the main group of bioactive principles [174]. Among them, the proanthocyanidins and other glycosylated derivatives of flavonoids are responsible for the pharmacological activity of hawthorn fruits, leaves and flowers [175]. Table I.3 summarizes the chemical composition generally found in the different parts and species of hawthorn plants [182].

1.2.1.1. Flavonoids

1.2.1.1.1. Flavones

Flavones are one of the most important subgroups of flavonoids of hawthorn. Some flavones have been reported in hawthorn which are a series of compounds whose aglycones are apigenin or luteolin (Figure I.21) [182]. Vitexin (apigenin-8-*C*-glucoside) and its derivatives such as vitexin-2''-*O*-rhamnoside have been found the most popular flavones in hawthorn [183,184]. Vitexin and vitexin-2''-*O*-rhamnoside (Figure I.16) were reported in *Crataegus laevigata*, *Crataegus rhipidopylla* and their hybrid *Crataegus macrocarpa* [184], while vitexin-2''-*O*-rhamnoside was also identified in the leaves of another species such as *Crataegus pinnatifida* var. *major* [183]. Vitexin, vitexin-2''-*O*-rhamnoside and isovitexin were also identified in the flowering tops of *Crataegus monogyna*, *Crataegus pentagyna* and *Crataegus laevigata* [182]. Besides, orientin and isoorientin were the main compounds in *Crataegus pentagyna* [185]. Furthermore, four flavonoid ketohexosefuranosides, pinnatifinoside A, B, C, and D were isolated and identified from the leaves of *Crataegus pinnatifida* [186].

Table I.3: Phenolic compounds reported in hawthorn (taken from [182]).

Species/varieties (site of collection)	Organ	Procyanidins	Flavonols	C-glycosyl flavones	Other phenolics
<i>C. pinnatifida</i> (China)	Fruit	(-)-Epicatechin; Dimers: B2, B5; Trimers: C1 and two unknowns; Tetramer: D1	Hyperoside; Isoquercetin; Rutin; Quercetin	Vitexin	Chlorogenic acid; Ideain
	Leave	(-)-Epicatechin	Hyperoside; Rutin; Quercetin	Vitexin	Six lignans
<i>C. pinnatifidavar Major</i> (China)	Fruit	(-)-Epicatechin; Dimers: B2, B5; Trimers: C1 and two unknowns; Tetramers: D1 and 7 unknowns; Pentamers: 4 unknowns; Hexamers: 2 unknowns; Glycosides: 2 hexosides of monomer, 7 hexosides of dimer, 1 hexoside of trimer, 2 hexosides of tetramer	Hyperoside; Isoquercetin; Quercetin; Quercetin-di-(rhamnosyl) hexoside; Quercetin-rhamnosylhexoside; Rutin	Vitexin	Chlorogenic acid; Ideain; Protocatechuic acid
	Leave	(-)-Epicatechin; Dimers: B2, B5; Trimers: C1	Hyperoside; Isoquercetin; Rutin; 4'''-rhamnosylrutin	2''-O-acetylvitexin; 3''-O-acetylvitexin; 6''-O-acetylvitexin; 4'''-O-glucosylvitexin; Pinnatifida A, B, C, D; Vitexin; Vitexin-2''-O-rhamnoside; Vitexin-4''-O-glucoside;	Chlorogenic acid
<i>C. brettschneideri</i> (China)	Fruit	(-)-Epicatechin; Dimers: B2, B5; Trimers: C1 and two unknowns; Tetramers: D1	Hyperoside; Isoquercetin		Chlorogenic acid; Ideain;
<i>C. scabrifolia</i> (China)	Fruit	(-)-Epicatechin; Dimer: B2, B5; Trimer: C1 and two unknowns; Tetramer: D1	Hyperoside; Isoquercetin; Rutin	Vitexin	
	Leave		Hyperoside; Isoquercetin; Rutin; 4'''-O-rhamnosylrutin	4''-O-glucosylvitexin; Vitexin; Vitexin-2''-O-rhamnoside;	
<i>C. cuneata</i> (China)	Fruit		Hyperoside		
	Leave		Hyperoside; Isoquercetin; Rutin; 4'''-O-rhamnosylrutin	4''-O-glucosylvitexin; Vitexin; Vitexin-2''-O-rhamnoside; Vitexin-4''-O-glucoside	Gallic acid; Hydroxylbenzoic acid; protocatechuic aldehyde
<i>C. pinnatifida var. Psilosa</i> (China, Korea)	Fruit		Hyperoside; Isoquercetin	Vitexin	
	Leave		Hyperoside; Quercetin-3-O-rhamnosylgalactoside	Vitexin; Vitexin-2''-O-rhamnoside	
<i>C. hupehensis</i> (China)	Fruit		Hyperoside; Rutin	Vitexin	
	Leave		Hyperoside; Rutin		Chlorogenic acid

<i>C. sanguinea</i> (China)	Fruit		Hyperoside; Rutin	Vitexin	
<i>C. maximowiczii</i> (China)	Fruit		Hyperoside; Rutin	Vitexin	
<i>C. wilsonii</i> (China)	Leave		Hyperoside; Rutin		Chlorogenic acid
<i>C. aurantia</i> (China)	Leave		Hyperoside; Rutin		Chlorogenic acid
<i>C. kansuensis</i> (China)	Fruit	(-)-Epicatechin	Hyperoside		
	Leave			Rutin	Chlorogenic acid
<i>C. grayana</i> (Finland)	Fruit	(-)-Epicatechin; Dimers: B2, B5; Trimers: C1 and two unknowns; Tetramers: D1	Hyperoside; two quercetin-pentosides; Methoxykaepferol-methylpentosylhexoside; Quercetin-hexoside acetate; Quercetin-rhamnosylhexoside		
	Leave	(-)-Epicatechin; Dimers: B2, B5; Trimers: C1 and two unknowns; Tetramers: D1	Hyperoside; two quercetin-pentosides; Methoxykaepferol-methylpentosylhexoside; Quercetin-hexoside acetate; Quercetin-rhamnosylhexoside; Methoxykaempferol-pentoside; Quercetin-rhamnosylhexoside		
<i>C. monogyna</i> (Europe)	Leave		Hyperoside; Isoquercetin; Rutin; 4''-O-rhamnosylrutin., Sexangularetin-3-O-glucoside; Kaempferol-3-O-glucoside	4''-avetylvitexin-2''-O-rhamnoside; 4''-O-glucosylvitexin; isovitexin; Vitexin; Vitexin-2''-O-rhamnoside;	Chlorogenic acid
	Pollen		Sexangularetin; Sexangularetin-3-neohesperidoside; Kaempferol-3-neohesperidoside		
<i>C. laevigata</i> (Europe)	Fruit	(-)-Epicatechin; Dimers: B2, B4 and B5; Trimers: C1, epicatechin-(4 β →6)-epicatechin-(4→8)-epicatechin, epicatechin-(4 β →8)-epicatechin-(4 β →6)-epicatechin; Tetramers: D1; Pentamers: (-)-epicatechin units linked through C-4 β /C-8 bonds.			

	Leave	(-)-Epicatechin; Dimers: B2, B4 and B5; Trimers: C1, epicatechin-(4 β →6)-epicatechin-(4→8)-epicatechin, epicatechin-(4 β →8)-epicatechin-(4 β →6)-epicatechin; Tetramers: D1; Pentamers: (-)-epicatechin units linked through C-4 β /C-8 bonds.	Hyperoside; Isoquercetin; Rutin	Acetylvitexin-2''-O-rhamnoside; Isovitexin; Vitexin; Vitexin-2''-O-rhamnoside	Chlorogenic acid; caffeic acid
	Flower	(-)-Epicatechin; Dimer: B2, B4 and B5; trimers: C1, epicatechin-(4 β →6)-epicatechin-(4→8)-epicatechin, epicatechin-(4 β →8)-epicatechin-(4 β →6)-epicatechin; Tetramers: D1; Pentamers: (-)-epicatechin units linked through C-4 β /C-8 bonds.	Hyperoside; Isoquercetin; Rutin		
<i>C. macrocarpa</i> (Europe)	Leave		Hyperoside; Isoquercetin; Rutin	Vitexin; Vitexin-2''-O-rhamnoside; (S)- and (R)-eriodictyol-7-O-glucoside; Luteolin-7-O- β -D-glucuronide	Chlorogenic acid
<i>C. azarolus.</i> <i>vararonia</i> (Tunisia)	Flower		Hyperoside; Isoquercetin; Rutin; Spiraeoside; Quercetin		Chlorogenic acid
<i>C. azarolus. var</i> (Tunisia)	Flower		Hyperoside; Isoquercetin; Rutin; Spiraeoside; Quercetin		Chlorogenic acid
<i>C. rhipipophylla</i> (Europe)	Leave		Hyperoside; Isoquercetin; Rutin	Isovitexin; Vitexin-2''-O-rhamnoside; (S)- and (R)-eriodictyol-7-O-glucoside; Luteolin-7-O- β -D-glucuronide	Chlorogenic acid
<i>C. pentagyna</i> (Europe)	Leave		Hyperoside; Isoquercetin; Rutin; Sexangularetin-3-O-glucoside	Isorientin; Isoorientin-2''-O-rhamnoside; Isovitexin; Orientin; Orientin-2''-O-rhamnoside; Vitexin; Vitexin-2''-O-rhamnoside.	

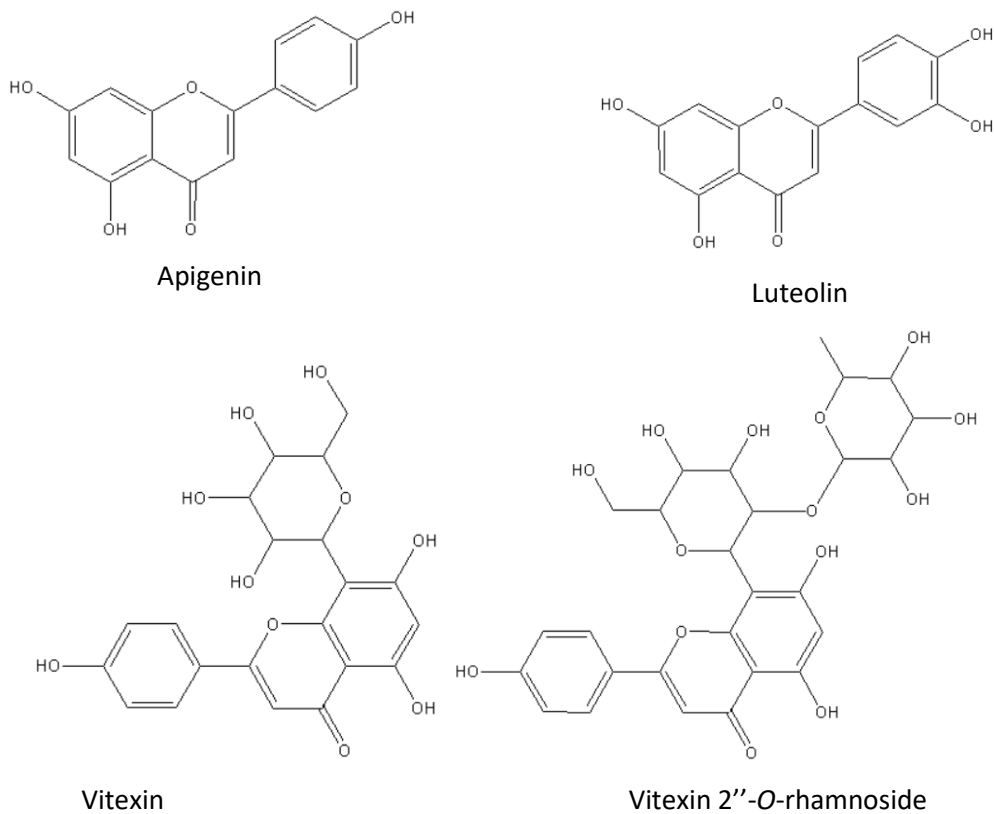
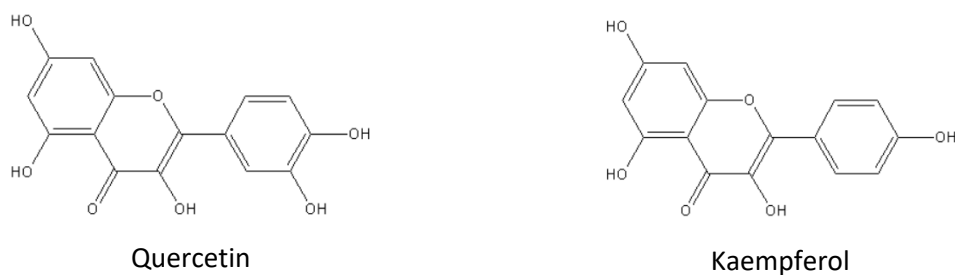


Figure I.16: Structure of some flavones in hawthorn (Source: Pubchem).

I.2.1.1.2. Flavonols

Flavonols are other important subclasses of flavonoids of hawthorn. Kaempferol, quercetin, and 8-methoxykaempferol were reported as main flavonol aglycones in hawthorn [187]. Hyperoside in *Crataegus monogyna*, *Crataegus oxyacantha* and *Crataegus laevigata* was found the major flavonol component [85]. Rutin, hyperoside, and quercetin were present in the leaves of *Crataegus pinnatifida* [188] (Figure I.17).



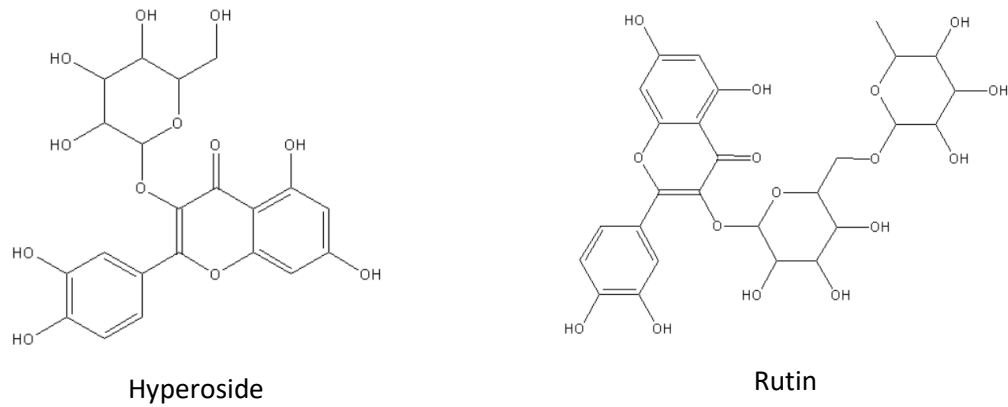


Figure I.17: Structure of some flavonols in hawthorn (Source: Pubchem).

I.2.1.1.3. Flava-3-ols (flavanols)

Some flavanols found in *Crataegus monogyna* are (\pm)-epicatechin and (\pm)-catechin, while (-)-epicatechin is an abundant component in the floral buds of *Crataegus monogyna*. Figure I.18 shows the structure of some flavanols present in hawthorn [98,189].

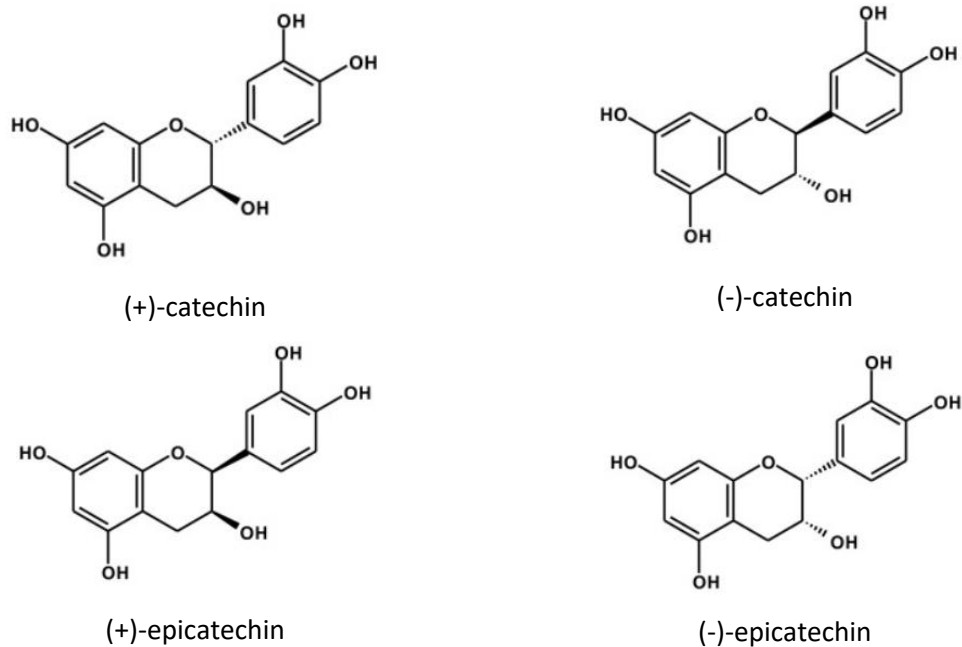
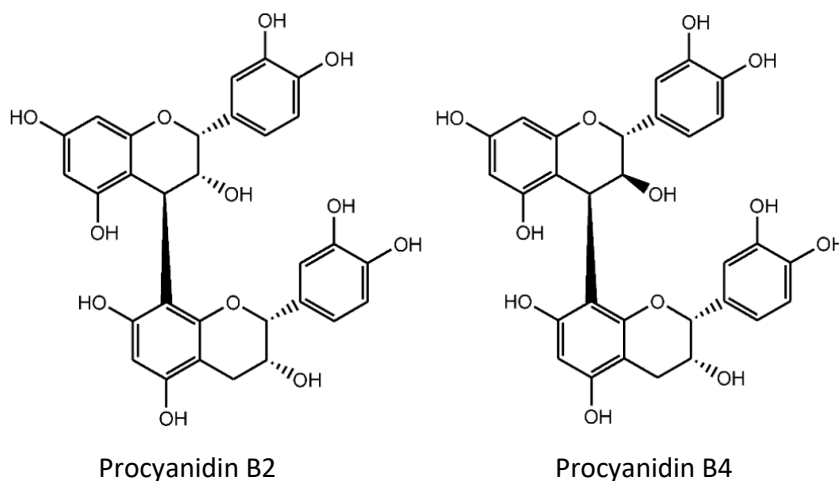


Figure I.18: Structure of some flavanols in hawthorn (taken from [190]).

I.2.1.1.4. Procyanidins and proanthocyanidin oligomers

Proanthocyanidins (condensed tannins), including the lesser bioactive and bioavailable polymers (four or more catechin moieties) represent a group of condensed flavan-3-ols, such as procyanidins, prodelphinidins and propelargonidins [117,191]. Oligomeric proanthocyanidins (OPCs) strictly refer to oligomers of catechins. OPCs are found in most plants and are considered the main active components. They are commonly used in the human diet [98]. Most proanthocyanidins (PAs) are linked between C-4 of upper unit and C-6 or C-8 of the next flavan A-ring which are often named as B-type PAs, while proanthocyanidins represented by an additional (2 β →O→7) interflavan bond are categorized as A-class PAs [192].

Up to now, over thirty oligomeric procyanidins have been isolated and identified in the leaves and fruits of hawthorn, the concentration of individual compounds show variation depending on the part and species of the plants [182]. Several dimers such as procyanidin B2, B4, B5, trimers such as procyanidin C1, epicatechin-(4 β →8)-epicatechin-(4 β →6)-epicatechin and epicatechin-(4 β →6)-epicatechin(4 β →8)-epicatechin, tetramer such as procyanidin D1 and hexamer F have been isolated and identified in the leaves, flowers and fruits of *Crataegus laevigata* [97] and *Crataegus pinnatifida* [193], while procyanidins B2 and epicatechin were identified as major phenolic compounds in *Crataegus pinnatifida* [194] and *Crataegus grayana* [195], as presented in Figure I.19.



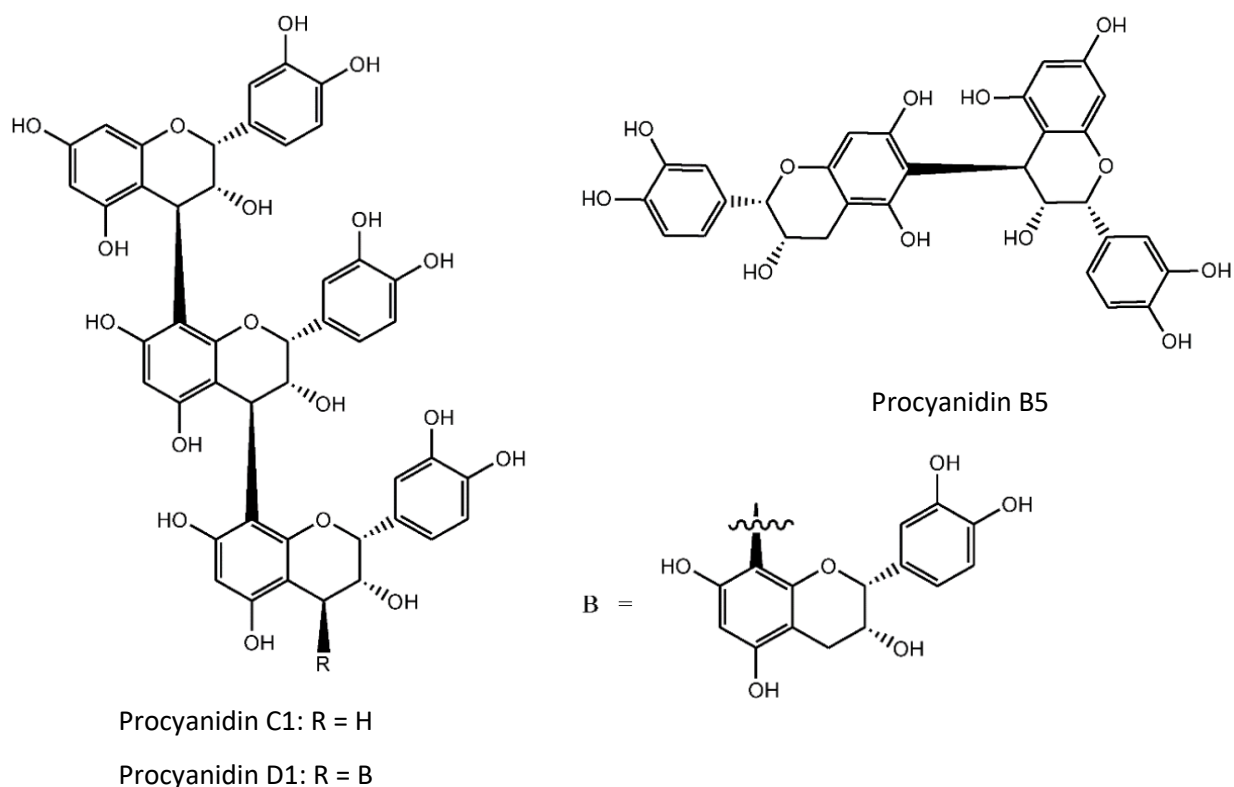


Figure I.19: Structure of several procyanidins (taken from [177]).

I.2.1.2. Others

Chlorogenic acid (or 3-O-caffeoylquinic acid) has been frequently found in the fruits and leaves of all hawthorn species [146,182]. Besides, its isomer 5-O-caffeoylquinic acid (or neochlorogenic acid) was identified only in the fruits of *Crataegus grayana* [195]. Seven phenolic acids, i.e. protocatechuic, *p*-hydroxybenzoic, caffeic, chlorogenic, ferulic, vanillic and syringic acid, were also found in the extracts of *Crataegus monogyna* leaves [92]. Figure I.20 presents the structure of chlorogenic acid and 5-O-caffeoylquinic acid.

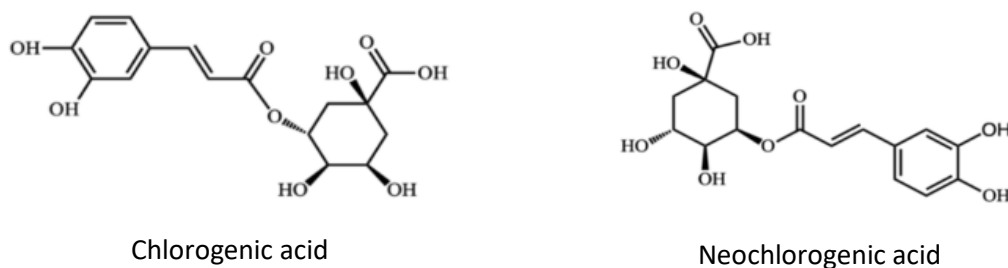


Figure I.20: Structure of chlorogenic acid and 5-O-caffeoylquinic acid (taken from [196]).

1.2.2. Pharmacological properties

1.2.2.1. Effects on Cardiovascular diseases and vascular system

Many recent clinical trials indicated that hawthorn extracts are used as dietary supplements and herbal medicines in the treatment of patients with chronic heart failure. Pittler et al. investigated eight trials including 632 patients with chronic heart failure (New York Heart Association (NYHA) classes I to III) [197]. The results of meta-analysis showed that hawthorn extract increased the maximal workload of the patients, several symptoms such as fatigue, listlessness and dyspnea were also improved considerably. Another randomised, placebo-controlled, double-blind clinical study reported the efficacy of *Crataegus* extract WS® 1442 in 40 male and female patients with congestive heart failure defined as NYHA class II and treated for 12 weeks. The data showed that *Crataegus* extract WS® 1442 was safe and well tolerated and was clinically effective to those patients [198]. Holubarsch et al. also concluded that WS® 1442 was safe and had no severe side effects observed [199].

Some publications reported the cardio-protective effects of hawthorn extract both in *in vitro* and *in vivo* studies. Swaminathan et al. [200] investigated the cardioprotective effect in a crystalloid perfused heart model of ischaemia/reperfusion injury. The results indicated that hawthorn extract was able to scavenge superoxide, hydroxyl and peroxy radical, improved the cardiac contractile function, lessened the size of myocardial infarct, and reduced the activities of creatine kinase and lactate dehydrogenase. The hawthorn extract may play an important role to inhibit the apoptotic pathways in order to protect the cardiovascular system. In another *in vivo* study, Jayalakshmi et al. [201] evaluated the cardioprotective effect of alcoholic extract hawthorn berries on isoproterenol-induced myocardial infarction. It was demonstrated on rats that hawthorn extract prevented an increased level of lipid peroxides, maintained the balance of antioxidant status and inhibited the development of coronary artery diseases.

Hawthorn extract showed beneficial effects on the coronary circulation. Schwinger et al. investigated the mode of inotropic action of *Crataegus* extract WS 1442 in human myocardium. They found that *Crataegus* extract WS 1442 increases the force of contraction in human myocardium [202]. Schussler et al. investigated the effects of the major flavonoids from *Crataegus* species on coronary flow, heart rate and velocity of contraction and relaxation [203]. As a result, the *O*-glycosides, named luteolin-7-glucoside, hyperoside and rutin at the concentration of 0.5 mmol/L, showed positive effect on the relaxation velocity and the coronary flow.

Alcoholic extracts from *Crataegus oxyacantha* can induce anti-arrhythmic effects against digoxin-induced arrhythmias in anesthetized Wistar rats [204]. The results showed that the duration of different arrhythmia types, such as premature atrial contractions, ventricular extrasystole, ventricular tachycardia, ventricular fibrillation in the experimental group was considerably shorter than that in the control group, it means that the arrhythmia symptoms were reduced (Table I.4). Arterial blood pressures in both experimental and control groups decreased, however, arterial blood pressure decreasing in the experimental group was much more compared to the control group (Figure I.21). Another study investigated the effect of chloroform, ethylacetate, and methanol extracts of the flowering tops of *Crataegus meyeri* on ischaemic arrhythmias in anaesthetized rats. The results exhibited the reduction of arrhythmia induced by myocardial ischaemia in open-anesthetized male rats [205]. Muller et al. indicated that *Crataegus* extract prolongs the action potential duration and delays the recovery of the maximum upstroke velocity in guinea-pig papillary muscle, meaning that *Crataegus* have some anti-arrhythmic properties [206].

Table I.4: Effect of ethanol extract of *Crataegus oxyacantha* on the digoxin-induced arrhythmias in experimental and control groups (taken from [204]).

Arrhythmias type	Duration of arrhythmias (min)		
	Control group (n = 7) Digoxin with 0.9% NaCl infusion	Experimental group (n = 8) Digoxin with extract infusion	P
Premature atrial contractions	13.45 ± 5.21	6.23 ± 2.53	<0.001
Ventricular extrasystole	48.56 ± 7.19	29.25 ± 1.98	<0.001
Ventricular tachycardia	41.25 ± 7.03	39.19 ± 8.02	<0.001
Ventricular fibrillation	23.12 ± 6.71	11.94 ± 4.46	<0.001

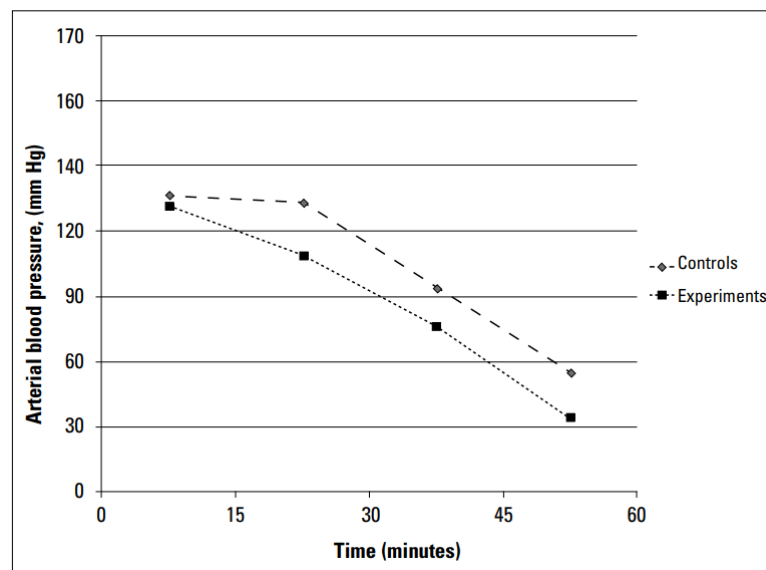


Figure I.21: Arterial blood pressure variation in the study population between the experimental group and control group (taken from [204]).

Endothelial dysfunction and inflammation has an important role in the growth of atherosclerosis and cardiovascular diseases. Hawthorn extracts may help to protect the

endothelial function and inhibit the atherosclerosis by regulating the apoptosis-associated genes expression [207]. Khodja et al. also showed that chronic intake of *Crataegus* reduces the prostanoid-mediated contractile responses and improves the increase of oxidation stress resulting in the prevention of the aging-related endothelial dysfunction [208]. The stenosis of coronary arteries due to atherosclerosis becomes clinically manifest in pathologies such as angina pectoris or cardiac infarction. Another *in vitro* study demonstrated that hawthorn extract inhibited the migration and proliferation of vascular smooth muscle cells, therefore, hawthorn extracts play a significant role to prevent angioplasty-related restenosis (reoccurrence of stenosis) [209].

1.2.2.2. Anti-hypertensive effect

The anti-hypertensive effect of hawthorn extracts has been investigated in *in vitro*, *in vivo* and in clinical studies. Some clinical trials indicated that hawthorn extract showed a decrease of moderate blood pressure. Walker et al. investigated the effect of hawthorn extract for hypertension in type 2 diabetes patients who were randomised to daily 1200 mg hawthorn extract or placebo for 16 weeks. The first randomized controlled trial demonstrated a hypotensive effect of hawthorn extract in diabetic patients [210], i.e. the diastolic blood pressure in hawthorn group showed greater reduction than that in the placebo group after 16 weeks (Figure 1.22). Belz et al. assessed the efficaciousness of camphor *Crataegus* berry combination (CCC) on orthostatic hypotension in patients with orthostatic dysregulation. It was demonstrated that CCC drops showed a decrease of the orthostatic fall in blood pressure compared with placebo and had a significant effect on diastolic blood pressure after 1, 3 and 5 minutes orthostasis [211]. Asgary et al. also indicated that hawthorn extract significantly decreased the systolic and diastolic blood pressure in patients with primary mild hypertension [212].

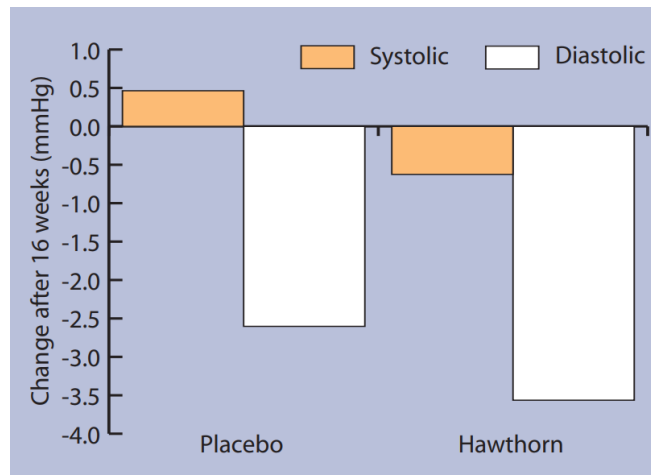


Figure I.22: Change in blood pressure after 16 weeks of daily supplementation with hawthorn extract compared with placebo. Diastolic blood pressure was significantly reduced in the hawthorn group compared to placebo group ($P = 0.035$) (taken from [210]).

Amel et al. investigated the hypotensive effect of leaves extracts of *Crataegus azarolus*. The results showed a decrease of mean arterial blood pressure in anesthetized rats, providing scientific support for treating the hypertension [77]. Another investigation focusing on the effects of the hyperoside-rich fraction obtained from *Crataegus tanacetifolia* leaves extracts on the blood pressure and the structure of the coronary artery wall in L-NAME-induced hypertensive rats. It was observed that the hyperoside-rich fraction lessened the increase in mean arterial blood pressure and also decreased the thickness of the media player of the coronary blood vessels caused by *N*-nitro-L-arginine methyl ester after 4 weeks treatment [213]. In China, Myakuru (MR) is an herbal medicine produced from three herbal components including *Crataegus pinnatifida* extract (85%), *Panaxnotoginseng* (commonly referred to Chinese ginseng or notoginseng) extract (10%) and *Ginkobiloba* extract (5%). Iwaoka et al. revealed the preventive effects of MR on hypertension in rats. They observed that systolic blood pressure in the MR-treated rats compared with the control group showed a significant decrease and cerebral blood flow in the MR-treated group was considerably higher than in the control group [214].

1.2.2.3. Anti-hyperlipidemic effect

Hawthorn extracts have shown their potential to decrease the low-density lipoprotein cholesterol, serum cholesterol and the plasma level of triacylglycerols in both animal models and studies in hyperlipidemic humans. Xu et al. investigated the effects of hawthorn fruit compounds on lowering blood lipid levels in atherosclerotic Apolipoprotein E-deficient mice [215]. The results indicated that hawthorn fruits significantly decreased the ratio between low-density lipoprotein cholesterol (LDL-C) and serum cholesterol (SC) as well as the reduction of triglyceride levels. Ye et al. evaluated the anti-hyperlipidemic effect induced by high-fat diet in mice using hawthorn fruit extract [216] and identified four compounds from hawthorn ethanolic extract (quercetin, hyperoside, rutin and chlorogenic acid) that were likely responsible for this property. They reported that those compounds have synergy effects on the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase that produces cholesterol and the lowering-lipid efficaciousness. Another investigation demonstrated that red ginseng and *Crataegii fructus* (CF, *Crataegus pinnatifida* fruits) showed a considerable decrease of blood triglyceride (TG) and total cholesterol levels in Triton WR-1339-induced hyperlipidemic mice and a reduction of serum TG levels in corn oil-induced hypertriglyceridemic mice [217] (Figure I.23). Kuo et al. investigated the effect of the extract from *Crataegus pinnatifida* fruits on obesity and dyslipidemia induced by high-fat diet on hamsters. It was demonstrated that the treatment by hawthorn extract decreased the food intake, the body weights, and the weights of adipose tissues of hamsters, and reduced the plasma levels of total cholesterol (TC), triglyceride (TG), LDL-cholesterol (LDL-C) [166]. Niu et al. also confirmed the improvement of fruit extract of *Crataegus pinnatifida* on obesity and hyperlipidemia in hamsters. They showed that the extract of *Crataegus pinnatifida* fruit enhances the expression of peroxisome proliferator-activated receptor alpha to facilitate β -oxidation-related enzymes in liver for lipid degradation and blood lipid reduction [218]. Akila et al. focused on the synergistic effect of alcoholic extracts obtained from berries of *Crataegus oxyacantha* on the lipid parameters in the growth of aortic lesions in diet-induced atherosclerosis on rats. Hawthorn extract

showed prevention of lipid increase in the serum and heart, leading to a considerable decrease in lipid accumulation in the liver and aorta [219].

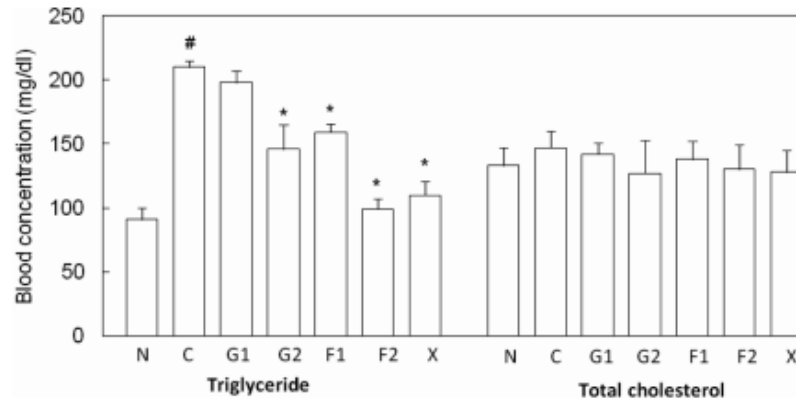


Figure 1.23: Effect of red ginseng (RG), and *Crataegii fructus* (CF), in Triton WR-1339-induced hyperlipidemic mice. The normal group (N) received a vehicle alone. The control group (C) was administered with Triton WR-1339 alone. The samples were orally G1, 50 mg/kg red ginseng; G2, 100mg mg/kg; F1, 50 mg/kg *Crataegii fructus*; F2, 100 mg/kg *Crataegii fructus*; X, 10 mg/kg Xenical; Values indicate mean \pm S.D (n=6). # Significantly different, compared with normal group ($p < 0.05$). *Significantly different, compared with control group ($p < 0.05$) (taken from [217]).

There are some *in vivo* and *in vitro* studies about the anti-hyperlipidemic effect of hawthorn extracts from flowering tops. Wang et al. indicated that the flavonoids fraction, which was extracted and isolated from the leaves of *Crataegus pinnatifida* revealed the inhibitory effects on triglyceride (TG) and glucose absorption and suppressed the accumulation of TG and free fatty acid *in vivo*. Besides, it also suppressed the gene expression of peroxisome proliferator-activated receptor-gamma (PPAR γ), sterol regulatory element-binding protein 1c (SREBP 1c), activating protein 2 (aP2) and adiponectin *in vitro*. Thus, *Crataegus pinnatifida* leaves may play a significant role in the treatment of type 2 diabetics and hyperlipidemics [220]. One *in vivo* study on a larval zebra fish model, showed that hawthorn leaves and flowers have a potential effect on the cardiac output as well as on intravascular cholesterol levels [221].

1.2.2.4. Antioxidant activity

A huge amount of investigations on the anti-oxidant and free radical scavenging activities of different *Crataegus* species has been published in the last decades. Bahorun et al. indicated that the highest Trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) values were observed when total polyphenol, flavonoids and proanthocyanidin were maximum. They also showed that the FRAP value has strong correlation with TPC, TFC and OPC, while the TEAC value is strongly associated with TFC and not with TPC and OPC [222]. Bernatoniene et al. concluded that epicatechin and catechin showed the highest contribution to radical scavenging activity compared to other bioactive components in both aqueous and ethanolic extracts of *Crataegus monogyna*. Moreover, ethanolic extracts contained higher TPC than aqueous extracts and thus revealed stronger radical scavenging activity [75]. Amel et al. indicated that ethyl acetate extract of *Crataegus azarolus* possessed the highest TPC and TFC contents. Correspondingly, this extract showed the highest antioxidant activity in several assays, such as DPPH radical scavenging assay, ABTS radical scavenging activity assay, and hydroxyl radical scavenging assay [77].

The anti-oxidant activity and free radical scavenging activities from different parts of hawthorn plant were also compared. Guo et al. investigated the antioxidant activity of peel, pulp and seed fractions of 28 Chinese fruits in ferric reducing/antioxidant power assay (FRAP assay). The results indicated that hawthorn pulp possesses the highest FRAP value and hawthorn peel and seed have the highest anti-oxidant activity among all fruits studied [223]. Keser et al. determined the anti-oxidant activity of hawthorn aqueous and ethanol extracts of leaves, flowers and ripened fruits. They showed that the aqueous and ethanol extracts of *Crataegus monogyna* fruits had the highest reducing power and metal chelating activity [224]. Froehlicher et al. compared the antioxidant properties of dried flowers, flowering tops (leaves and flowers), fresh and dried fruits of hawthorn using 2,2'-azinobis (3-ethylbenzothiazoline- 6-sulphonic acid assays (ABTS⁺), DPPH radical scavenging assays

and Cu²⁺-induced human low density lipoprotein (LDL) oxidation assays. Antioxidant activity was found higher for dried flowers and flowering tops than for fresh and dried fruits and the antioxidant activity had the best correlation with TPC [225].

1.2.2.5. Anti-inflammatory effect

Many researches focused on the anti-inflammatory effects of hawthorn extracts both *in vivo* and *in vitro*. Tadic et al. have been found that hyperoside and isoquercetin are the major flavonoids in the ethanolic extract of hawthorn berries and revealed a strong anti-inflammatory effect in a model of carrageenan-induced rat paw oedema[85]. It was seen that the anti-inflammatory effects of the water fraction of hawthorn fruit might be attributed to the down-regulation of cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF- α), interleukin 1 β (IL-1 β), and interleukin 6 (IL-6) expression in LPS-stimulated RAW 264.7 cells [226] (Figure I.24). Kao et al. also demonstrated that hawthorn extract exhibits the potential anti-inflammatory activity both *in vitro* and *in vivo* [227]. *In vitro*, the extract from dried fruits of *Crataegus pinnatifida* reduced the release of prostaglandin E₂ (PGE₂) and nitric oxide induced by lipopolysaccharide (LPS) in macrophage RAW 264.7 cells. *In vivo*, after 5 days, rats pretreated with hawthorn extract showed significant reduction of plasma levels of the hepatic enzyme markers alanine aminotransferase and aspartate aminotransferase induced by LPS. The extract also reduced the neutrophil infiltration and liver necrosis induced by LPS. In addition, it was indicated that the extract lessened the hepatic expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) induced by LPS in rats.

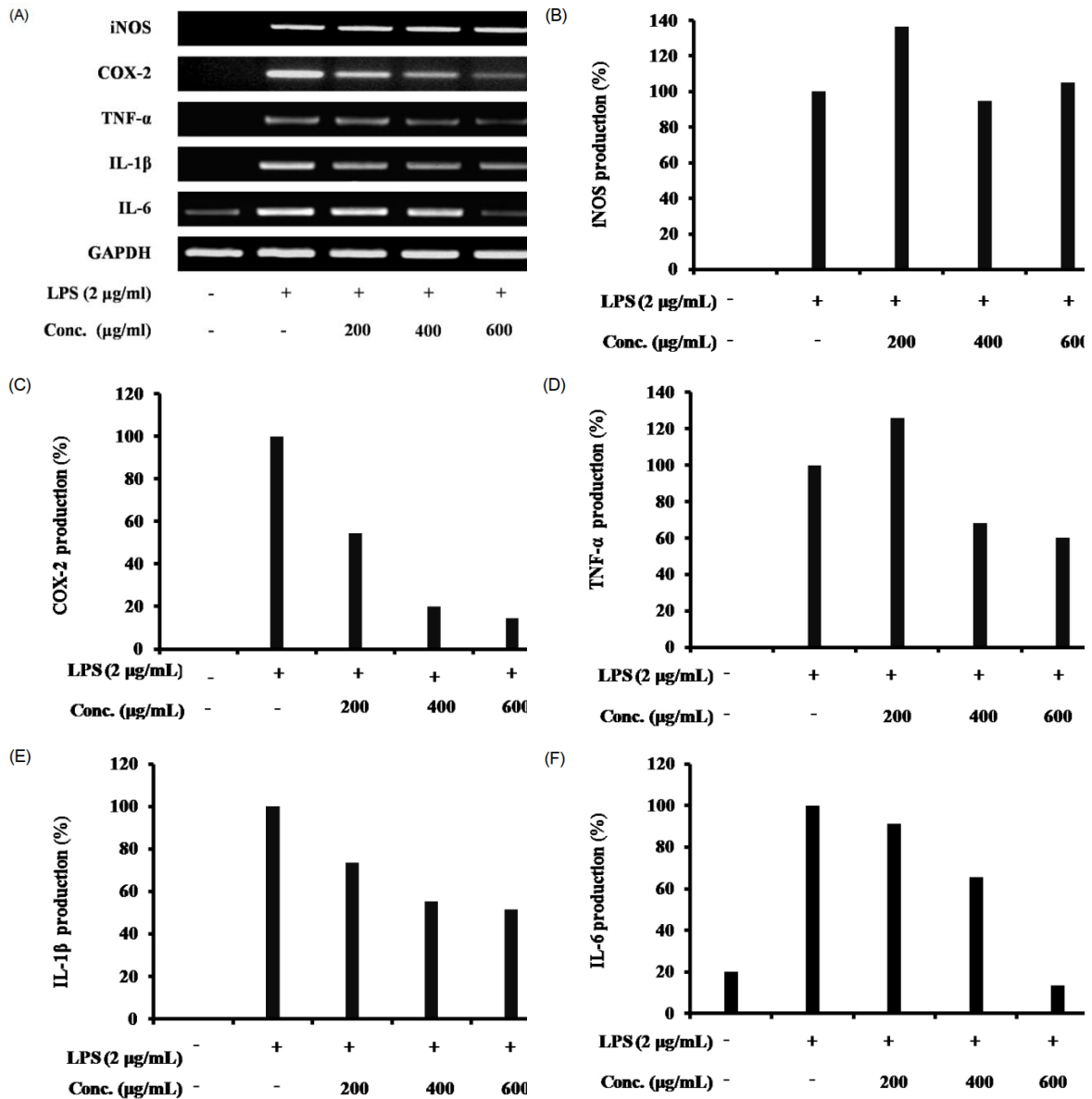


Figure 1.24: Effect of hawthorn fruit water fraction on nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF- α), interleukin 6 (IL-6) and interleukin 18 (IL-18) mRNA production in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 2 $\mu\text{g/ml}$ of lipopolysaccharide (LPS) and various concentrations of hawthorn fruit fraction for 24h. (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of the expression of iNOS, COX-2, TNF- α , IL-18, IL-6 mRNA; (B), (C), (D), (E) and (F) Quantification of iNOS, COX-2, TNF- α , IL-18, IL-6 expression levels were achieved with densitometric measurement (taken from [226]).

There are some investigations of anti-inflammatory effect in the leaves of hawthorn extract. Zeynep et al. investigated the anti-inflammatory effects of the ethanol extract from *Crataegus orientalis* leaves. The results showed that the dry extract of *Crataegus orientalis* leaves plays a significant role in the treatment of painful and inflammatory diseases [228]. Carmen et al. indicated that a main triterpene fraction named cycloartenol, isolated from the twigs, stems and leaves of *Crataegus monogyna* showed a decrease of hind-paw oedema, in agreement with anti-inflammatory property [229].

1.2.2.6. Other effects

Belkhir et al. demonstrated that the hawthorn extract from *Crataegus azarolus* leaf and fruit peel possesses a highly antibacterial activity, especially against *Staphylococcus aureus* and *Streptococcus faecalis* [230]. Orhan et al. determined the antibacterial, antifungal and antiviral activities of the extract from several *Crataegus* species including *Crataegus aronia* var. *aronia*, *Crataegus monogyna* ssp. and *Crataegus pseudoheterophylla*. Data showed that the extract is strongly effective against *Candida albicans* and *Herpes simplex* virus [79].

Tadic et al. investigated the gastro-protective activity of hawthorn extract using an ethanol-induced acute stress ulcer in rats with ranitidine as a reference drug. The results revealed that hawthorn ethanol extract produced dose-dependent gastro-protective activity [85].

1.3. Black currant (*Ribes nigrum* L.)

Blackcurrant (*Ribes nigrum* L.) is a woody shrub belonging to the genus *Ribes* classified in the *Saxifragaceae* family, but taxonomic studies placed the genus in the *Grosulariaceae* family due to morphological characteristics such as inferior ovaries, syncarpous gymnosperm and fresh fruit [231]. Blackcurrant leaves are alternate, simple, from 3 to 5 cm broad and long, with five palmate lobes and a serrated margin. The flowers

are produced in racemes known as “strigs” up to 8 cm long containing ten to twenty flowers, each about 8 mm in diameter. Each flower has a hairy calyx with yellow glands. All parts of the plant are strongly aromatic. Blackcurrant is widely cultivated across temperate Europe, Russia, New Zealand, parts of Asia and to a lesser extent North America [160] (presented in Figure I.25).

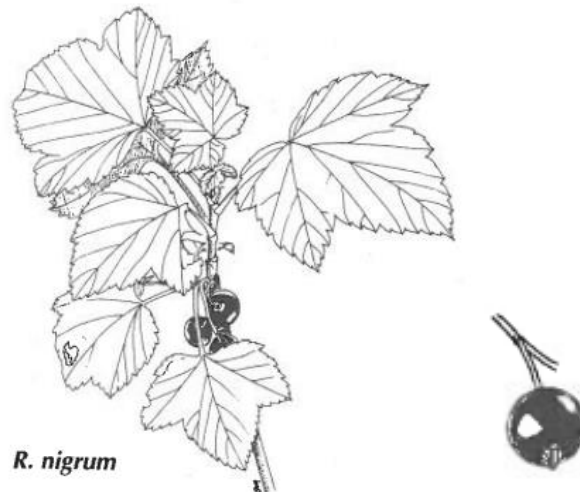


Figure I.25: Blackcurrant leaves and fruits (taken from [176]).

Blackcurrant contains a valuable source of bioactive compounds especially anthocyanins, proanthocyanidins, phenolic acids derivatives and other beneficial phenolic compounds including flavonoids such as flavonols (glycosides of myricetin, quercetin, kaempferol and isorhamnetin) and favan-3-ols. Moreover, recent investigations indicated that blackcurrant shows high contents of vitamin C which contributes along with bioactive phenolic compounds to the high antioxidant activity [232]. Since ancient times, blackcurrant leaves have been used in European folk medicines to treat rheumatism, arthritis and respiratory problems [233]. Nowadays, many studies have been reported showing that health promoting properties of blackcurrant can be attributed to its anti-oxidant, anti-inflammatory, and anti-microbial activities, as well as vasomodulatory, anti-haemostatic

and muscle-relaxing effects, improvement of visual function and neuroprotective and Cancer-preventive activities [3,4,16,17,160,234,235].

1.3.1. Chemical composition

1.3.1.1. Phenolic acids

Mattila et al. indicated that common hydroxybenzoic acids such as p-hydroxybenzoic, protocatechuic, vanillic, and gallic acids have been found in blackcurrant berries, while protocatechuic and gallic acids are the major ones [236]. Another study demonstrated that hydroxycinnamic acids including p-coumaric, caffeic, ferulic and sinapic acids were present in blackcurrant leaves and berries, while p-coumaric acid is the most abundant phenolic acid [17,237,238]. The esters of hydroxycinnamic acids such as chlorogenic acid (5-*O*-caffeoylquinic acid, neochlorogenic acid (3-*O*-caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-caffeoylquinic acid) are present in the buds, leaves, and fruits of blackcurrant [239,240] (see their structures in Figures I.2 and I.20).

1.3.1.2. Flavonoids

1.3.1.2.1. Anthocyanins

Anthocyanins play an important role in plant and are responsible for the color of fruits, vegetables and other parts of the plant. The anthocyanin content may vary and depend on many factors including genetic and agronomic ones [231]. Slimestad et al. have reported fifteen anthocyanin structures found in the extract of blackcurrant berries, including the 3-*O*-glucosides and the 3-*O*-rutinosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin, cyanidin 3-*O*-arabinoside, and the 3-*O*-(6''-p coumaroylglucoside)s of cyanidin and delphinidin. The 3-*O*-glucosides and the 3-*O*-rutinosides of delphinidin and cyanidin are the most abundant anthocyanins (Figure I.26) [235,241–243]. Vagiri et al. identified these four major anthocyanins in the buds, leaves and fruits of black currant. In buds and leaves, the 3-*O*-glucoside and the 3-*O*-rutinoside of cyanidin are the most abundant anthocyanins [240].

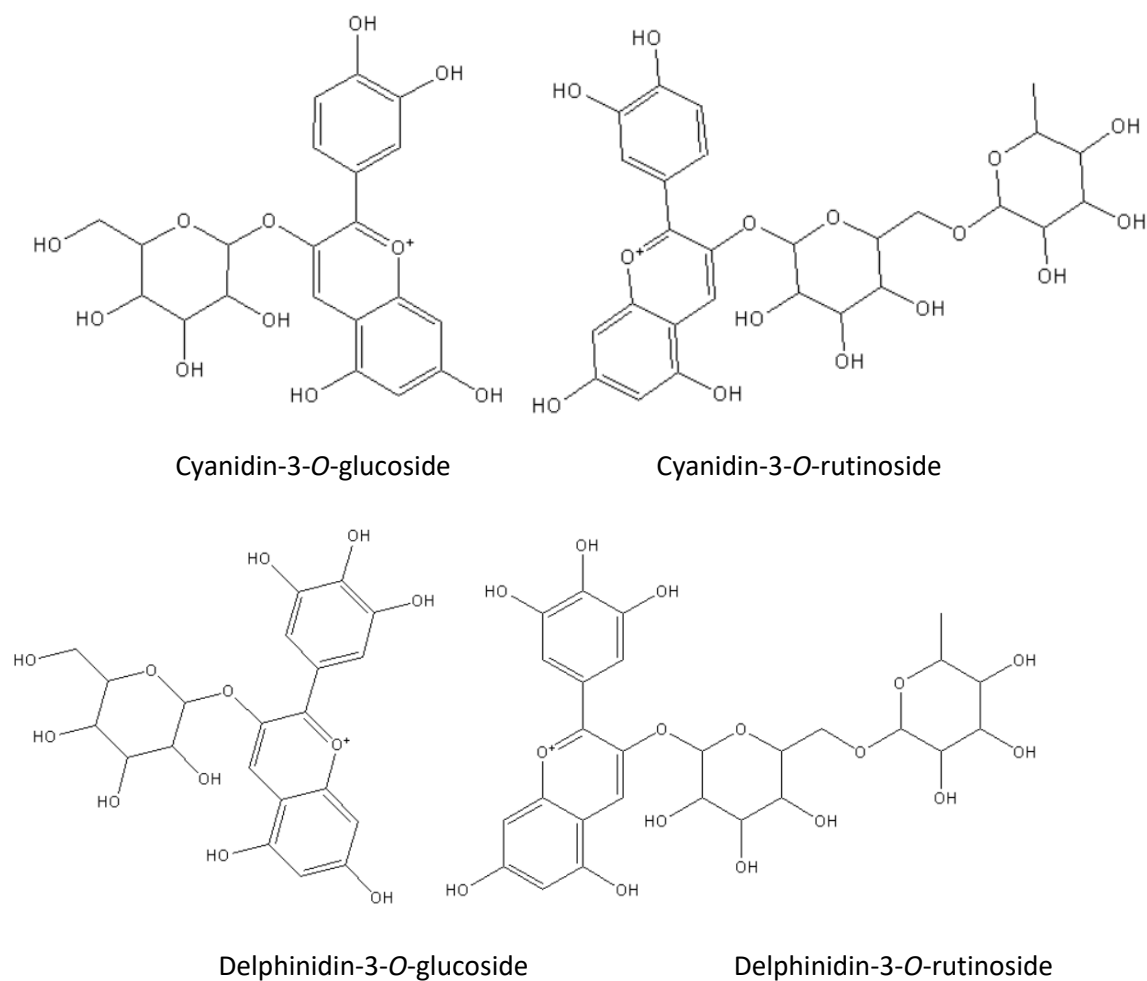


Figure I.26: The major anthocyanins present in black currant (Source: Pubchem).

I.3.1.2.2. Flavonols

Flavonols are another main class of flavonoids found in black currant. Recent studies indicated that quercetin, myricetin, kaempferol and isorhamnetin are the most frequent flavonols reported in blackcurrant (Figures I.17 and I.27). They are present as glycosides with mono-, di-, tri- and tetrasaccharides of glucose, galactose, rutinose, xylose and glucuronic acid [242,244–247].

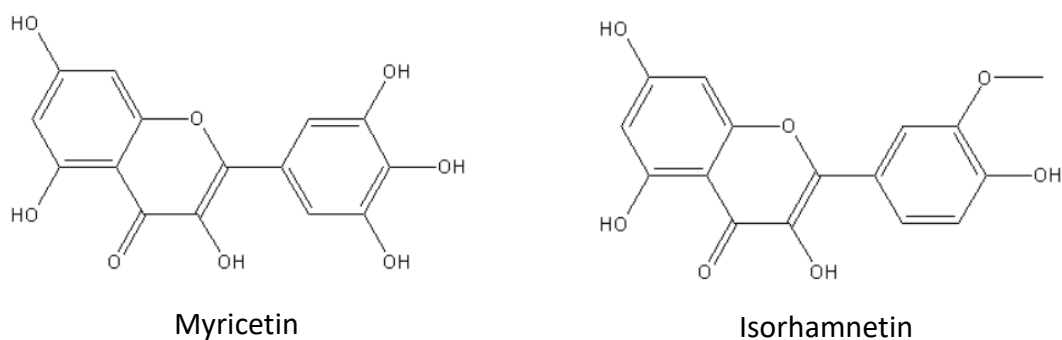


Figure I.27: The major flavonols present in black currant (Source: Pubchem).

Other flavonols such as quercetin-3-(6''-malonyl)-glucoside, quercetin-3-O-glucosyl-6''-acetate, myricetinmalonylglucoside, kaempferolmalonylglucoside have been found and identified in the extracts of blackcurrant buds, leaves and berries by Vagiri et al and Oszmianski et al. [234,239,244].

I.3.1.2.3. Flavanols or flavan-3-ols

(+)-catechin and epigallocatechin have been identified in berries of various blackcurrant varieties by Gavrilona et al. and Vagiri et al. [234,248]. Let us remind that condensed tannins or proanthocyanidins are oligomers or polymers of flavan-3-ols. Proanthocyanidins containing (epi)catechin or (epi)gallocatechin as subunits are called procyanidins or prodelphinidins, respectively. Wu et al. reported that those proanthocyanidins existing as monomers, dimers, trimers, tetramers, and polymers have been identified and quantified by HPLC-ESI-MS/MS coupled with a diode array and/or fluorescent detector in the extracts of mixed solvent (acetone/water/acetic acid) in seven cultivars of *Ribes nigrum* [249,250]. Tabart et al. indicated that the most common flava-3-ols including epigallocatechin, gallocatechin, catechin, and epicatechin have been found in the extracts of black currant buds, leaves and berries [251] (Figures I.18 and I.28).

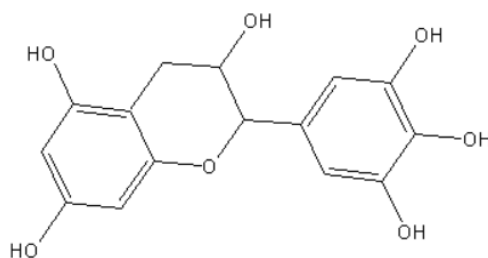


Figure I.28: Structure of epigallocatechin (Source: Pubchem).

I.3.1.2.4. Other flavonoids

Aureusidin is an aurone, a minor subclass of flavonoids, that has been identified in blackcurrant seeds by Yinrong Lu et al. [245] (Figure I.29).

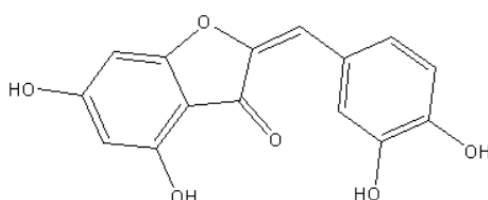


Figure I.29: Structure of aureusidin (Source: Pubchem).

I.3.2. Pharmacological properties

I.3.2.1. Effects on cardiovascular diseases and vascular system

Many articles reported the therapeutic potential of blackcurrant concerning cardiovascular-associated diseases both *in vivo* and *in vitro*. Edirisinghe et al. investigated the effect of blackcurrant anthocyanins on the activation of endothelial nitric oxide synthase (eNOS) *in vitro* in human endothelial cells. The results showed that the four anthocyanins from blackcurrant juice concentrates including cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside are an evidence for the significant effects on phosphorylation of protein kinase B (p-Akt) and endothelial nitric oxide synthase (p-eNOS). It means that blackcurrant juice concentrates activated endothelial nitric oxide synthase (eNOS) via protein kinase B (Akt)/phosphatidylinositol-3 (PI3) kinase pathway *in vitro* in human umbilical vein

endothelial cells (HUVECs) [252] (Figure I.30). Iwasaki-Kurashige et al. indicated that two anthocyanins (delphinidin-3-rutinoside, delphinidin-3-glucoside) isolated from commercial blackcurrant juice reduced hind-limb perfusion pressure that can be mediated by endothelial nitric oxide (NO) and H_2O_2 . In addition, it was demonstrated that blackcurrant delphinidin reduced peripheral vascular resistance in a rat hind-limb perfusion model [253]. Another study investigated the effect of black currant concentrate (BCC) on smooth muscle in the model of norepinephrine-precontracted thoracic aortas of rats. The results showed that in the rat aorta, BCC improves the synthesis of NO resulting in the endothelium-dependent vasorelaxation via the H1-receptors on the endothelium. It suggests that it plays a significant role to increase the blood circulation which is useful to prevent the occurrence of a myocardial infarction or stroke [254].

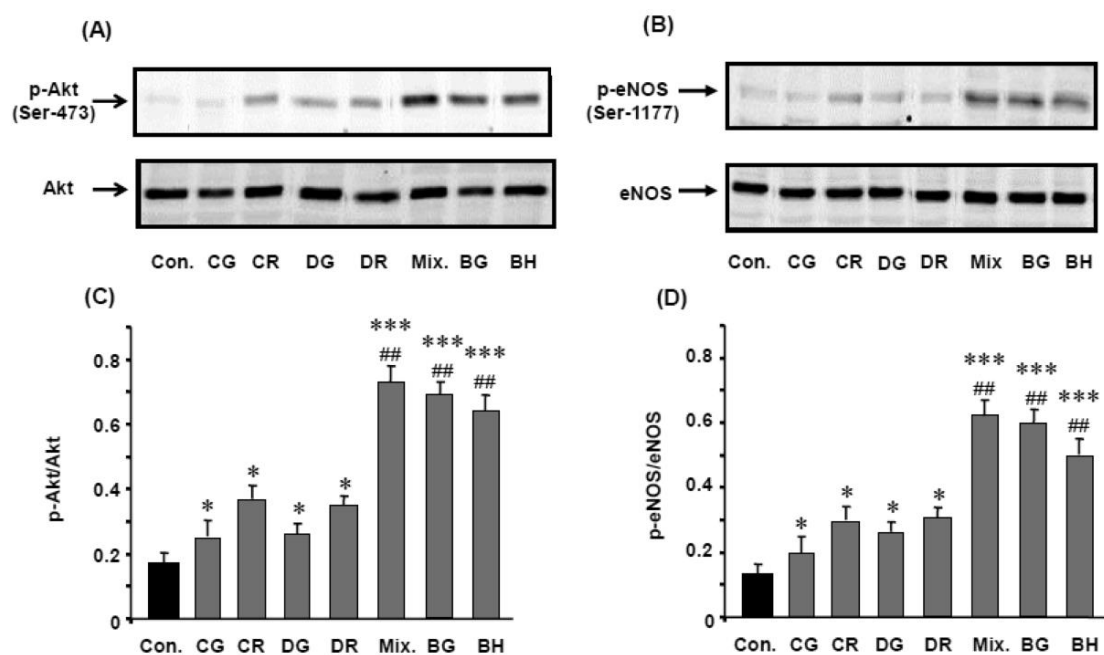


Figure I.30: Effect of major anthocyanins present in blackcurrant juice concentrates on the activation of Akt and eNOS in vitro in HUVECs. HUVEC cells were treated with commercially available cyanidin-3-O-glucoside (CG; 2.3 $\mu\text{g/ml}$), cyanidin-3-O-rutinoside (CR; 20 $\mu\text{g/ml}$), delphinidin-3-O-glucoside (DG; 5.8 $\mu\text{g/ml}$) and delphinidin-3-O-rutinoside (DR; 29.6 $\mu\text{g/ml}$) and a mixture of all four (Mix), and the levels of p-Akt and p-eNOS were compared with PBS control (con.) and Ben Gairn (BG) and Ben Hope (BH) concentrates. Representative blot shows that all four major anthocyanins significantly increased (A) p-Akt and (B) p-eNOS compared to PBS control at the corresponding concentration found in the Ben Gairn and Ben Hope (1 $\mu\text{g/ml}$) that produced maximum p-Akt and p-eNOS ($P < 0.05$). The mixture of all four major anthocyanins produced significantly increased p-Akt and p-eNOS compared to each individual anthocyanin alone ($P < 0.01$). Phosphorylated Akt and

eNOS in response to the mixture of all four major anthocyanins were similar to the ones produced by Ben Gaim and Ben Hope (1 µg/ml). The histograms shown in the both panels C and D are those obtained after quantification of the blots using densitometry (n=3) for p-Akt and p-eNOS, respectively. The ordinates are the relative ratios of the phosphorylated and non-phosphorylated form of each enzyme. (*) P<0.05 and (***) P<0.001, significant compared to control (n=3); ## P<0.01, significant compared to CG, CR, DG and DR (n=3) (taken from [252]).

There are also some clinical studies on the cardiovascular system. Katsumura et al. investigated the effects of blackcurrant anthocyanin intake on peripheral muscle circulation during typing work in humans. In that study, the results indicated that the blackcurrant anthocyanin components including delphinidin 3-rutinoside, delphinidin 3-glucoside, cyanidin 3-rutinoside, and cyanidin 3-glucoside can decrease muscle fatigue and enhance shoulder stiffness by improving peripheral blood flow [255]. Another clinical trial focuses on the effect of black currant seed oil containing α-linolenic, γ-linolenic, linoleic and stearidonic acid on the fatty acid profiles of plasma lipids and concentrations of serum total and lipoprotein lipids, plasma glucose and insulin. It was noted that blackcurrant seed oil supplementation plays a crucial role on serum lipid profile which had a beneficial effect on low-density lipoprotein (LDL) cholesterol levels [256].

1.3.2.2. Anti-oxidant activity

Ehala et al. evaluated the content of phenolic compounds and their antioxidant activity of six different berries including blackcurrant (*Ribes nigrum*). The results showed that the antioxidant of blackcurrant berries have the highest antioxidant capacity by using ABTS radical cation decolorization assay [257]. Another study also indicated that the antioxidant activity of blackcurrant berries containing the most abundant components, i.e. anthocyanins (delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside and cyanidin-3-O-rutinoside), flavonols (myricetin-3-O-rutinoside and myricetin-3-O-glucuronide) and vitamin C, showed the highest antioxidant abilities compared with other berries (blueberries, raspberries, redcurrants and cranberries) by using FRAP assay [258]. Kapasakalidis et al. investigated the total phenol and anthocyanin contents of blackcurrant pomace and

blackcurrant press residue (BPR) extracts and their antioxidant activity. The antioxidant property of BPR extracts determined by scavenging ABTS radical cation assay reveals much higher values than that in the pomace extracts in both two solvents used (solvent A: 3% formic acid in methanol, solvent B: methanol/water/acetic acid (40:55:5)), however, after acid hydrolysis, the former showed much lower than the later (Figure I.31), it means that hydrolysis released a considerable percentage of antioxidant components in pomace. Correspondingly, the total phenol of BPR extracts is also much higher than that in the pomace extracts. Interestingly, BPR anthocyanins have a significant contribution (more than 70%) to the obtained high radical scavenging capacity of the corresponding extracts [16].

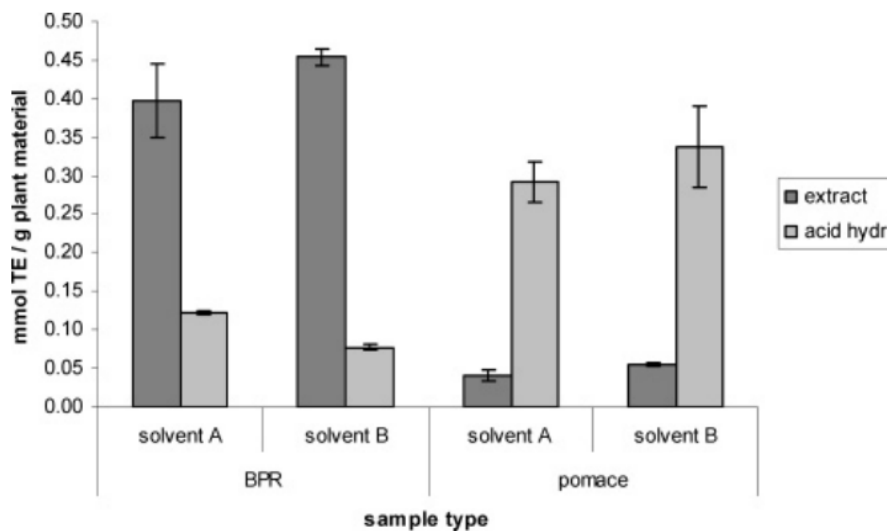


Figure I.31: ABTS radical scavenging activity of blackcurrant plant material, (solvent A: 3% formic acid in methanol, solvent B: methanol/water/acetic acid (40:55:5), mmol Trolox equivalent (TE)/g plant material (taken from [16]).

Some investigations on the anti-oxidant and free radical scavenging activities of blackcurrant leaves have been recently published. Nour et al. investigated the phenolic profile and compared the influence of different extraction methods on the antioxidant activity prepared from various cultivars of blackcurrant leaves. The main phenolic compounds are gallic, chlorogenic, caffeic, p-coumaric, ferulic, sinapic and salicylic acids, and the three flavonoids rutin, myricetin and quercetin. The total phenolic content

correlated with the antioxidant capacity of the corresponding extracts. The antioxidant capacity in 40% ethanol extracts showed the highest values ranged from 257.8 $\mu\text{mol Trolox/g dry weight}$ to 386.8 $\mu\text{mol Trolox/g dry weight}$, followed by that in 80°C water extracts ranged from 222.0 $\mu\text{mol Trolox/g dry weight}$ to 285.6 $\mu\text{mol Trolox/g dry weight}$ [17].

1.3.2.3. Cancer preventive properties

Several investigations reported the cancer-preventive potential of blackcurrants *in vitro*. Olsson et al. investigated the effects of 10 different extracts of fruits and berries on cell proliferation of colon cancer cells HT29 and breast cancer cells MCF-7. The results indicated that all the extracts reduced the proliferation of both colon cancer cells HT29 and breast cancer cells MCF-7 using the four different concentrations of dry extract (0.025, 0.05, 0.25 and 0.5 % of plant dry matter of total weight in the wells (weight approximated to be equal to volume in the wells). In MCF-7 cells, blackcurrant showed one of the highest inhibition effects for the cell proliferation in the two highest concentrations of extract and, interestingly, the inhibition of proliferation may be contributed by the blackcurrant anthocyanins [259] (Figure I.32). Wu et al. made an investigation by comparing the effects of different berry extracts on cell viability and expression of markers of cell proliferation and apoptosis in human colon cancer HT-29 cells. It could be seen that blackcurrant shows the highest levels of cell growth inhibition [260].

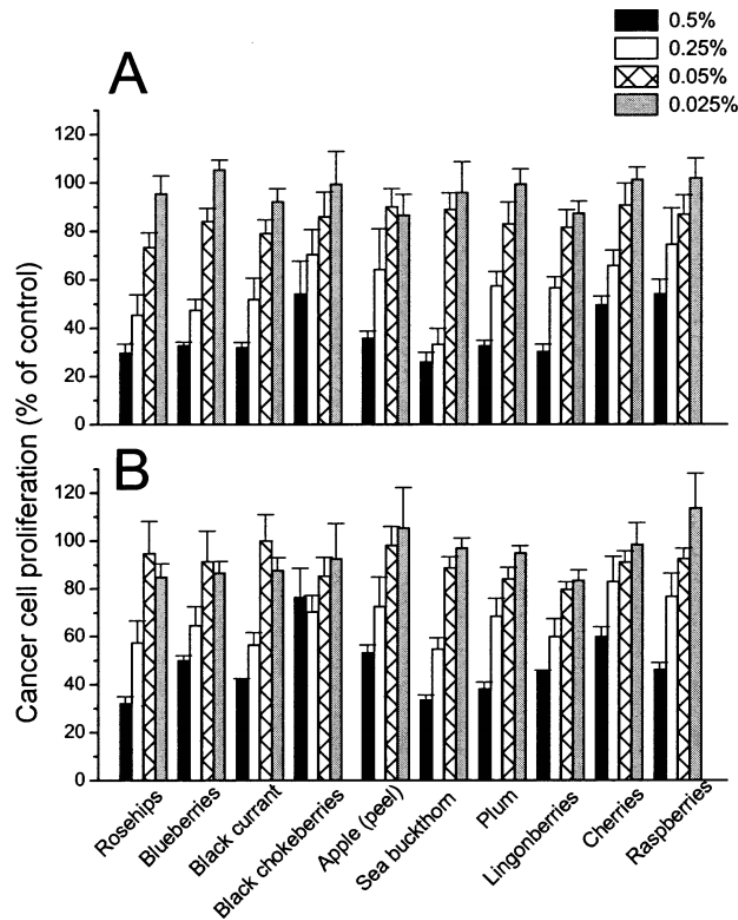
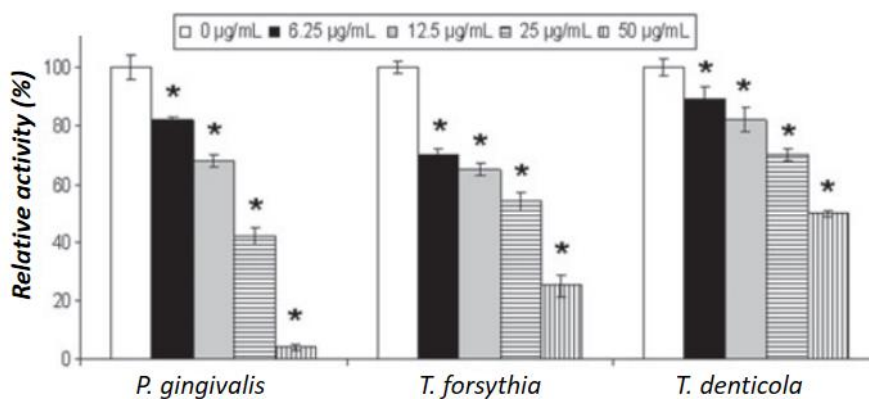


Figure I.32: (A) Colon cancer cell HT29 and (B) breast cancer cell MCF-7 proliferation. Results are presented as means \pm SD based on three independent replicates for each extract, and the proliferation tests were repeated on three different occasions (taken from [259]).

Other investigations evaluated the potential effects of blackcurrant on cancer cells *in vivo*. Takata et al. reported the antitumor activity of blackcurrant polysaccharide by an enzymatic treatment. The results demonstrated that blackcurrant polysaccharide reduced a tumor weight in a Ehrlich carcinoma model in mice [261]. Bishayee et al. examined the chemopreventive effects of an anthocyanin-rich blackcurrant skin extract against diethylnitrosamine (DENa)-induced hepatocellular carcinogenesis in rats. It was shown that the anthocyanin-rich blackcurrant skin extract revealed a significant inhibition of incidence, multiplicity, size and volume of hepatocyte nodules. These results supported the development of blackcurrant bioactive components for the chemopreventive agents of human liver cancer [262].

1.3.2.4. Anti-microbial activity

Blackcurrant fruit extracts have shown potential antimicrobial activity. Santos et al. investigated the inhibition of host- and bacteria-derived proteinases by major anthocyanins of blackcurrant extract such as cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside. The results demonstrated that blackcurrant anthocyanin extracts inhibited significantly *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* proteinases at different concentrations of extract (6.25, 12.5, 25 and 50 µg/mL) (Figure I.33). Data show that the natural compounds may play an important role in the adjunctive treatments of periodontal disease [263]. Another experiment evaluated the antiviral activities of blackcurrant fruit extracts against influenza virus types A and B. It can be seen that not only the extract inhibits the growth of both two viruses but also inactivates them directly [264]. Suzutani et al. evaluated the anti-herpesvirus activity of black currant fruit extracts. The results revealed that the extracts showed an inhibition of the herpes simplex virus type 1 attachment onto the cell membrane and also the prevention of the plaque formation of herpes simplex virus types 1 and 2, and varicella-zoster virus [265].



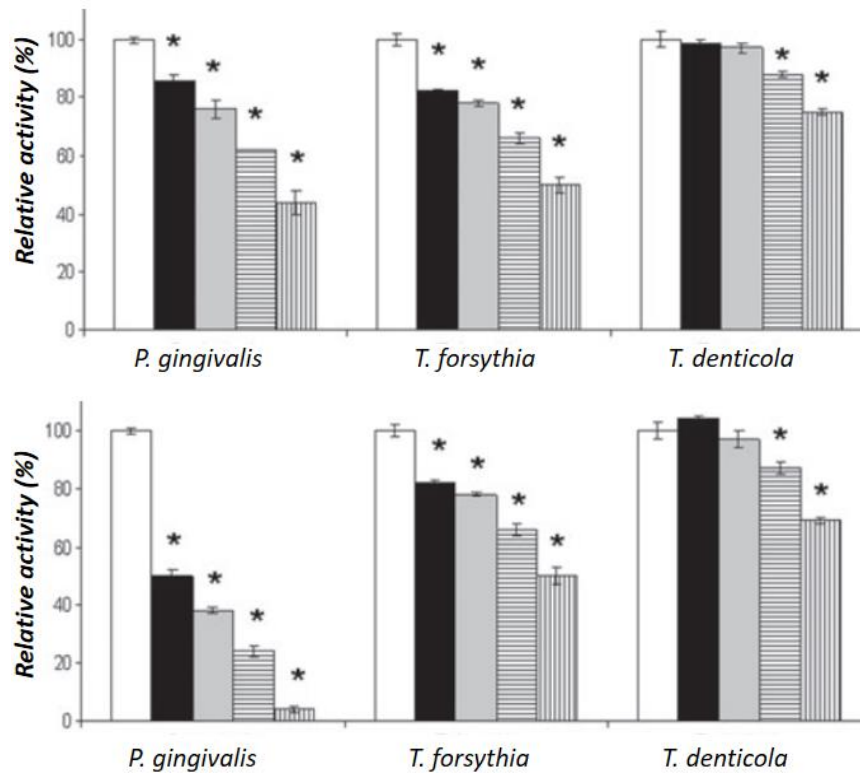


Figure I.33: Inhibitory effect of blackcurrant anthocyanins on bacterial proteinase activity. A value of 100% (or at 0 $\mu\text{g}/\text{mL}$ of anthocyanins) was assigned to degradation observed after incubation (for 4h) of substrate with *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*) or *Treponema denticola* (*T. denticola*) proteinases in the absence of anthocyanins. Bars marked with asterisk (*) indicated significant inhibition of enzyme activity when compared with the untreated control ($p < 0.05$). (A) Cyanidin-3-O-glucoside, (B) Cyanidin-3-O-rutinoside, (C) Delphinidin-3-O-rutinoside (taken from [263]).

There are also some studies reporting the antimicrobial activity of blackcurrant leaves extracts. Steavic et al. investigated the antimicrobial activity of the essential oil with the major volatile compounds such as δ -3-carene, β -caryophyllene, sabinene, *cis*- β -ocimene, α terpinolene obtained from blackcurrant leaves. Essential oils showed antimicrobial activity against 14 micro-organisms such as *Escherichia coli*, *Salmonella typhimurium*, *Streptococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Pseudomonas tolaasii*, *Proteus mirabilis*, *Bacillus subtilis*, *Micrococcus luteus*, *Micrococcus flavus*, *Listeria monocytogenes*, *Candida albicans*, *Trichophyton mentagrophytes*,

Epidermophyton floccosum [233]. Opera et al. also demonstrated that the essential oil of blackcurrant buds extracted from three different varieties revealed antibacterial ability against *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [266]. Another plant extracts obtained from wild blackcurrant leaves exhibited potential therapeutic to fight the infection of influenza virus type A [267].

1.3.2.5. Anti-inflammatory activity

The anti-inflammatory activity is another important pharmacological property of blackcurrant leaves. There is a huge amount of investigations focused on the anti-inflammatory effects of black currant leaves both *in vivo* and *in vitro*. Tabart et al. demonstrated that the blackcurrant leaf extract showed anti-inflammatory capacities by inhibiting the myeloperoxidase (MPO) activity and reactive oxygen species (ROS) production on activated neutrophils *in vitro* [268]. Another investigation suggested that polyphenol-rich blackcurrant extract supplementation reduced obesity-induced inflammation in adipose tissue and splenocytes *in vivo*, meaning that blackcurrant leaves extract can prevent inflammation in diet-induced obese mice [269]. Garbacki et al. investigated the anti-inflammatory effects of proanthocyanidins isolated from *Ribes nigrum* leaves on carrageenin acute inflammatory reactions induced in rats. Blackcurrant proanthocyanidins extracts revealed the inhibition of paw oedema which was efficient after 2 h by pretreatment of the animals with proanthocyanidins (at 10, 30, 60 and 100 mg/kg⁻¹) using carrageenin in a dose and time-dependent manner (Figure I.34) [270]. Proanthocyanidins also inhibited the carrageenin-induced pleurisy, by reducing (i) lung injury, (ii) pleural exudate formation, (iii) polymorphonuclear cell infiltration, (iv) pleural exudate levels of TNF- α , IL-1 β , and (v) pleural exudate levels of nitrite/ nitrate (NOx) [270].

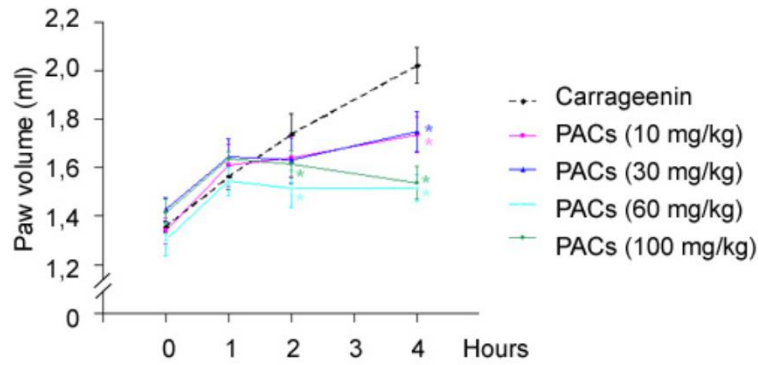


Figure I.34: Time course of inflammatory reaction induced by injection of carrageenin 1% in rat hind paw and its antagonism by PACs (10, 30, 60 and 100 mg/kg-1). Inflammation is expressed as the increase of the rat paw volume (ml) from 0 to 4 h following injection of carrageenin. The volume of the paw was reduced by PACs at the four doses tested and the inhibition is time and dose-dependant. Each value is the mean \pm s.e. mean of $n = 6$ experiments. * $P < 0.05$ versus carrageenan (taken from [270]).

I.3.2.6. Other activities

Falin et al. investigated the efficacy of blackcurrant oil for the treatment of hyperlipidemia. It was concluded that the blackcurrant oil showed an increase of the serum high density lipoprotein-cholesterol (HDL-C) level and also a decrease of triglyceride levels and total cholesterol in hyperlipidemic patients who have a lower body mass index [271].

I.4. *Chrysanthellum americanum*

Chrysanthellum americanum belongs to the genus *Chrysanthellum*, the tribe *Coreopsideae* is classified in the *Asteraceae* family [272]. To date, the name of *Chrysanthellum* has been confused, indeed the name of the genus *Chrysanthellum* is a diminutive of *Chrysanthemum* derived from the Greek word “chrusos”, gold and “anthesis” chamomile called “golden chamomile” [20]. In addition, according to Wikipedia, *Chrysanthellum* is a genus of flowering plants in the *Chrysanthemum* family [273]. According to French pharmacopoeia, *Chrysanthellum americanum* is an herbaceous plant of 10 to 30 cm, slender stems, round in section, with pinnately lobed and alternate leaves, and yellow flowers from 3 to 5 mm in diameter at flowering and up to 8-10 mm at maturity period (Figure I.35). It grows mainly in the mountainous regions or moderate altitude areas

in Africa from Senegal to Nigeria and South America from Southern Mexico to Northern Brazil [18,20].

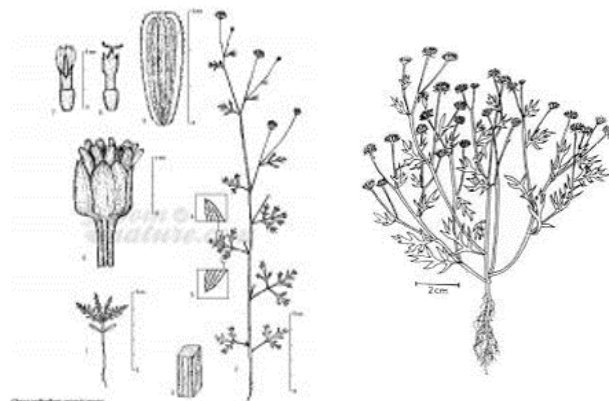


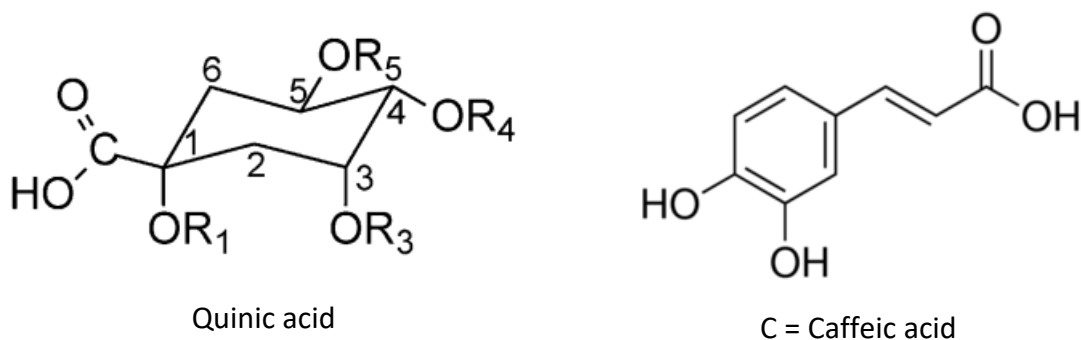
Figure I.35: *Chrysanthellum americanum* flowers and flowering tops (taken from [176]).

Chrysanthellum americanum has been traditionally used for its significant wound healing properties in the African and American folk medicine and in the treatment of fever, hepatitis, jaundice and dysentery [19]. In Cuba traditional medicine, it has been used for gastro-intestinal pains, rheumatism and kidney [18]. Nowadays, several studies have been reported that the *Chrysanthellum americanum* health promoting properties can be attributed to its anti-oxidant and anti-microbial activities, as well as its hepatic protector, venous endothelium and lipid lowering action [18–21,274].

1.4.1. Chemical composition

1.4.1.1. Phenolic acids

Chlorogenic, caffeic and quinic acid have been reported in *Chrysanthellum americanum* [20]. To date, there are very few published articles of *Chrysanthellum americanum*. However, a large number of publications in the *Chrysanthemum* has been recently investigated. Among them, dicaffeoylquinic acids are the most abundant phenolic acids presented in *Chrysanthemum* and there are six isomers existing in nature (Figure I.36) [275,276].

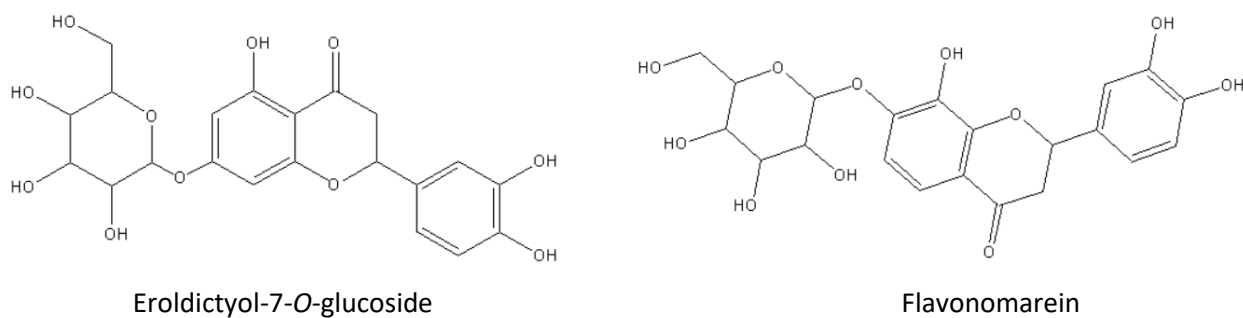


Name	R ₁	R ₃	R ₄	R ₅
1,3-di- <i>O</i> - caffeoylquinic acid	C	C	H	H
1,4-di- <i>O</i> - caffeoylquinic acid	C	H	C	H
1,5-di- <i>O</i> - caffeoylquinic acid	C	H	H	C
3,4-di- <i>O</i> - caffeoylquinic acid	H	C	C	H
3,5-di- <i>O</i> - caffeoylquinic acid	H	C	H	C
4,5-di- <i>O</i> - caffeoylquinic acid	H	H	C	C

Figure I.36: Structure of six isomers of dicaffeoylquinic acids (taken and modified from [277]).

1.4.1.2. Flavonoids

There are two flavanones such eriodictyol-7-*O*- glucoside and isookanin-7-*O*- glucoside or flavonomarein, one flavone (luteolin-7-*O*-glucoside), one chalcone (okanin-4'-*O*-glucoside or marein and one aurone (maritimetin-6-*O*-glucoside or maritimein reported in the *Chrysanthellum americanum* [20] (Figure I.37).



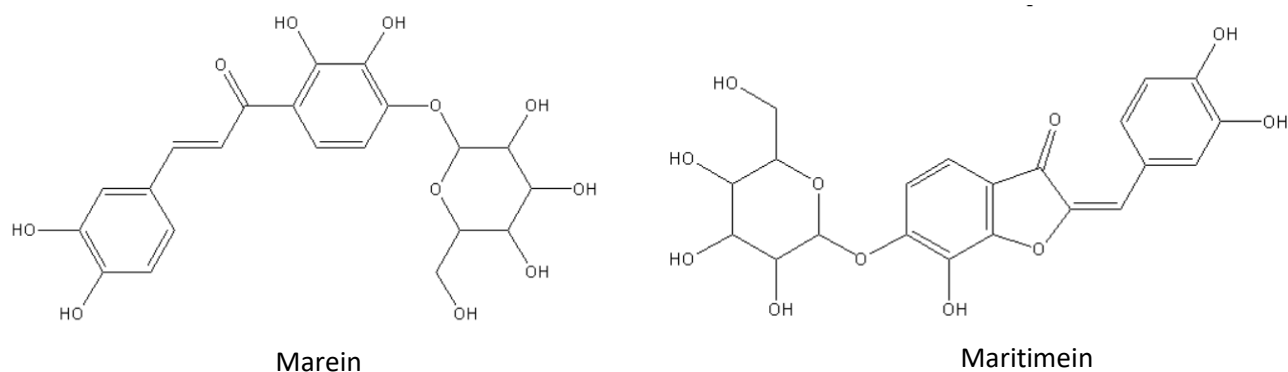


Figure 1.37: Structure of several flavonoids present in *Chrysanthellum americanum* (Source: Pubchem).

1.4.2. Pharmacological properties

There are several studies focused on the composition of essential oil, antimicrobial and antioxidant activities of volatile oil in the aerial parts of *Chrysanthellum americanum*. Mevy et al. indicated that the essential oil with the main components as caryophyllene oxide, hexa-2,4-dienol, β -caryophyllene, α -pinene and verbenol showed potential antifungal activity against *Candida albicans*, *Saccharomyces cerevisiae* and those volatile oils revealed slightly antioxidant activities [19]. Guenne et al. also confirmed that the extracts from the whole-plant of *Chrysanthellum americanum* were promising sources to treat several bacterial infection diseases [274].

Chrysanthellum americanum is able to stimulate the potential of hepatic detoxification and regenerate the damaged hepatocytes. It also may play an important role on lowering the triglyceride levels and total cholesterol [18,20]. In addition, *Chrysanthellum americanum* is also offered in the preventive or curative treatment of vascular disorders (arterial disease of the lower limbs, capillaritis, retinal and choroidal disorders of vascular origin). Indeed, it has positive effects on capillary permeability and fragility, as well as on peripheral microcirculation, thanks to its vitamin P properties [278]. Patients with arteritis of the lower limbs saw their walking perimeter increase [279]. It is important to note that

the effects of the drug were not fully manifested until after a certain period of time. Two patients had improved retinal circulation, another had improved headache and ringing in the ears. Three times, venous insufficiency responded quickly to the treatment. In clinical pharmacology, a work in the context of preliminary trials in humans was carried out, with a preparation containing 100 mg of CA extract [280]. The authors found a significant decrease in permeability after one month of treatment at a rate of 500 mg per day in 25 patients. For the same patients, capillary resistance increased significantly while the increase was even more marked one month after stopping the therapy and two months later, it was still significant. The effects of the product on microcirculation were considered to be positive. The mean blood flow to the lower limb in patients with chronic thrombosing arteritis increased by 30% after one month of treatment and by 53% after 2 months. Work was also carried out in 29 patients with retinal and choroidal disorders, who were administered 400 mg per day of extract [281]. Overall, a positive result was found in 24 out of 29 cases. Another clinical evaluation focused on 26 arteritic patients who received 300 mg per day of CA extract for 3 months compared to 32 others to whom only a placebo was administered [281]. Patients treated report an improvement in their symptoms and an increase in their spontaneous activity. There was an excellent correlation between the clinical improvement and the improvement of the blood flow measured by Doppler effect both in the lower limbs and in the supra-aortic arteries. It is the richness of CA in flavonoids and saponosides that gives beneficial action on the circulatory system. Finally, it relieved heavy legs by its venotonic action.

I.5. Tea creaming

I.5.1. Introduction

Tea creaming is composed of insoluble particles occurring in various kinds of tea, such as green tea or black tea, especially formed in the ready-to-drink tea products when the tea solution cools down. Tea creaming formation shows a cloudy or hazy appearance which can be observed as undesirable precipitations [25,26]. Tea creaming consists of

nanoparticles (with a size from 10 to 400 nm) of great interest for pharmaceutical purposes [282], together with larger particles. Its formation depends on many factors, such as chemical composition of individual input tea material, infusion solid concentration, and temperature. The amount of formed tea creaming increased as well as these components increasingly dissolved into the tea infusion at high temperature extracted and gradually precipitated when cooling [27]. Recent study indicated that the nanoparticles can play a significant role in biomedical sciences including drug delivery, nutraceuticals and production of biocompatible materials [282].

1.5.2. Chemical composition

Most of investigations on chemical composition of tea creaming focus on green and black tea. Kim et al. indicated that caffeine and 12 phenolic compounds including phenolic acids, tea catechins and flavonol glycosides have been found in original green tea infusion (obtained from drying 15 ml of tea infusion at 85°C for 12h) and postcream infusion (obtained after 12h incubation and holding temperature and centrifuged at 7°C for 30 minutes to remove insoluble tea creaming), based on the concentration of individual compositions in original green tea infusion and post cream infusion, the amount of insoluble tea creaming was determined as presented in Table I.38. Caffeine and tea catechin such as (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) are the main components [25].

Table I.38: Concentration of polyphenolic compounds and caffeine in original green tea infusion and post cream infusion (clear extract) after 12 hours let at 4°C and concentration difference present in insoluble tea creaming^a (taken from [25]).

compound	original infusion (mg/L)	postcream infusion (mg/L)	tea cream (mg)
1. gallic acid	26.3 ± 2.8	3.73 ± 0.3	22.6
2. (-)-epigallocatechin (EGC)	128 ± 2.2	102 ± 5.1	26.0
3. caffeine	603 ± 9.8	549 ± 12.5	54.6
4. (-)-epigallocatechin gallate (EGCG)	435 ± 8.1	397 ± 15.8	37.9
5. (-)-epicatechin (EC)	84.8 ± 4.0	84.2 ± 1.1	0.6
6. (-)-gallocatechin gallate (GCG)	19.5 ± 0.7	17.0 ± 0.9	2.5
7. (-)-epicatechin gallate (ECG)	99.2 ± 4.2	97.2 ± 1.8	2.0
8. myricetin 3-glycoside-1	29.8 ± 2.0	22.2 ± 0.9	7.6
9. myricetin 3-glycoside-2	39.8 ± 2.6	32.4 ± 2.3	7.4
10. quercetin 3-rutinoside (rutin)	119 ± 4.1	89.0 ± 2.8	29.8
11. quercetin 3-glucoside	21.3 ± 2.8	21.3 ± 0.1	0.1
12. kaempferol 3-glucoside	19.1 ± 0.6	19.0 ± 1.0	0.1
13. kaempferol 3-rutinoside	26.3 ± 0.9	25.2 ± 1.0	1.1

^aListed compounds 8–13 are expressed as mg/L rutin equivalents.

Lin et al. have been reported a comparative study on the chemical composition of tea creaming issued from green and black tea (Table I.6) [283].

Table I.39: Creaming concentration and creaming percentages of major chemical components in green tea and black tea (mean ±SD) (taken from [283]).

Components	Creaming concentration (mg/mL)		Creaming percentage (%)	
	Green tea	Black tea	Green tea	Black tea
Total solid	8.49 ± 0.43 ^a	7.77 ± 0.21 ^b	30.74 ± 2.84	32.87 ± 0.70
Tea polyphenols	3.91 ± 0.20	–	41.11 ± 4.57	–
Proteins	1.91 ± 0.08	1.77 ± 0.12	51.02 ± 2.08	54.96 ± 2.78
Methylxanthines	1.93 ± 0.42 ^a	0.82 ± 0.01 ^b	50.50 ± 0.32 ^b	54.96 ± 0.80 ^a
Carbohydrates	0.30 ± 0.02 ^b	0.42 ± 0.05 ^a	19.05 ± 2.03 ^b	25.15 ± 2.89 ^a
Amino acids	0.08 ± 0.00 ^b	0.19 ± 0.03 ^a	4.21 ± 0.17 ^b	10.98 ± 1.75 ^a
GA	0.01 ± 0.00 ^b	0.02 ± 0.00 ^a	3.23 ± 0.31 ^b	5.84 ± 0.24 ^a
GC	0.12 ± 0.02 ^a	0.04 ± 0.01 ^b	39.45 ± 1.75 ^a	19.96 ± 1.32 ^b
EGC	0.07 ± 0.02	0.09 ± 0.01	13.24 ± 0.80 ^b	41.92 ± 0.83 ^a
C	0.09 ± 0.02 ^a	0.05 ± 0.00 ^b	12.25 ± 1.47 ^b	27.12 ± 0.73 ^a
EGCG	1.39 ± 0.24 ^a	0.08 ± 0.01 ^b	35.31 ± 0.59 ^b	45.88 ± 2.93 ^a
EC	0.05 ± 0.01 ^a	0.02 ± 0.00 ^b	6.37 ± 0.38 ^b	13.64 ± 1.33 ^a
GCG	0.52 ± 0.12 ^a	0.02 ± 0.00 ^b	55.55 ± 1.23 ^a	48.34 ± 1.96 ^b
ECG	1.18 ± 0.25 ^a	0.23 ± 0.00 ^b	34.27 ± 0.07 ^b	62.20 ± 2.63 ^a
CG	0.22 ± 0.04 ^a	0.01 ± 0.00 ^b	67.70 ± 2.46 ^a	55.59 ± 9.10 ^b
Caffeine	1.84 ± 0.40 ^a	0.77 ± 0.01 ^b	51.97 ± 0.23 ^b	55.69 ± 0.79 ^a
Theobromine	0.08 ± 0.02 ^a	0.04 ± 0.00 ^b	35.53 ± 2.26 ^a	42.92 ± 0.83 ^a
Theophylline	0.00 ± 0.00 ^b	0.02 ± 0.00 ^a	8.86 ± 0.90 ^b	59.82 ± 2.61 ^a
TFs	–	0.08 ± 0.00	–	70.05 ± 7.79
TRs	–	1.85 ± 0.62	–	39.43 ± 12.92
TBs	–	1.99 ± 0.00	–	33.17 ± 0.72

Different lower case letter (a and b) for the concentration and percentage of tea components denoted significant differences ($P < 0.05$) between green tea and black tea. Abbreviations: EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; EGC, epigallocatechin; GC, gallocatechin; EC, epicatechin; C (-) catechin; GA, gallic acid; TFs, theaflavins; TBs, thearubigins; SD, standard deviation.

As can be seen from Table I.39, the major components of tea creaming in green tea are proteins, methylxanthines, gallic catechin (GC), epigallocatechin (EGCG), gallic catechin gallate (GCG) and caffeine, while proteins, methylxanthines, caffeine, theaflavins (TFs), thearubigins (TBs) are the main compounds of tea creaming in black tea. Chemical composition of tea creaming in black tea has been confirmed by Bee et al. and Jobstl et al. [26,284].

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CHAPTER II:
OPTIMIZING WATER-BASED EXTRACTION OF
BIOACTIVE PRINCIPLES OF HAWTHORN:
FROM EXPERIMENTAL LABORATORY RESEARCH
TO HOMEMADE PREPARATIONS

CHAPTER II: OPTIMIZING WATER-BASED EXTRACTION OF BIOACTIVE PRINCIPLES OF HAWTHORN: FROM EXPERIMENTAL LABORATORY RESEARCH TO HOMEMADE PREPARATIONS

Molecules, 2019, 24(23), 4420

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Keywords: hawthorn; water-based extraction; procyanidin; polyphenol; flavonoid; standardization; extraction mode; infusion; granulometry.

Abstract:

Hawthorn (*Crataegus*) is used for its cardiotonic, hypotensive, vasodilative, sedative, antiatherosclerotic and antihyperlipidemic properties. One of the main goals of this work is to find a well-defined optimized extraction protocol, usable by each of us, and leading to repeatable, controlled and quantified daily uptake of active components from hawthorn, at a drinkable temperature (below 60 °C). A thorough investigation of the extraction mode in water (infusion, maceration, percolation, ultrasounds, microwaves) on the yield of extraction and the amount of phenolic compounds, flavonoids and proanthocyanidin oligomers, and on the UHPLC profiles of the extracted compounds, was carried out. High-resolution Fourier transform ion cyclotron resonance mass spectrometry was also implemented to discriminate the different samples and conditions of extraction. The quantitative and qualitative aspects of the extraction as well as the kinetics of extraction were studied, not only according to the part (flowers or leaves), the state (fresh or dried) and the granulometry of the dry plant, but also the stirring speed, the temperature, the

extraction time, as well as the volume of the container (cup, mug or bowl) and the use of infusion bags.

II.1. Introduction

The extraction of bioactive principles from medicinal plants is often poorly controlled and depends on a large number of factors, such as extraction temperature, extraction time, particle size of the dry plant, and amount and origin of plant introduced into the extraction solvent [1]. This is particularly true when people are doing plant infusions at home, with a poor control of the experimental conditions of extraction leading to low repeatability/reproducibility. Outside the laboratory, neither the plant weight, the granulometry, the temperature, nor the water volume are usually well controlled. A consequence of this matter of fact is that the intake dose of biologically relevant components extracted from the medicinal plant may significantly vary and remain uncontrolled. This is one of the reasons why the Western medicine is often reticent to promote the use of home-prepared medicinal plant and may prefer the prescription of standardized and commercialized plant-based extracts. However, standardized extracts of plants and / or plant-based medicines are generally expensive, restricting the access to a limited part of the population [2-3]. It seems therefore crucial to investigate the impact of the experimental conditions of extraction, including conditions close to home-prepared extraction, on the daily intake dose and its reproducibility. It is also important to normalize it in comparison with standardized medicines. Moreover, some recent studies demonstrated that tea drinkers in modern life have more oesophagus cancer compared to the rest of the population because they drink too hot ($> 60^{\circ}\text{C}$) [4-5]. It is consequently important to study and to optimize simple and straightforward protocols of extraction using a minimum of equipment, leading to repeatable, quantifiable and safe daily uptakes of active components from a given medicinal plants.

Hawthorn (*Crataegus*) is a bushy shrub, usually spiny, with light green leaves, white umbellate flowers, and edible red fruits [6]. It is readily available in the wild in temperate areas of Eurasia and North America, with over 280 species listed. In traditional Chinese medicine, the fruit is used for its stimulating properties of digestion and gastric function and for the improvement of blood circulation [6-12]. In Europe and North America, flowering

tops (leaves and flowers) are used for their astringent, antispasmodic, cardiotoxic, diuretic, hypotensive, vasodilative, sedative, antiatherosclerotic, and antihyperlipidemic properties [6-9, 12-23]. Most of the experimental works published on *Crataegus* focuses on the extraction, quantification, and identification of phenolic compounds, flavonoids, and tannins to which the merit of these pharmacological effects is attributed [10, 12, 24-58]. These bioactive principles are usually extracted in ethanol, methanol, or alcohol/water mixtures at different temperatures and extraction times and using various extraction modes, essentially Soxhlet [30, 45, 46, 52, 56, 58-68], maceration [24-28, 31, 33, 38, 43, 47, 49-51, 57, 60, 63, 69-78], or ultrasonic [29, 32, 35, 36, 39-42, 56, 60, 63, 77, 79-83], but also decoction [33, 84, 85], infusion [33, 44, 61, 66, 84], percolation [10, 28, 53, 86, 87], microwave [60], or even supercritical carbon dioxide without any solvent [77, 88, 89]. Ultrasonic and microwave were found the most efficient extraction modes [60, 63, 77]. The influence of *Crataegus* species [26, 27, 35, 41, 43, 46, 65, 70, 73, 79, 83, 89, 94], the harvest area [20, 27, 32, 36, 41, 54-56, 65, 70, 79, 94], and the plant organ (flowering tops, flowers, leaves, fruits at various states of ripening) [8, 25, 27, 32, 35-37, 40, 41, 43, 47, 48, 51, 56, 61, 65, 66, 70, 72-74, 77, 82, 84, 89-94], were also largely studied in the literature. Before extraction, the plant is generally dried, crushed, and powdered using a grinder (typically a mortar or a coffee grinder, when mentioned) [24, 25, 28, 29, 35, 36, 38-43, 45-51, 56, 57, 59-61, 63-66, 68, 70-72, 75, 76, 78-82, 85, 86, 93-96], but, to our knowledge, no article deals with the influence of the plant granulometry on the extraction yield. The use of an organic solvent (typically ethyl acetate) or the presence of an alcoholic co-solvent with water was found to improve the extraction yield of bioactive principles, compared to extraction in pure water [28, 29, 38, 53, 60, 61, 78, 81, 87, 94]. Actually, few articles focus on the extraction modes in water [28-30, 33, 37, 41, 44, 53, 62, 66, 76, 79, 84, 85, 87, 96] and none is addressing all the parameters in a single study for the investigation of hawthorn extraction mode and hawthorn extracts analysis. *Crataegus* extracts have been studied and are still currently studied in clinical trials, showing their effectiveness in treating mild heart failure without side effects [6-9, 14, 15, 17-20, 22]. Other biological tests have been performed on animals to investigate the impact of hawthorn extracts on various illnesses, including cancers [33, 55], atherosclerosis [48, 58, 75], thrombosis [52, 59], cataract [97], anxiety [45], heart diseases [13, 68, 72, 75, 95], stomach diseases [10], neurological diseases [29], liver diseases [48, 67, 72], or microbial diseases [10, 43, 50, 73].

In this work, a thorough investigation of the extraction mode in water (infusion, maceration, percolation, ultrasounds, microwaves) on the yield of extraction and the amount of phenolic compounds, flavonoids, and proanthocyanidin oligomers, and on the UHPLC profiles of the extracted compounds, was carried out on hawthorn dry plants. The quantitative and qualitative aspects of the extraction, as well as the kinetic of extraction were studied according to the part (flowering tops or flowers), the state (fresh or dried), and the granulometry of the dry plant (from ~200 μm to 5 mm). The impact of the extraction parameters such as the stirring speed (250 to 1000 rpm), the temperature (20 to 100°C), the extraction time (5 to 30 min), as well as practical parameters such as the volume of the container (cup, mug or bowl) and the use (or not) of infusion bags have been also investigated. High-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) was also implemented to get more qualitative information on the chemical compositions allowing discriminating the different samples and conditions of extraction.

One of the main goals of this work is to find a well-defined optimized extraction protocol, usable by each of us, and leading to repeatable, controlled and quantified daily uptake of active components from hawthorn, at a drinkable temperature (60 °C).

II.2. Results and Discussion

Five different modes (infusion, maceration, ultrasonic, microwave, and percolation) were compared to extract the water-soluble bioactive components contained in hawthorn. This study was voluntarily restricted to water as extracting solvent since the final optimized protocol should be useable by anyone and kept as simple as possible (absence of non-drinkable solvents). The protocols of extraction according to each extraction mode are described in the experimental part (see sections II.3.3 to II.3.7, a picture of each experimental setup is also provided in supporting information of chapter II, see Figure SI-II.2). The temperature of extraction, the stirring speed, the extraction time, the plant part (flowering tops or flowers) and nature (dry, fresh), and the granulometry were studied. Table II.1 gathers the different modalities that were studied in this work for each experimental parameter according to the extraction mode. For each experimental conditions of extraction, the kinetic of extraction have been first studied by monitoring the

UV absorbance upon the extraction time (section II.3.1). Then, the extraction yields were determined and the specific contents in polyphenols, flavonoids and procyanidin oligomers were measured by complexation and spectrophotometry at 10 min and 30 min extraction times. All extractions were always performed in triplicate (3 independent extractions). Analyses using UHPLC-ESI-MS and (-)ESI-FT-ICR-MS were also performed to get a better insight in the differences between extraction modes / natures of the plant.

Table II.1. List of the experimental parameters and their modalities investigated in this work for each extraction mode.

Parameter of extraction	Extraction mode				
	Infusion	Maceration	Ultrasonic	Microwave	Percolation
Temperature (°C)	Decreasing from 90 upon time	20, 40, 60, 80	20, 40, 60	96 (300W)	100
Stirring speed (rpm)	250, 500, 750, 1000 (magnetic stirring)	500 (magnetic stirring)	250 (mechanical stirring)	No	No
Extraction time (min)	5, 10, 30	5, 10, 30	5, 10, 30	5, 10, 30	5, 10
Plant state	fresh, fresh after 1 year, dry	fresh, dry	dry	dry	dry
Plant granulometry	raw, grinded (1 mm, 2 mm, coarse, fine, ultrafine 10" and 30")	raw, grinded 1 mm	raw, grinded 1 mm	raw, grinded 1 mm	raw, grinded 1 mm

II.2.1. Influence of the extraction mode on the kinetics of extraction and on the global extraction yields using raw dry plants

The kinetics of extraction were compared on raw (non-grinded) hawthorn (lot n° 20335) for infusion, maceration, and US-assisted extraction modes by monitoring the UV absorbance at 198 nm (Figure II.1). 100 µL aliquot were taken from the reactor at different extraction times and diluted in 4 mL (or 8 mL to avoid saturation of the UV detector) water (see section II.3.9). The monitoring wavelength was chosen to maximize the number of components that can be detected. Such simple experiments are very useful in practice to optimize the extraction time and the yield of extraction. The effect of stirring speed (magnetic stirring) was studied for the infusion mode, while the effect of temperature was investigated for maceration and ultrasonic-assisted modes. The kinetics of extraction could not be studied for MW-assisted and percolation modes because the equipment did not

allow simple and repeated sampling during the extraction. An extraction time of 30 min was considered as a maximum reasonable extraction time, therefore the UV monitoring was stop after 30 min. Extraction yields (in mass % of the introduced plant) were determined on independent experiments from the UV monitoring, by evaporation and freeze-drying of the whole extract at 10 min (or 30 min) extraction times.

In the case of infusion mode (see Figure II.1A), the temperature decreased in the reactor from about 90°C to 40°C after 30 min extraction at 500 rpm. The drinkable temperature (60°C) to avoid any side effect in the health, such as increasing risk of esophageal carcinoma [4, 5], was reached after 10 min extraction (250 mL water). The increase in stirring speed from 250 rpm to 1000 rpm increases the kinetics of extraction, as seen on the absorbance monitoring (Figure II.1A), while the maximum absorbance (and the extraction yield) were less affected by the stirring speed. All the kinetic curves (absorbance $A(t)$ vs extraction time t) were fitted according to the following equation (see plain lines in Figure II.1) using Excel solver:

$$A(t) = A_{\infty} - A_1 \exp\left(-\frac{t}{\tau_1}\right) - (A_{\infty} - A_1) \exp\left(-\frac{t}{\tau_2}\right) \quad (1)$$

where A_{∞} is the maximum absorbance at infinite extraction time, A_1 is a fitting parameter corresponding to an intermediate extraction plateau and t_1 and t_2 are two characteristic extraction times. These two characteristics times were required to get a better fitting of the experimental curves, as observed by a first plateau of absorbance located between 8 and 10 min depending on the stirring speed. All fitting parameters are given in Supporting Information (see Table SI-II.2). To allow a fast comparison of the kinetics of extraction between extraction conditions, it was found convenient to simply calculate from the fitting curve, the time $t_{70\%}$ to get 70% of the absorbance value at highest extraction time (30 min). For infusions, $t_{70\%}$ is about 2 times lower at 1000 rpm stirring speed (4 min) vs 9 min at 250 rpm (see Table II.2). The extraction yield was found similar at 10 and 30 min extraction times and about 16% (expressed in mass % of the solid extract compared to the initial mass of dry plant), showing that 10 min infusion are sufficient to extract the maximum bioactive principles from dry raw hawthorn plant.

As for the maceration mode, the same experimental set-up as the one used for infusion was used except that the extraction temperature was kept constant using a temperature sensor and regulator (see section II.2.4). The stirring speed was set to 500 rpm (magnetic stirring) and different temperatures were investigated (20, 40, 60 and 80 °C). The corresponding UV kinetic monitoring are displayed in Figure II.1B. The kinetics of extraction were slower than for infusions, but similar for all maceration temperatures, with a typical

$t_{70\%}$ of about 12-13 min regardless of the temperature (Table II.2). Even at 80°C maceration, the kinetics is significantly slower than for infusion and the extraction yield at 30 min (16%) is similar to infusion at the same stirring speed, despite a ~17% higher absorbance at 30 min compared to infusion mode. The extraction yields and maximum absorbance at 30 min rapidly dropped with decreasing extraction temperature (only 9.7% yield at 20°C for 30 min extraction). By selecting the maceration temperature at 60°C (drinkable temperature), the extraction yield at 10 min was found lower (12%) than for infusion mode (16%) after 10 min corresponding to the same final temperature (60°C).

In the case of US-assisted mode, the stirring speed was set to 250 rpm (mechanical stirring). The higher the temperature, i.e. from 20°C to 60°C, the faster the extraction kinetic, as expected. Similarly to infusion mode, $t_{70\%}$ was less than 10 min, regardless of the temperature (Table II.2). The absorbance values at 30 min were about 2 times higher than maceration mode at the same temperature. At 40°C and 60°C, the absorbance at 30 min were also higher than for infusion mode. By selecting the temperature at 60°C (drinkable temperature), the extraction yield was found lower at 10 min (17%) than at 30 min (21%) extraction time, but still higher than for the maceration mode at the same temperature (12% after 10 min), similar to infusion after 10 min (16%) but significantly higher after 30 min.

As for the MW-assisted mode, the power was set to 300 W. The temperature increased rapidly to reach 78°C at 5 min, 95°C at 10 min and 97°C at 30 min. The extraction yields at 10 and 30 min (17% and 22%) were found similar to those for US-assisted mode at 60°C, regardless of the extraction time (Table II.2), but with much higher final temperatures in the case of MW-assisted mode.

As for the percolation mode, the extraction yield at 10 min was found even slightly higher (18%) than that for US-assisted and MW-assisted modes (17%) (Table II.2). This can be explained by the high temperature (100°C) which is set from the beginning of the extraction. No yield value was obtained at 30' extraction time, because of the clogging at the end of the extraction.

In conclusion of this part, at 10 min extraction time on raw materials, infusion, US, MW and percolation modes lead to relatively similar extraction yields comprised between 16% and 18%. However, at 30 min extraction time, US and MW modes were significantly more efficient (21-22%) than infusion (16%) or macerations.

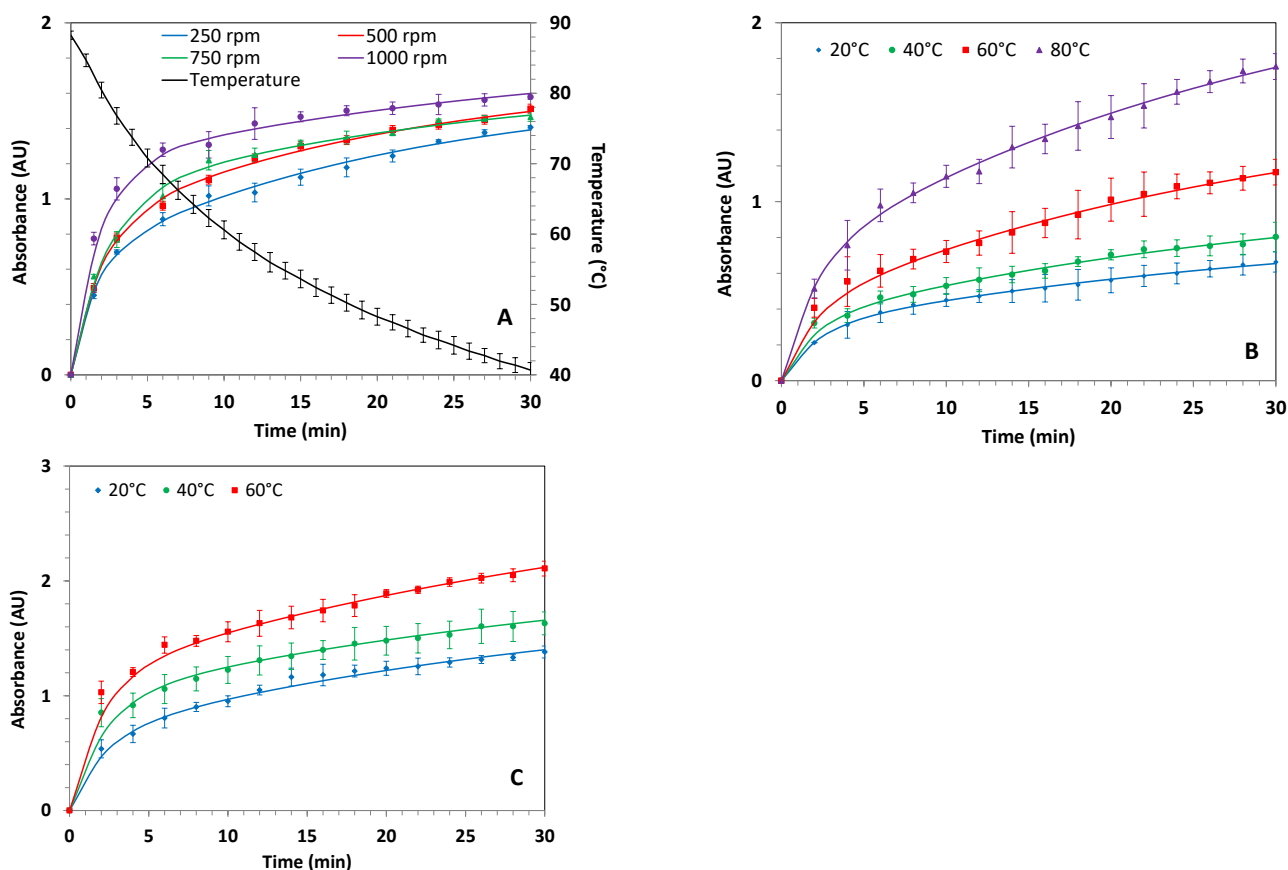


Figure II.1. Kinetics of extraction of raw flowering tops hawthorn (lot n°20335) monitored by UV absorbance at 198 nm for various extraction modes. A: Infusion mode at 250 rpm, 500 rpm, 750 rpm and 1000 rpm stirring speed, with the corresponding temperature profile obtained at 500 rpm. B: Maceration mode at 20°C, 40°C, 60°C and 80°C and at 500 rpm stirring speed. C: Ultrasonic (US) mode at 20°C, 40°C and 60°C and at 250 rpm stirring speed. In all cases, 2.5 g of raw hawthorn in 250 mL water was used. 100 μ L of solution were taken and added to 4 mL ultrapure water before each UV measurement. Error bars are \pm one SD on $n=3$ repetitions of independent extractions. If the absorbance values were above 1.7, dilution in 8 mL (instead of 4 mL) was used but the experimental values are then multiplied by 2 to allow comparison with dilutions in 4 mL.

Table II.2. Physicochemical characteristics of the flowering top hawthorn extracts depending on the extraction mode, the extraction time and the plant granulometry. In all cases, 2.5 g of hawthorn material in 250 mL water was used. For kinetic UV monitoring, 100 μ L of solution were taken and added to 4 mL water before UV measurement, except for ^a, where the 100 μ L were added to 8 mL water to avoid spectrometer saturation (values reported in the table are multiplied by a factor 2 for better comparison). ^b: \pm one standard deviation calculated on $n = 3$ repetitions. ^c: in mg eq. GA/g dry plant, \pm one standard deviation calculated on $n = 3$ repetitions. ^d: in mg eq. Q/g dry plant, \pm one standard deviation calculated on $n = 3$ repetitions. ^e: in mg eq. CY/g dry plant, \pm one standard deviation calculated on $n = 3$ repetitions. ^f: in mg / g dry plant, \pm one standard deviation calculated on $n = 3$ repetitions. Lot number for raw and grinded flowering tops materials: 20335. Lot number for flowers only: 20334. Fresh flowering tops: harvested in 2017.

Plant nature & granulometry	Extraction mode	Experimental conditions	$t_{70\%}$ (min)	A(t) at 30 min	10 min extraction time					30 min extraction time			
					Extraction yield (%) ^b	TPC ^c	TFC ^d	OPC ^e	Vitexin O-rhamnoside ^f	Extraction yield (%) ^b	TPC ^c	TFC ^d	OPC ^e
Flowering tops (Raw)	Infusion	250 rpm	9	1.41	-	-	-	-	-	14.29 \pm 0.56	-	-	-
		500 rpm	8	1.51	15.64 \pm 0.83	18.90 \pm 1.72	2.33 \pm 0.19	1.24 \pm 0.10	5.25 \pm 0.20	16.14 \pm 0.45	18.78 \pm 0.68	2.47 \pm 0.07	1.70 \pm 0.16
		750 rpm	6	1.47	-	-	-	-	-	15.03 \pm 0.71	-	-	-
		1000 rpm	4	1.58	-	-	-	-	-	16.02 \pm 0.89	-	-	-
	Maceration (at 500 rpm)	20°C	12	0.66	-	-	-	-	-	9.75 \pm 0.56	-	-	-
		40°C	12	0.80	-	-	-	-	-	11.87 \pm 0.75	-	-	-
		60°C	13	1.17	12.02 \pm 0.81	12.57 \pm 0.74	1.47 \pm 0.15	1.06 \pm 0.02	3.72 \pm 0.25	14.18 \pm 0.67	14.60 \pm 1.67	1.86 \pm 0.18	1.10 \pm 0.07

	80°C	13	1.76	-	-	-	-	-	16.19 ±1.07	-	-	-
	20°C	10	1.38	-	-	-	-	-	13.65 ±1.22	-	-	-
US (at 250 rpm)	40°C	8	1.63	-	-	-	-	-	14.48 ±1.19	-	-	-
	60°C	8	2.14 ^a	17.21 ±0.53	21.17 ±2.57	2.46 ±0.03	2.37 ±0.20	5.99 ±0.12	20.78 ±1.09	24.17 ±0.57	2.75 ±0.05	2.46 ±0.06
MW	300W	-	-	17.30 ±1.67	22.69 ±1.30	2.80 ±0.06	2.35 ±0.20	6.66 ±0.15	21.57 ±0.20	30.14 ±0.44	3.21 ±0.17	3.82 ±0.08
Percolation	-	-	-	18.16 ±1.34	23.87 ±2.14	2.89 ±0.12	3.01 ±0.22	6.19 ±0.49	-	-	-	-
	250 rpm	< 1.5	2.27 ^a	-	-	-	-	-	-	-	-	-
Infusion	500 rpm	< 1.5	2.41 ^a	22.20 ±0.59	32.79 ±0.67	3.45 ±0.20	3.93 ±0.09	7.63 ±0.10	23.19 ±0.66	34.67 ±0.93	3.56 ±0.06	4.63 ±0.39
	750 rpm	< 1.5	2.43 ^a	-	-	-	-	-	-	-	-	-
	1000 rpm	< 1.5	2.52 ^a	-	-	-	-	-	-	-	-	-
	20°C	12	1.76	-	-	-	-	-	19.11 ±0.35	-	-	-
Flowering tops (Grinded 1 mm)	Maceration (at 500 rpm)	40°C	4.5	2.07 ^a	-	-	-	-	19.20 ±0.48	-	-	-
		60°C	< 1.5	2.30 ^a	20.59 ±0.29	28.45 ±0.26	3.12 ±0.07	3.52 ±0.14	6.97 ±0.16	21.18 ±1.29	30.28 ±0.37	3.20 ±0.07

		80°C	< 1.5	2.58 ^a	-	-	-	-	-	23.30 ±0.78	-	-	-
		20°C	-	-	-	-	-	-	-	14.28 ±1.67	-	-	-
	US (at 250 rpm)	40°C	-	-	-	-	-	-	-	17.50 ±1.72	-	-	-
		60°C	< 1.5	3.05 ^a	24.24 ±0.98	33.58 ±1.23	3.64 ±0.47	4.29 ±0.10	7.92 ±0.26	25.10 ±1.86	33.26 ±1.72	3.74 ±0.17	4.32 ±0.19
	MW	300W	-	-	23.28 ±0.42	34.73 ±1.57	3.93 ±0.17	4.04 ±0.08	8.01 ±0.11	23.81 ±1.37	37.31 ±0.87	3.85 ±0.05	4.73 ±0.21
	Percolation	-	-	-	18.98 ±0.64	27.15 ±1.78	2.95 ±0.20	3.62 ±0.13	6.57 ±0.36	-	-	-	-
Flowering tops (Fresh)	Infusion	500 rpm	16.5	0.72	10.07 ±1.02	8.28 ±0.66	1.19 ±0.05	0.41 ±0.04	5.14 ±0.41	12.27 ±1.22	10.41 ±0.66	1.60 ±0.19	0.86 ±0.08
	Maceration	60°C, 500 rpm	13	0.58	6.69 ±1.84	2.94 ±1.38	0.63 ±0.06	0.14 ±0.04	-	11.09 ±0.78	9.80 ±0.89	1.35 ±0.09	0.65 ±0.05
Flowers only (Raw)	Infusion	500 rpm	-	-	10.18 ±0.87	11.50 ±0.32	1.66 ±0.01	0.66 ±0.05	2.19 ±0.03	-	-	-	-

II.2.2. Influence of the plant grinding on the extraction kinetics and on the global extraction yield

In a second set of experiments, similar extractions were performed on grinded (using 1 mm mesh size grinder) hawthorn flowering tops of the same lot (lot n° 20335). Clearly, the grinded plant led to much faster kinetics of extraction with $t_{70\%}$ lower than 1.5 min (Table II.2), for all extraction modes, regardless of the stirring speed and temperature, except for the two lowest maceration temperatures (20°C and 40°C). The kinetics of extraction were so fast that it was not possible to accurately determine $t_{70\%}$ (see Figure SI-II.3 for the UV monitoring). Extraction yields were similar at 10 and 30 min extraction times due to fast extractions (see Table II.2), but slightly higher values were obtained for MW and US-assisted modes (23-25%) than for infusion mode (22-23%) and maceration mode (20-21%). The extraction yields obtained from grinded material were much higher for all extraction modes compared to raw materials (+42% for infusion and ultrasonic extractions; +34% for microwaves and up to +71% for maceration at 60°C), except for percolation.

Figure II.2 displayed the UV absorbance obtained at 198 nm for 10 min extraction time as a function of the extraction yields at the same extraction time for the 5 extraction modes, for raw/grinded plants. There is a linear correlation between the UV absorbance and the extraction yield. However, surprisingly, this correlation does not extrapolate to zero extraction yield at zero absorbance, suggesting that 7-8% of the initial mass of the dry plant correspond to some easily extracted UV-transparent compounds. Similar trends/results were obtained at 5 min and 30 min extraction times (see Figure SI-II.4). From Figure II.2, the most efficient extraction mode appears to be the US-assisted mode at 60°C closely followed by MW and infusion modes, for grinded materials.

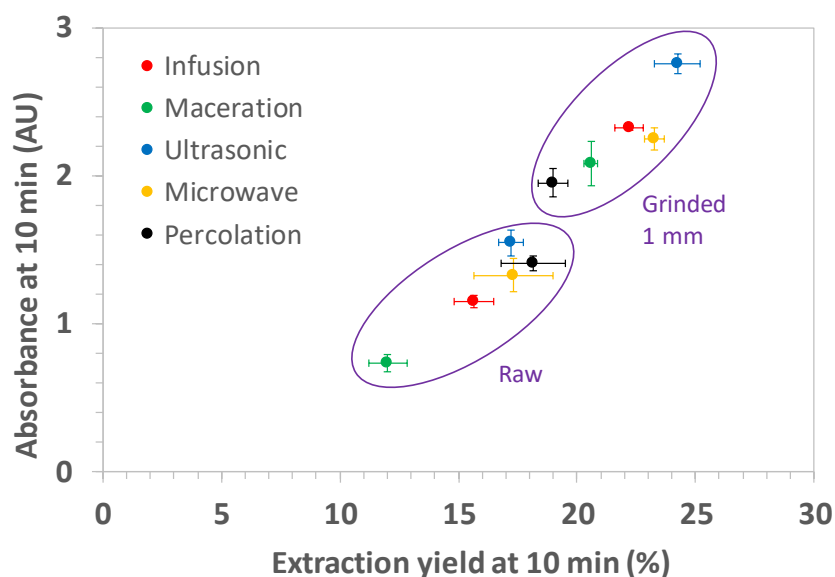


Figure II.2. UV absorbance values at 198 nm versus the extraction yield at 10 min extraction time for various extraction modes. Maceration and ultrasonic assisted modes at 60°C. In all cases, 2.5 g of hawthorn material in 250 mL water was used. Dry hawthorn lot number: 20335. Grinded material with 1 mm grid mesh size. Error bars are \pm one SD on $n = 3$ repetitions of independent extractions.

Compared to the other experimental parameters (extraction mode, stirring speed, and to a lower extent, extraction temperature), the plant granulometry is, by far, the most important factor to speed-up the kinetics and to maximize the yield of extraction. Between the lowest value (maceration on raw material) and the highest value (ultrasonic on grinded plants), obtained at the same drinkable temperature (60°C), a factor of 2 was found on the extraction yield, demonstrating the importance of the protocol of extraction, even for similar temperature of extraction.

A good news from this study, in a practical point of view, is that the infusion mode appears as the best compromise among all the extraction modes because (i) it is probably the most simple mode to implement, (ii) the kinetics of extraction was very fast and the extraction yield at 10 min was not very different from the best values obtained in this study (22% for infusion vs 23-24% for MW and US –assisted modes; non-significant differences between extraction modes at 0.97 confidence level by one-way ANOVA), and (iii) the temperature profile of infusion is much more gentle than for MW, which is preferable to avoid temperature degradation of biologically active components.

II.2.3. Quantification of total polyphenol, flavonoid and proanthocyanidin contents.

Comparison with commercialized standardized extracts and antioxidant activity

After extraction, the plant extracts were evaporated, lyophilized and kept in the dark at -18°C for better conservation prior to analysis. The values for the total amounts of polyphenol (TPC), flavonoid (TFC) and proanthocyanidin oligomers (OPC) contents in the dry plant extracts were determined by colorimetric methods (see sections II.3.10 to II.3.12) and expressed as equivalent content in gallic acid (GA) for TPC, quercetin (Q) for TFC and cyanidin (CY) for OPC. The numerical values are reported in Table II.2 for 10 min and 30 min extraction times ($n=3$ repetitions on 3 independent extractions).

As expected, the extraction yields of polyphenols, flavonoids and proanthocyanidin oligomers were much higher for grinded material than for raw material. TPC (resp. TFC) contents at 10 min extraction varied between 12.6 and 34.7 mg eq. GA / g dry plant (resp. 1.47 and 3.93 mg eq. Q / g dry plant), when comparing the worst (maceration, 60°C, raw) and the best (microwaves, grinded) figures of merits. This represents an enhanced extraction factor (EF) of about 2.7, which is even raised up to 3.8 for OPC. This improvement in the extraction of the components of interest (TPC, TFC and OPC) is even more pronounced than the increase in the global extraction yield, which is only affected by a factor of ~ 2 . Clearly, the grinding of the dry plant is the most important parameter to increase the extraction yields for all the quantified components. At 10 min extraction time, for grinded dry hawthorn, 27-35 mg equivalent GA (TPC)/ g dry plant, 2.9-4.0 mg equivalent Q (TFC)/ g dry plant and 3.6-4.3 mg equivalent CY (OPC) / g dry plant were extracted, which is in good agreement with the values usually reported in the literature for the same plant [20, 24, 33, 34, 53, 61]. MW and US modes give the best extraction yields, either for raw and grinded plants, in good agreement with the literature [60], but the differences with infusion mode are only limited (only 5 to 10% differences on TPC, TFC and OPC extraction yields).

As a matter of comparison with commercialized standardized extracts (see Table II.3), the dry extract content and the TPC, TFC and OPC contents obtained from a single infusion (at 500 rpm) of 2.5 g of 1 mm grinded hawthorn in 250 mL water were determined. One infusion brings about 555 mg of dry extract containing 82 mg equivalent GA (TPC), 8.6 mg

equivalent Q (TFC) and 9.8 mg equivalent CY (OPC). These values are, for instance, similar to the dry extract content of a Cardio Max WS1442® tablet (450 mg per tablet) and similar to the TFC intake given by 5 mL of standardized hawthorn plant extract (EPS Phytoprevent®, 7.5-12.5 mg eq. Q). The contents in TPC, TFC and OPC given by the suppliers are gathered in Table II.3 (when known) for 4 different standardized commercial products (tablets of dry hawthorn extracts Faros 300 LI132® and Cardio Max WS1442®, EPS Phytoprevent® or Crataegisan® Bioforce extracts), even if the standardization is not always performed using the same compounds of reference. On the whole, it can be concluded that one to two infusions per day of 2.5 g of grinded dry hawthorn flowering top provide similar quantities of hawthorn extracts and TPC/TFC/OPC contents compared to the advised posology of the standardized formulations.

The antioxidant activity quantified by the Trolox equivalent antioxidant capacity (TEAC) assay is displayed in Figure II.3, expressed either per g of dry extract (in blue) or per 2.5 g of plant corresponding to one extraction experiment (in red). Interestingly, except for the extracts issued from fresh hawthorn (see next section for more explanations), TEAC values of all the extracts were found between 140 and 160 mg eq. Trolox / g dry extract. These results showed that the antioxidant activities were directly related to the overall extracted quantity, whatever the extraction mode and/or the plant granulometry (raw, grinded). The ranking of the antioxidant activity expressed for 2.5 g of plant (corresponding to one intake), is in good agreement with the ranking of the extraction yields previously discussed with the best figures of merits obtained for grinded material combined with ultrasonic, microwaves or infusion.

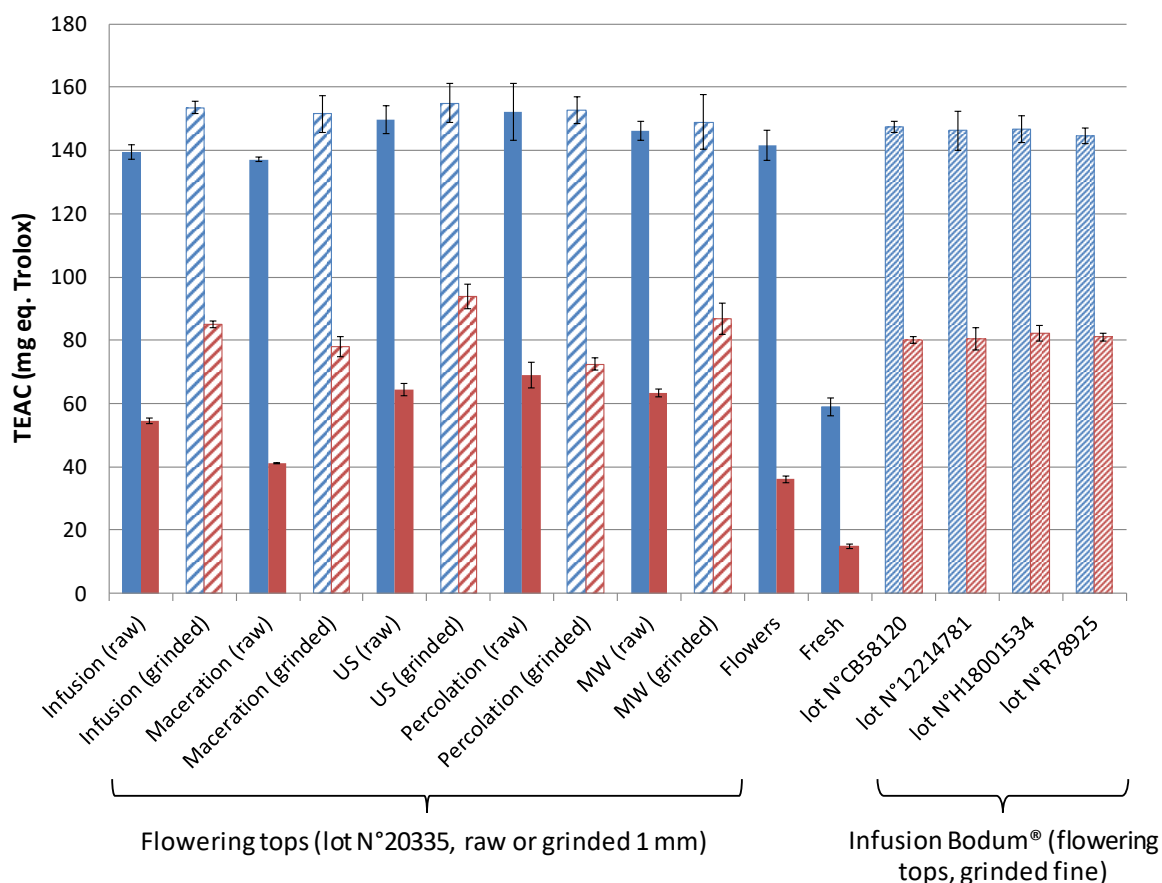


Figure II.3. Antioxidant activities of hawthorn extracts obtained by TEAC assays. Dry plant: Lot N°20335 (flowering tops) and 20334 (flowers only), except for infusion Bodum® (as indicated on the graph). In blue: TEAC in mg eq. Trolox per g of extract. In red: TEAC in mg eq. Trolox per 2.5 g plant. US and maceration modes performed at 60°C. Hatched lines = grinded materials.

II.2.4. Quantification Influence of the extraction mode and the nature / state of the hawthorn on the extracted UHPLC profiles

To get a better insight into the differences between the samples obtained from various extraction modes and from diverse natures of hawthorn (flowering tops vs flowers, dry vs fresh, raw vs grinded), reversed phase UHPLC and positive mode UHPLC-MS analysis were performed (see section II.3.13 for more details) according to previously published method used for hawthorn extracts [39]. Figure II.4 displays the chromatographic profiles obtained for some of the samples presented in Table SI-II.1 (see Figure SI-II.5 for the other UHPLC profiles). The corresponding relative peak area distributions are provided in Figure II.5 for the 11 main components detected at 273 nm in UHPLC and identified by UHPLC-ESI-MS coupling (positive mode) in the same conditions of elution (see Figure SI-II.6 for the peak area distributions issued from all the UHPLC profiles analyzed). Each relative peak area displayed in Figure II.5 was calculated by dividing the peak area of each component by the

sum of the peak area of the 12 identified components. These values are average values calculated on 3 independent extractions. Table II.4 contains the list with names and molar masses of these identified components. Figure SI-II.7 gives the chemical structure of all compounds identified.

Regarding the influence of the extraction mode on the UHPLC profiles, Figures II.4 and II. 5 show that the profiles are very similar for infusion, maceration, US, and percolation modes with a majority (by decreasing order of peak area) of vitexin-2-O-rhamnoside (peak 8), chlorogenic acid (peak 3), hyperoside (peak 9), and isoquercetin (peak 11). The two remarkable differences concern the relative content in cyanidin (peak 1), which is significantly lower for the US mode; and a lower relative content in chlorogenic acid for MW mode. The quantification of vitexin-2-O-rhamnoside (peak 8) by external calibration, using a commercially available standard, confirmed that the extraction mode did not significantly change the extracted quantity of this major component (~5-7 mg/g of dry plant for all extraction modes, see Table II.2), except a slightly higher content for MW mode (8.01 mg/g dry plant).

The influence of the nature (flowering tops vs flowers), the state (dry vs fresh), and the granulometry (raw vs grinded) of the plant on the UHPLC profiles was also investigated. Raw and grinded extracts were compared on the same lot (n°20335) by infusion extraction. Dry flowers (without leaves) was also compared to dry flowering tops using infusion extraction. Extracts obtained by infusion of fresh flowering tops harvested in April 2017 on Oléron Island (located on the French oceanic west coast, see Figure SI-II.1 for more details) were analyzed and compared to infusion of the same plant after one year drying.

Figure II.4 revealed (i) similar profiles between raw and grinded flowering tops but with a higher content in procyanidins B2 and C1 (peaks 4 and 6) for grinded plant, in good agreement with the higher OPC content (3.93 vs 1.24 mg eq. CY / g of plant); (ii) higher content in hyperoside in dry flowers but lower contents in apigenin-C-hexoside and vitexin-2-O-rhamnoside (the latter being confirmed by external calibration, 2.19 mg / g of dry plant) compared to dry flowering tops; (iii) much higher content (confirmed by external calibration, 5.1 mg / g of fresh weight plant) of vitexin-2-O-rhamnoside and very low

contents in apigenin-C-hexoside and procyanidins (in agreement with low OPC values, 0.41 mg eq. CY / g of plant) in freshly harvested flowering tops. Interestingly, the differences observed in the UHPLC profiles between the fresh and the dry flowering tops (different lots) tends to vanish after one year drying of the 'fresh' flowering tops, with increasing contents in epicatechin, hyperoside, apigenin-C-hexoside and procyanidins for the dry plant.

Table II.3. Comparison of the TPC, TFC and OPC contents between commercially available standardized extracts (as given by the supplier) and infusions at 10 min extraction time (as determined in this work). Q = quercetin, CY = cyanidin, GA = gallic acid, HY = hyperoside, epiCAT = epicatechin. ^a as for 2017.

Standardized extracts	Uptake quantity	Dry extract content	Dry plant:dry extract ratio	Plant organ	Excipients	Extraction solvent	TPC	TFC	OPC	Price (€)	Treatment cost / day (cents)	Cost / 30 days (€) ^a
EPS Phyto-prevent®	5-10 mL	900 mg / 5 mL	-	leaves & flowers	Glycerol	-	-	7.5-12.5 mg eq. Q / 5 mL (0.8-1.4 %)	-	19.89 / 150 mL	66.3 / 5 mL	19.9
WS1442® crataegutt novo 450 (orCardiplant® 450 or Cardio Max WS 1442®)	1-2 Tablets	450 mg / 1 tablet	4.0-6.6 : 1	leaves & flowers	Glucose, SiO ₂ , Fe ₂ O ₃ , TiO ₂ , sucrose, gelatine, macrogol, citricacid, ...	EtOH (45 %)	-	-	78-90.6 mg eq. epiCAT / 1 tablet (17.3-20.1 %)	16.13 / 50 tablets	64.5 / 2 tablets	19.4
Crataegisan® Bioforce	30-90 drops (0.75-2.25 mL)	690 mg / 90 drops	3.2 : 1	fruits	EtOH (46-54%)	EtOH (50 %)	12.7 mg eq. GA / 90 drops (1.84 %)	-	6.4 mg / 90 drops (0.93 %)	10.50 / 50 mL	52.5 / 90 drops	15.8

Faros 300® LI 132	3 Tablets	300 mg / 1 tablet	4.0-7.0 : 1	leaves, flowers & fruits	Glucose, SiO ₂ , lactose, TiO ₂ , sucrose, gelatine, macrogol, ...	MeOH (70 %)	-	6.6 mg eq. HY / 1 tablet (2.2 %)	-	23.25 / 100 tablets	69.7 / 3 tablets	20.9
Infusion (lot N°20335)	1-2 infusions	555 mg / 10 min infusion (from 2.5 g grinded dry plant)	4.3-4.6 : 1	leaves & flowers	Water	Water	82 mg eq. GA / 1 infusion	8.6 mg eq. Q / 1 infusion	9.8 mg eq. CY / 1 infusion	29.25 / 1 kg	7.3 / 1 infusion	2.2

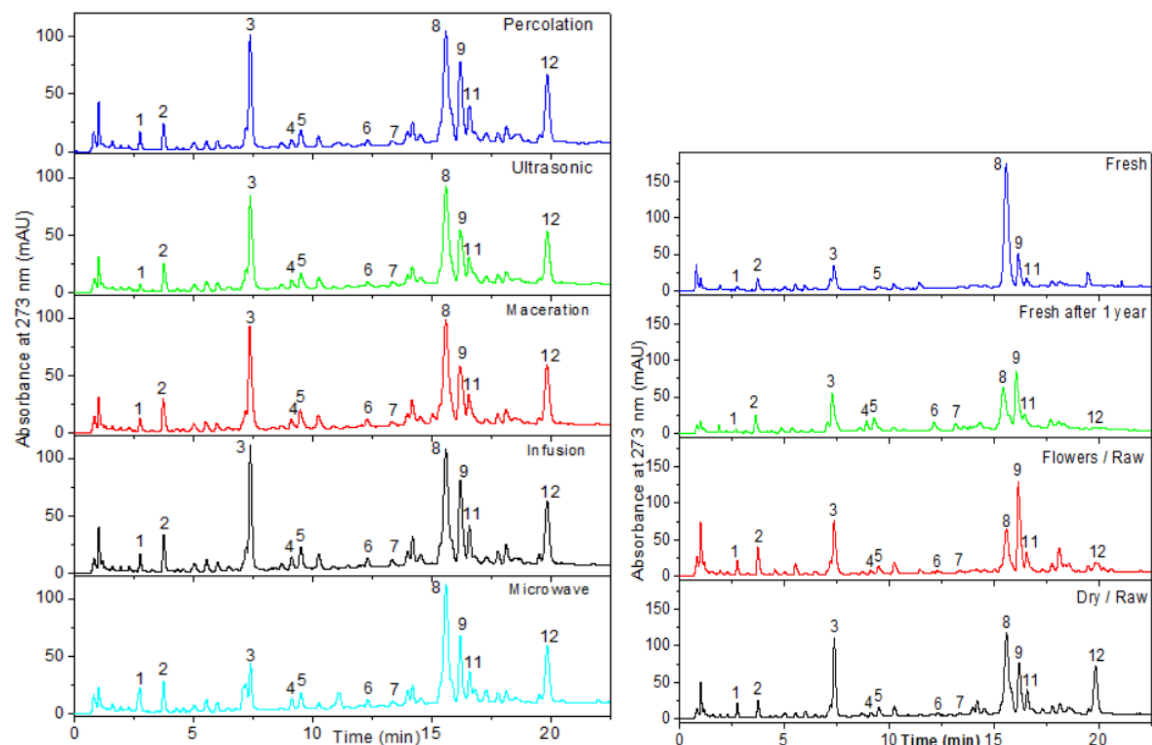


Figure II.4. UHPLC profiles of different hawthorn extracts obtained from different extraction modes for 1 mm grinded hawthorn flowering tops (on the left) and from infusion of different parts (flowering tops vs flowers) or different states (fresh vs dry) of hawthorn (on the right), as indicated on the figure. See experimental part for the different extraction protocols. Experimental conditions: Luna® Omega polar C18 column (1.6 μm , 100 \times 2.1 mm), binary solvent system: water/formic acid (1‰, v/v) as solvent A and acetonitrile/formic acid (1‰, v/v) as solvent B. Gradient program: 5 % B, then increase of B to 100 % in 30 min with a convex increase, flow rate: 0.4 mL.min⁻¹, injection volume: 4 μL . Column temperature: 35°C. UV monitoring at 273 nm. UV-Vis spectra recorded between 200 and 550 nm. Lot number for raw and grinded flowering tops materials: 20335. Lot number for flowers: 20334. Fresh flowering tops: harvested in 2017 and extracted one week after (fresh) or one year after (fresh after one year). Peak identification: 1=Cyanidin, 2=5-O-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7=cinnamtannin A2, 8=vitexin-2-O-rhamnoside, 9=hyperoside, 11=isoquercetin, 12=apigenin C-hexoside.

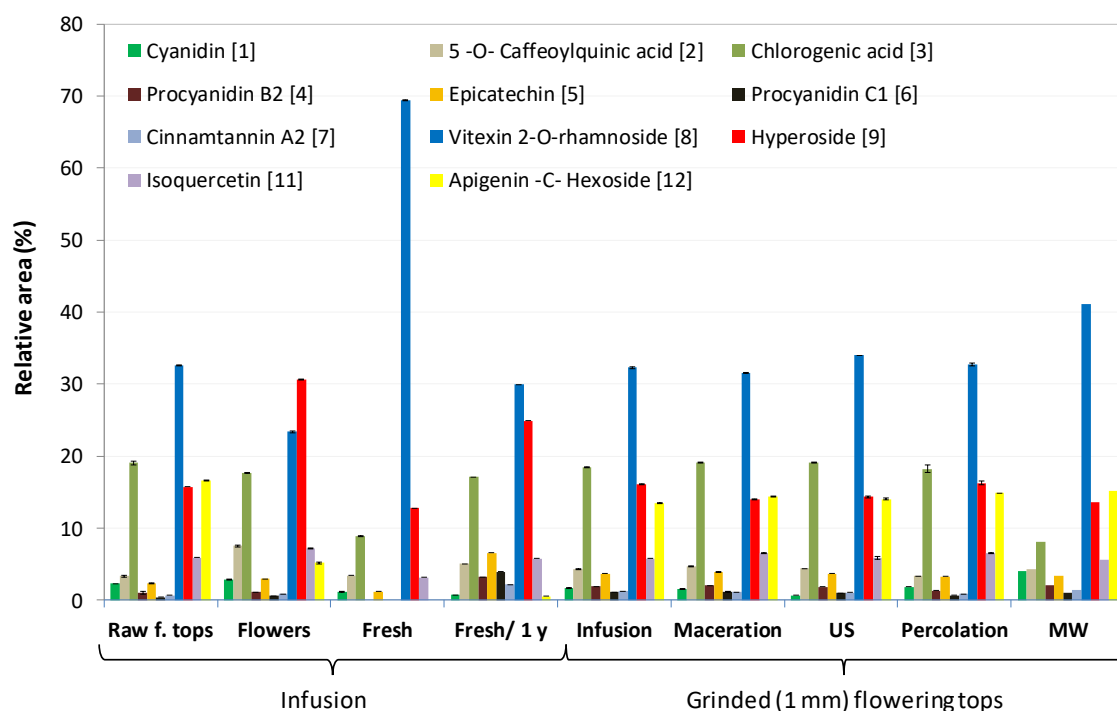


Figure II.5. Relative peak area distributions for the main identified chromatographic peaks according to the extraction mode for grinded (1 mm, lot n°20335) hawthorn (infusion, maceration, US, percolation, MW) or according to the nature of hawthorn by infusion (raw dry flowering tops (lot n°20335), fresh flowering tops, flowers only (lot n°20334)). Same experimental conditions as in Figure II.4. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 12 identified components. Error bars: \pm one standard deviation calculated on $n=3$ repetitions.

Table II.4. Peak identification of the main compounds detected by UHPLC in the various hawthorn extracts. λ_{max} are the local maximum of absorbance on the UV spectrum. $[M+H]^+$ column provides the m/z value of the precursor ion. Other ions column gives the m/z value of fragments detected in the MS spectra. Identification method using UV spectrum and ESI(+) spectrum of the pure standards (R) or a secondary standard mixture (R1, *Crataegus* spp. Extract).

Peak	Retention time (min)	λ_{max} (nm)	$[M+H]^+$	Other ions in the spectrum	Identified compound	Standard used for identification	Ref.
1	2.71	204, 218, 260	288		Cyanidin	R	[40]
2	3.73	218, 236, 324	355	377, 711	5-O-Caffeoylquinic acid	R1	[40]
3	7.49	219, 238, 325	355	377, 711	Chlorogenic acid (3-O-caffeoylquinic acid)	R1	[40]
4	9.1	227, 280	579	427, 289	Procyanidin B2	R	[40]

5	9.45	224 279	291	147, 139, 123	Epicatechin	R	[40]
6	12.24	280	867	579	Procyanidin C1	R	[40]
7	13.37	219, 280	1155	287, 413, 575	Cinnamtannin A2	R	[40]
8	15.59	216, 269, 338	579	433, 313	Vitexin 2-O-rhamnoside	R1	[40]
9	16.13	220, 256, 353	465	303	Hyperoside	R1	[40]
10	16.35	219, 280	577	289	Procyanidin A2	R	[40]
11	16.52	202, 257, 353	303	621	Isoquercetin	R	[40]
12	19.85	268, 337	433	621	Apigenin-C-hexoside	R1	[40]

II.2.5. Influence of extraction mode and the nature / state of the hawthorn studied by ESI FT-ICR-MS in negative mode

In this part, the discussion is essentially based on the grinded/raw, fresh/dry, and flower/flowering tops samples obtained with the previous extraction modes (all samples analyzed by ESI FT-ICR-MS are reported in Table SI-II.1). Mass spectra achieved for these samples are given in Figure SI-II.8 in duplicate (two independent extractions), with the corresponding global composition description in heteroatom classes and van Krevelen diagram. This latter graph is obtained by plotting the achieved raw formulae according to their H/C and O/C. Depending on the plot location and as illustrated in Figure II.6, it is possible to distinguish some area corresponding to biochemical families such as lipids, polyphenols, amino acids, carbohydrates [106, 107].

In this study, most of the samples led to the same global chemical description as illustrated in Figure SI-II.8. The achieved van Krevelen diagrams evidence some biochemical families (Figure II.6 and Figure SI-II.8). Among the CHO class, some carbohydrates and polyphenols are detected whereas in the CHON one, some amino acids, with possibly some amino sugars, are observed. Regarding the global composition description (Figure SI-II.8 – pie charts), only samples obtained from infusion of fresh flowering tops and dry flowers

present a slightly different composition. The achieved global composition description of samples obtained from dry flowering tops is very similar with CHO, CHOS, CHON, and CHOCI families representing respectively, on average, $63.2 \pm 1.4\%$, $4.7 \pm 0.5\%$, $25.7 \pm 1.3\%$, and $6.4 \pm 1.4\%$ of the total assigned features. For the fresh flowering tops infusion, the distribution changes to 58.2%, 4.2%, 26.9%, and 10.7% whereas for the samples from dry flowers infusion, it is 54.1%, 2.6%, 37.8%, and 5.5%. The higher number of chlorinated species in the samples from fresh flowering tops can be explained by the harvesting location, near Atlantic Ocean which can favor the presence of NaCl salt and chloride adduct during MS analysis. Regarding the flower samples, a higher number of CHON species, which are likely amino acids and amino sugars, characterizes them. These variations in global composition is confirmed by the PCA of the data obtained by (-) ESI FT-ICR MS analysis (Figure II.7). The PCA demonstrates the higher variability in the composition of the fresh flowering top and dry flower samples against the dry flowering tops samples.

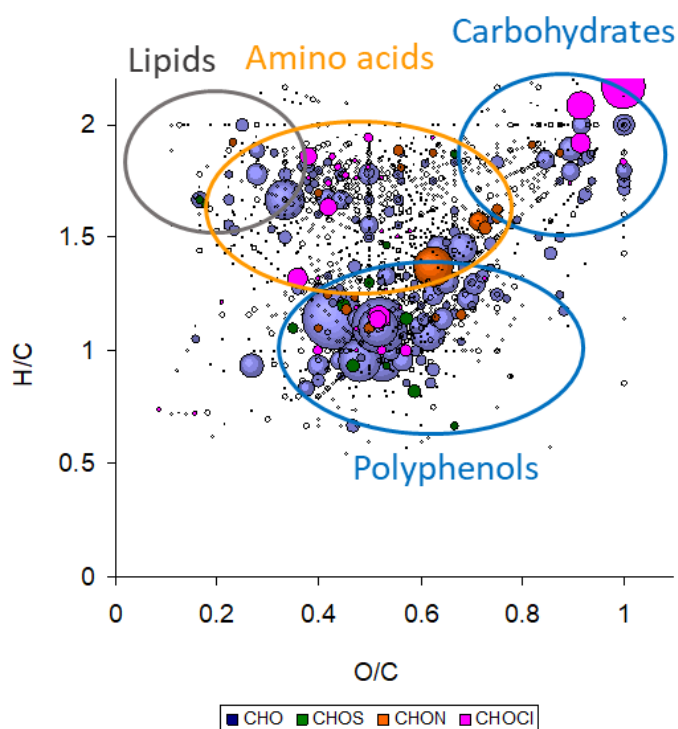


Figure II.6. Typical van Krevelen diagram achieved by (-) ESI FT-ICR MS analysis of hawthorn sample with area of distinguished biochemical compounds. The size of the bubble is relative to the peak intensity.

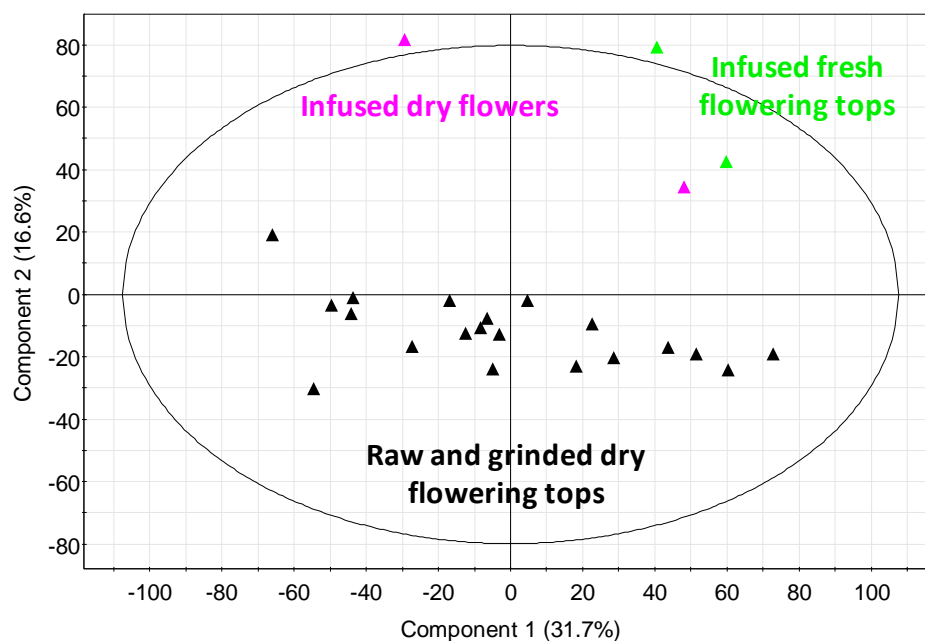


Figure II.7. PCA score plot of all mass features from hawthorn extracts measured by (-) ESI FT-ICR MS, in duplicate, obtained by various extraction modes and from different state/nature of plant: infused dry flower (pink), infused fresh flowering tops (green), and raw and grinded dry flowering tops extracted by infusion, maceration, ultrasonic, percolation, and microwave (black).

Due to significant composition similarity between the samples, HCA with heatmap (Figure SI-II.9) have been carried out to determine the features specific to a sample according to its characteristics (dry/fresh, raw/grinded, flower/flowering tops).

First, samples from fresh flowering tops were compared to the dry flowering top samples. Features specifically observed in each class were extracted, represented according to heteroatom class, and plotted on van Krevelen diagram (Figure II.8A). Moreover, the putative compounds obtained for these extracted features are also reported in Table SI-II.4. Features specific to the dry flowering top samples are mainly CHO species and the van Krevelen diagram indicates they are carbohydrates and more importantly polyphenols. Amongst these CHO assignments, one features at m/z 353.087809 was intensely detected and it can be associated to chlorogenic acid or 5-O-caffeoylquinic acid. This observation was confirmed by a more intense peak 3 (chlorogenic acid) in UHPLC-UV analysis (Figure II.4) in dry flowering tops compared to fresh flowering tops. The other putative compounds are flavonoids associated to one or more sugar. Regarding the fresh samples, the heteroatom

class distribution is more heterogeneous than with the dry samples, with more CHON and CHOCl species. This is in agreement with the previous global elemental composition description achieved for this sample. Concerning the former class of CHON compounds, their location on the van Krevelen diagram corresponds to amino acids and amino sugars. The extracted CHO species are lipids, carbohydrates, and polyphenols. Sucrose was found to be a possible component as well as some flavonoids bounded or not to a carbohydrate. Procyanidin A2 is also one matching component of the extracted features.

The same procedure was done with the dry samples but coming from, on the one hand, the flowers (without leaves) and, on the other, the flowering tops (Figure II.8B). The features specific to flowering tops samples are mainly CHO compounds. The van Krevelen diagram demonstrates they are carbohydrates and more importantly, polyphenols. Some putative features were found, which concern flavonoids linked to a sugar. Concerning the samples from flower infusion, the heteroatom class distribution shows that a significant part of the specific features belongs to CHON class. These nitrogen-containing species can be regarded as amino acids or amino sugars. In addition, two features are likely to be tryptophan and glutamate. CHO components were also evidenced and they are related to lipids and polyphenols. For this latter class of compounds, some matches have been found with flavonoids linked to sugars such as shaftoside (apigenin 6-C-beta-D-glucoopyranosyl-8-C-alpha-L-arabinopyranoside) and quercetin pentoside.

A PLS-DA was done between raw and grinded flowering tops samples. Extracted features relative to the grinded samples were plotted on van Krevelen diagram (Figure II.8C). Regarding the CHO class, most of the compounds are polyphenol species. Some putative species were found such as procyanidin A2 and B2. Flavonoid compounds, with catechin and pinnatifida, were also putatively assigned. Some CHON compounds were also present, which correspond to amino acids, with glutamate putatively assigned. The CHOCl compounds extracted for this class are in the sugar and polyphenol areas of the van Krevelen diagram. The extracted features of the raw sample class are mainly CHO and CHON

compounds. The CHO species are not related to particular biochemical compound families. A few components are sugars and polyphenols but low-intensity detected. A broader range of amino acids was achieved from this PLS-DA class. Such observations are in agreement with those obtained in UHPLC-UV with MS coupling (Figure II.4) that demonstrated higher OPC in grinded samples than in the raw ones.

The (-) ESI FT-ICR MS analysis enabled to achieve a more extensive description of the samples. Despite strong similarities between the samples, it has been possible to extract features specific to some characteristics of sample preparation such as the granulometry or the plant organ. Thus, this approach ensured to confirm previous observations obtained in UHPLC-UV with MS coupling and to extend it to other putatively assigned species. The additional studies highlighted composition differences between the dry flowering top samples vs the fresh ones and the dry flowers ones. The achieved results were consistent with the global composition with a significant amount of CHON species for the flower samples and, to a lesser extent, the fresh ones. In addition to the all the CHO species detected by (-) ESI FT-ICR MS, the information obtained on the nitrogen-containing compounds is complementary to what obtained with the previous analytical method. This demonstrates the complementarity of FT-ICR MS for the exhaustive characterization of the hawthorn samples.

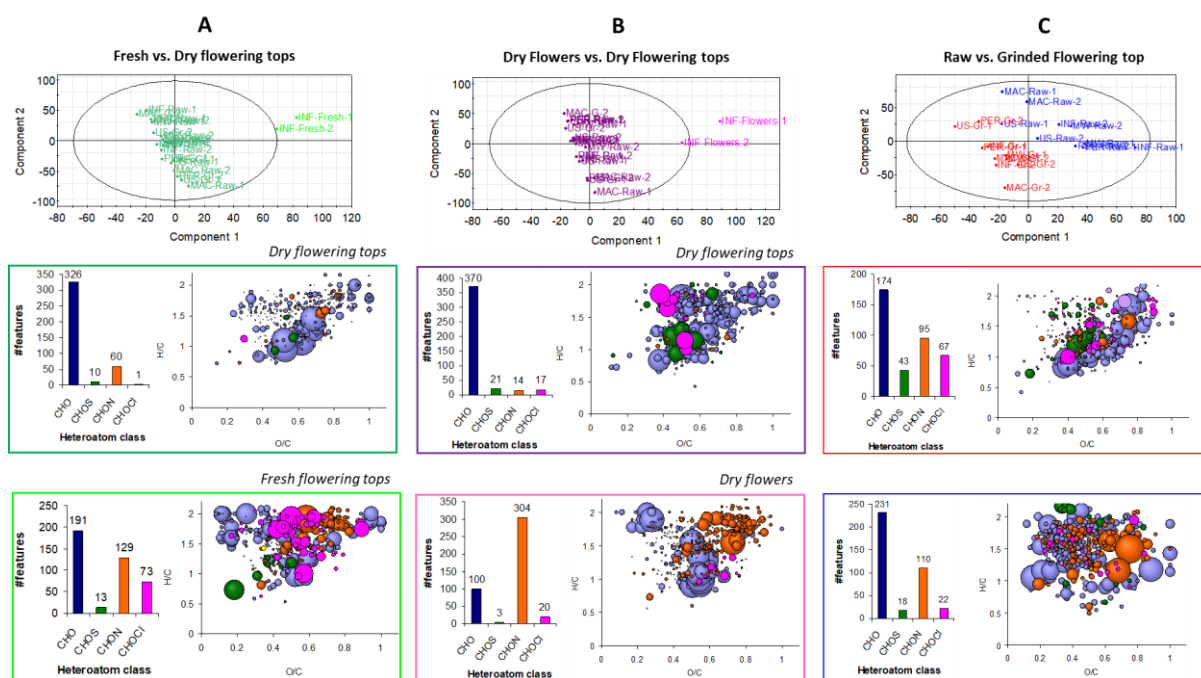


Figure II.8. Score plot of PLS-DA of hawthorn samples analysed by (-) ESI FT-ICR MS with (A) Fresh vs. Dry Flowering tops, (B) Dry Flowering tops vs. Dry Flowers, and (C) Raw vs. Grinded samples. Close to 400 assignments specific to each class were extracted and represented on bar chart according to their heteroatom class and on van Krevelen diagram. The bubble size is relative to peak intensity.

II.2.6. Optimization of the homemade infusion protocol and characterization of the plant granulometry

One of the main objective of this work was to develop a straightforward extraction protocol that can be accurately reproduced at home, maximizing the extraction of TPC/TFC/OPC within a minimum of time, and that should be kept as simple as possible (either in terms of manipulations or in the material required for the extraction). As we wanted to stick to real-life conditions / applications, the following experimental parameters were investigated to optimize the home-made protocol: (i) the size of the container (cup, mug or bowl, see Figure SI-II.10 for the dimensions and shapes) and therefore, the volume of introduced water (125, 250 and 405 mL, respectively); (ii) the presence or absence of magnetic stirring; (iii) the plant granulometry varied by using different affordable coffee grinders; and (iv) the use (or not) of a tea bag. Since the infusion mode was found to be one of the best compromise, as far as grinded materials are used, the optimized protocol has

been based on infusion. Temperature decrease profiles (see Figure SI-II.11 in supporting information of chapter II) can be correctly fitted (for natural convection experiments only) using a relatively simple model that takes into account the nature and geometry of the recipient. This model is based on the numerical resolution of the system of differential equations corresponding to the instantaneous material and thermal balances using a Mathcad script courteously provided by Condoret [108]. For example, 58% of the heat is evacuated via the vertical wall, 32% by evaporation for the mug. On the other hand, when the horizontal surface is greatly increased, the evaporation contributes for more than 65% of the energy loss.

As the plant granulometry is the main factor influencing the kinetics and the yield of extraction, it was crucial to find a simple way to grind the plants in a reproducible and affordable manner. Two electric coffee grinders were used and compared. The Delonghi (Model KG79) grinder, equipped with a burr-grinding wheel, was used at the two extremes positions, namely coarse and fine positions to get different granulometry. For a finer grinding, a Bosch electric grinder (Model MK6003) equipped with a fast rotating stainless steel chopping blade was used with two different grinding times (10 s and 30 s, by shaking the grinder simultaneously). For comparison, a grinding laboratory equipment (Ika, Model MF10 basic) was also used with two different grid sizes (1 mm and 2 mm). Figure SI-II.12 shows the pictures of the hawthorn material on a graph paper, before (raw) and after grinding. The density of the grinded material was measured using a graduated test tube (see section II.3.2 for more details), which is a very simple way to estimate the grinded plant density. This experimental parameter could be useful to optimize the grinding protocol, since it is related to the size distribution for polydisperse and non-spherical samples. Clearly, as seen in Figure II.8A, the density tends to increase with lower granulometry, which was in the order of: coarse < fine < grinded 2 mm < grinded 1 mm < ultrafine 10'' < ultrafine 30''. To get better quantitative data, the size distribution of the plant particle was determined by laser granulometry in dry phase (see section II.3.2) and the corresponding distributions are presented in Figure II.9A (see also Figure SI-II.12 for the repetitions). Volume size

distributions (given in diameter) are very broad with typical sizes ranging between 10-20 μm for the smaller particles, up to 500-700 μm for the largest one. Most of the distributions display a bimodal curve with a main mode between 180-300 μm , and a much less intense mode (or shoulder) at smaller sizes between 10 and 80 μm . The 'fine' distribution was close to the 2 mm grinded material; while the ultra-fine 10'' and 30'' present much lower granulometry. Figure II.9 represents the correlation between the grinded dry plant density and the particle diameters taken at different deciles of the distribution (D_{10} is the first decile, D_{50} is the median value, and D_{90} is the 9th decile). Interestingly, the most regular correlation between the plant density and the particle diameter is obtained with D_{10} . Such correlation could be useful to get a rough estimation of the hawthorn granulometry from simple determination of the dry plant density before or after grinding.

Regarding the choice of the recipient, the global extraction yield was found higher (about 10-15% more) for bowl and mug than cup, suggesting that a higher volume of water can extract higher amount of compounds (Table II.6). It is worth noting that this effect has nothing to do with the differences in the temperature profiles according to the recipient (see Figure SI-II.11), which is in the order of cup < bowl < mug (from lower to higher temperature at 10 min extraction time). TPC, TFC and OPC values were in the order of cup < mug < bowl, with more than 50% increase for the bowl compared to the cup. TPC, TFC and OPC values obtained for bowl using 250 mL of water were found in the same range as for the previously used three-necked flask. Using a 1 mm or 2 mm grinder did not change the extraction results significantly.

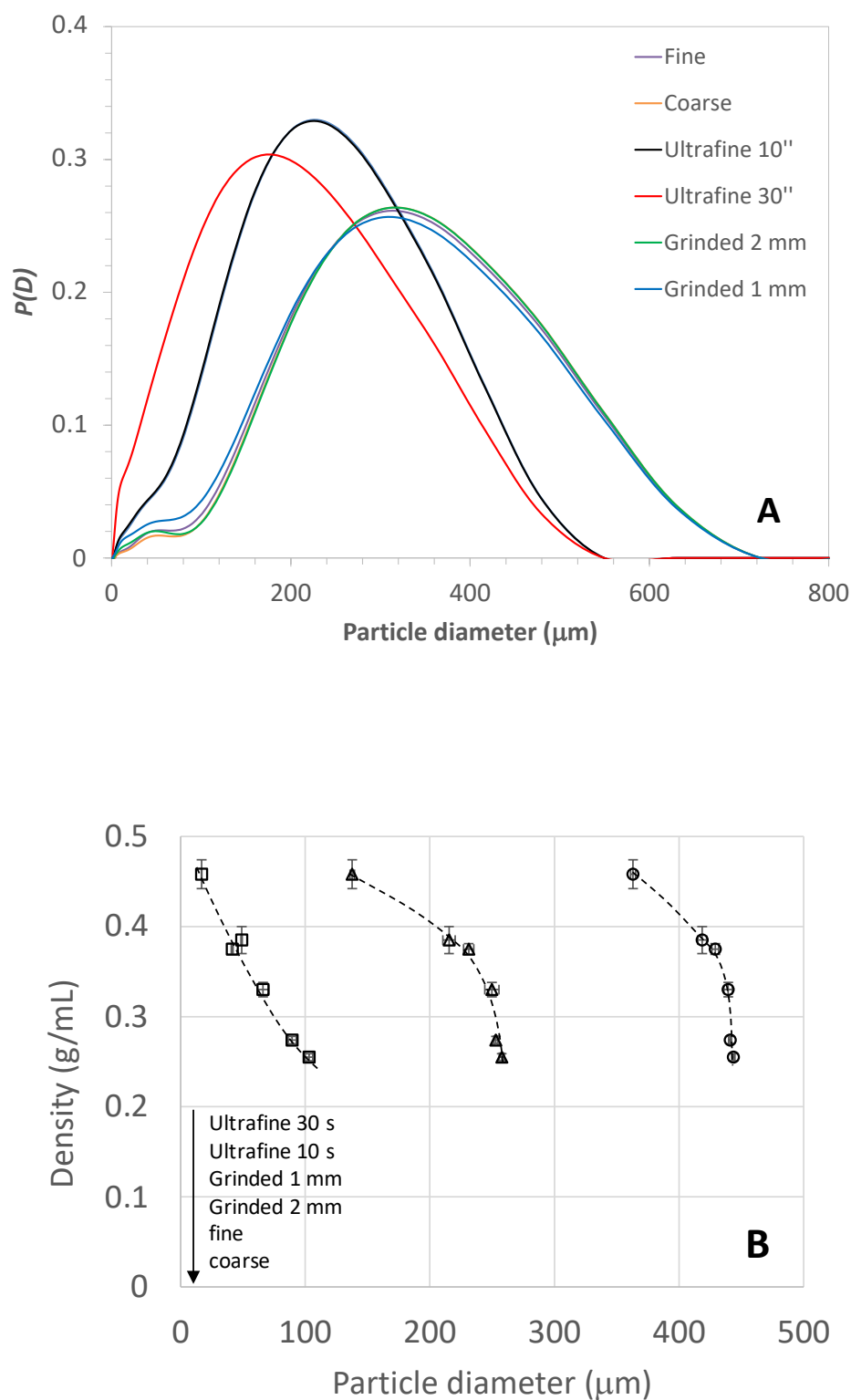


Figure II.9. Relative size distributions of raw and grinded hawthorn materials obtained by laser granulometry in dry mode (A) and variation of the density of hawthorn materials as a function of the particle diameter (B). D_{10} (\square), D_{50} (Δ) and D_{90} (\circ) with D_x being the $(x/10)^{\text{th}}$ decile of the distribution. See section II.3.2 for more details on the experimental conditions. Pictures of the hawthorn materials taken on a millimeter paper are provided in Supporting Information (see Figure SI-II.12).

The use of a Cilia® bag to avoid the plant particles to disperse into the extracted solution for practical reasons should be avoided since it decreased both the extraction yield and the TPC, TFC and OPC contents (Table II.5). This effect can be explained either by a lower diffusion of the extracted components from the inside to the outside part of the bag, or by a retention of a significant part of the extracted soluble compounds onto the surface of the paper bag. As a matter of comparison, an infusion in a mug with a Cilia bag is similar to an infusion in a cup without Cilia bag. When the granulometry of the plant is too fine (ultrafine 10''), the extraction yields of all components drop to values even lower than for raw materials, suggesting that there is a critical particle size value under which the pores of the bag are clogged.

Finally, the simplest and optimized way to perform an infusion at home, without using tea bag, is to use a 'French-press' coffee maker able to receive at least 250 mL of water (see Figure SI-II.2). This allows avoiding the use of a filter bag, the plant being freely floating in the recipient during the infusion, while the piston permits to push the residual solid parts of the plant to the bottom of the recipient, at the end of the extraction, before serving. It is worth noting that the granulometry of the grinded plant between fine (DeLonghi grinder), coarse (DeLonghi grinder) and ultrafine 10'' (Bosch grinder) did not significantly impact the extraction yield and the quantities of TPC, TFC and OPC that were extracted (see Table II.5).

The effect of stirring was also investigated by comparing the same extraction with and without magnetic stirring. As far as grinded materials are concerned (fine position or smaller sizes), the effect of this parameter was negligible: a short manual stirring of the Bodum® pot at the beginning and at the end of the extraction being enough to obtain quantitative extraction of the water-soluble components.

Table II.5. Extraction yield, TPC, TFC and OPC values in hawthorn extracts issued from infusion mode at 10 min extraction time. Influence of the particle size (plant state), the stirring, the use of a Cilia® bag, the nature of the container and the nature of the plant (different lot of dry flowering tops, dry flowers or fresh flowering tops). Experimentally, 2.5 g of hawthorn material in 125 mL (resp. 250 mL and 405 mL) water was used for cup (resp. mug or Bodum® and bowl recipients). ▲: see Table II.2 for more information. * Manual stirring was ensured manually by rotating the recipient at the beginning of the extraction and 10' later before filtration.

Cilia® bag	Stirring	Lot number	Plant organs	Plant material	Container	TPC mg eq. GA ▲	TFC mg eq. Q ▲	OPC mg eq. CY ▲	Extraction yield (%)	Vitexin 2-O- rhamnoside (mg/g dry plant)
No	Yes	55849	Flowering tops	Grinded 1 mm	Cup	26.6 ±1.6	2.88 ±0.17	2.24 ±0.18	19.8 ±0.9	-
					Mug	33.5 ±1.5	3.71 ±0.19	3.40 ±0.16	21.7 ±2.0	-
					Bowl	35.9 ± 0.9	4.27 ±0.19	3.77 ±0.23	23.1 ±0.2	-
				Grinded 2 mm	Cup	26.9 ±0.7	2.57 ±0.10	2.23 ±0.09	21.5 ±0.1	-
					Mug	32.2 ±0.4	3.42 ±0.23	3.07 ±0.02	24.1 ±0.2	-
					Bowl	35.3 ±1.2	3.92 ±0.21	3.56 ±0.22	24.2 ±0.3	-
Yes	Yes	55849	Flowering tops	Grinded 1 mm	Mug	24.9 ±0.2	3.01 ±0.22	2.29 ±0.05	16.9 ±1.0	-
Yes	Yes	CB58120	Flowering tops	Raw		13.6 ±0.3	1.71 ±0.02	0.70 ±0.06	16.8 ±0.1	-
				Fine	Mug	20.6 ±1.6	3.02 ±0.11	1.72 ±0.08	22.3 ±0.5	-
				Ultrafine 10"		10.2 ±0.4	1.45 ±0.11	0.73 ±0.05	11.7 ±0.3	-

No	Yes	CB58120	Flowering tops	Fine	Bodum®	21.6 ±0.4	3.13 ±0.10	1.81 ±0.09	22.5 ±0.3	-
				Fine		20.1 ±0.4	2.86 ±0.02	1.81 ±0.05	21.7 ±0.1	5.62 ±0.3
		CB58120		Coarse		21.8 ±0.1	2.46 ±0.15	1.64 ±0.07	21.1 ±0.4	-
No	No*		Flowering tops	Ultrafine 10"	Bodum®	21.3 ±0.4	2.98 ±0.16	1.85 ±0.06	21.7 ±0.3	-
		H18001534		Fine		34.2 ±1.8	3.67 ±0.21	1.76 ±0.05	22.4 ±0.5	3.29 ±0.07
		1221478		Fine		28.0 ±1.3	3.66 ±0.19	1.64 ±0.06	22.0 ±0.3	2.35 ±0.19
		R78925		Fine		23.8 ±0.9	2.98 ±0.15	1.21 ±0.06	22.4 ±0.1	5.60 ±1.5
No	No*	20334	Flowers	Fine	Bodum®	37.2 ±0.7	3.46 ±0.09	1.96 ±0.05	21.7 ±0.6	3.36 ±0.33
No	No*	-	Fresh (after 1 year)	Fine	Bodum®	44.6 ±1.3	4.06 ±0.14	4.24 ±0.17	27.8 ±0.5	7.12 ±0.33

II.2.7. Variability between hawthorn lots

The global extraction yield has been compared on 4 different lots of dry flowering tops. Table II.5 shows that the extraction yield was almost the same (about 22%, non-significant effect of the lot number by one-way ANOVA at 0.95 confidence level) for all the lots. On the contrary, significant variations of the TPC (20-34 mg eq. GA), TFC (2.9-3.7 mg eq. Q) and OPC (1.2-1.8 mg eq. CY), expressed per g of dry plant, were observed and confirmed by one-way ANOVA at 0.95 confidence level. Therefore, the total amount of dry extract is almost constant but the repartition between the different classes of components can differ from one lot to the other. The change in composition was already observed on a given lot between fresh and dry flowering tops (see section II.3.4). We can conclude that, not only the lot but also the maturation of the plant can affect the repartition between the different classes of components. Interestingly, the maximum quantities of TFC, TPC, OPC (resp. 44, 4.1 and 4.2 mg) were obtained on the flowering top harvested on Oléron Island after one year drying. Figure SI-II.14 and Figure SI-II.15 display the chromatograms and the peak area repartition, respectively, for the different lots. Similar compounds are detected on the UHPLC profiles but some differences are mainly observed on the relative proportion of the different components. We can notice that hyperoside was more abundant in flowers than in flowering tops.

II.3. Materials and Methods

II.3.1. Chemicals

Different lots of dry hawthorn flowering tops (20335, 55849, CB58120, H18001534, 1221478, R78925) or dry flowers (20334) raw materials (*Crataegus oxyacantha*, origin France) were purchased from France Herboristerie (Noidans-Lès-Vesoul, France). Fresh *Crataegus monogyna* flowering tops were harvested on april 24, 2017 (see Figure SI-II.1 for exact localization and picture of the fresh flowering tops) on a wild isolated tree on Oléron Island (France). Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), aluminium chloride hexahydrate ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$), (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH), methanol (CH_3OH), hydrochloric acid (HCl), n-butanol ($\text{CH}_3-(\text{CH}_2)_3-\text{OH}$), ammonium iron(III) sulfate dodecahydrate ($\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$), gallic acid (GA), quercetin (Q), and cyanidin chloride (CY) were purchased from Merck (Saint-Quentin Fallavier, France). *Crataegus* spp. extract standard (R1) was purchased from HWI Group (Rülzheim, Germany) standardized at 29 mg of vitexin 2-O-rhamnoside per g of extract. Procyanidin A2, B2 and C1, epicatechin, cinnamtannin A2 and isoquercetin were purchased from Phytolab (Vestenbergsgreuth, Germany). Ultrapure water was obtained using a MilliQ system from Millipore (Molsheim, France). Cilia[®] bags (size L) were purchased from Casino local

supermarket (Montpellier, France). EPS Phytoprevent® (standardized fresh hawthorn fluid extract, standardized at 7.5-12.5 mg flavonoids as equivalent quercetin in 5 mL) containing 900 mg dry flowering tops extract / 5 mL glycerol, was purchased from Pilege (Oréed'Anjou, France). Crataegisan® Bioforceethanolic plant extract (46-54% EtOH) containing 690 mg tincture of fresh hawthorn fruits in 2.25 mL, standardized at 12.7 mg polyphenol and 6.4 mg oligomericprocyanidins, was purchased from Vogel (Colmar, France). WS1442® crataegutt novo 450 tablets (also named Cardiplant® 450 or Cardio Max WS 1442®) containing 450 mg dry plant extract each and standardized at 78-90.6 mg oligomericprocyanidins as equivalent epicatechin (17.3-20.1% in dry extract) were purchased from Schwabe Pharmaceuticals (Karlsruhe, Germany). Faros® 300 LI 132 tablets containing 300 mg dry plant extract each and standardized at 6.6 mg flavonoids as equivalent hyperoside (2.25% in dry extract) were purchased from LichtwerPharma (Berlin, Germany).

II.3.2. Grinded hawthorn, density and granulometry

Dry hawthorn plants were grinded using three different grinders. 'Coarse' and 'fine' hawthorn materials were obtained by grinding 2 g of raw material using Delonghi (Model KG79, Treviso, Italy) grinder at the position named 'coarse' and 'fine', respectively. 'Ultrafine 10 s or 30s' hawthorn materials were obtained by grinding 2 g of raw material using the Bosch grinder (Model MKM6003, Munich, Germany) at different manual shaking times (10 s and 30 s) as indicated in the text. '1 mm' and '2 mm' hawthorn materials were obtained by grinding required amount of raw material on a laboratory Ika grinder (Ika-Werke GmbH, Model MF10 basic, Staufen, Germany). The density of each hawthorn material was simply determined by measuring the volume occupied by 2 g hawthorn material in a 10 mL (or 25 mL) graduated test tube ($n=3$ determinations). Distribution in size of each hawthorn material was determined by dry laser Malvern granulometer (Malvern Panalytical, Royston, United Kingdom).

II.3.3. Infusion extraction

Infusion extraction was performed using a 500 mL three-necked flask equipped with an olive magnetic stirrer (Figure SI-II.2A). 2.5 g of dry plant were placed and 250 mL of boiled ultrapure water were added. Four different mixing speeds were tested, namely 250 rpm, 500 rpm, 750 rpm and 1000 rpm. The decrease in temperature was measured upon time using temperature sensor (Ebro EB120-IF, Ingolstadt, Germany). Three extraction times were investigated, namely 5 min, 10 min and 30 min. After filtration of the plant residues using Whatman filter paper placed on a Büchner funnel and a vacuum pump (KNF Model N820FT.18, Freiburg, Germany), the extract was concentrated using a

rotary evaporator (until 10 mL volume) and finally freeze-dried (Cryotec Model CRIOS-80, Saint-Gély-du-Fesc, France). Lyophilized dry extracts were stored at 4°C. Each extraction experiment was carried out in triplicate.

II.3.4. Maceration extraction

Maceration extraction was performed using a 500 mL three-necked flask equipped with an olive magnetic stirrer, an oil bath, and a heating magnetic stirrer with a digital thermo-regulator (Fisher Scientific Model FB15002, Illkirch, France) (Figure SI-II.2B). 2.5 g of dry plant were placed and 250 mL of water were added. Four different temperatures were tested, namely 20°C, 40°C, 60°C and 80°C. The mixture was stirred at 500 rpm. Three extraction times were investigated, namely 5 min, 10 min and 30 min. After filtration, the extract was concentrated and finally freeze-dried (as described in section II.2.3). Each extraction experiment was carried out in triplicate.

II.3.5. Ultrasound-assisted extraction

Ultrasound-assisted (US) extraction was conducted with ultrasonic homogenizer (UIP 1000 hdT, 1kW, Hielscher Ultrasonics GmbH, Germany). Experiments have been performed in a double jacket reactor of 1 L volume (Figure SI-II.2D) connected with a mechanical stirrer (IKA RSC classic, Germany) and a temperature sensor. Temperature was maintained constant with a cooling system connected to the double-jacket reactor. 2.5 g of dry plant were placed in the double jacket reactor and 250 mL of water were added. Three different temperatures were tested, namely 20°C, 40°C and 60°C. The mixture was mechanically stirred at 250 rpm. Three extraction times were investigated, namely 5 min, 10 min and 30 min. After filtration, the extract was concentrated and finally freeze-dried (see section II.2.3). Each extraction experiment was carried out in triplicate.

II.3.6. Microwave-assisted extraction

Microwave-assisted (MW) extraction was performed on a monomode Microwave apparatus using a closed-vessel system (NEOS-GR, Milestone Srl, Italy) (Figure SI-II.2E). 2.5 g of dry plant were placed in a 500 mL flask containing 250 mL of water. The flask was then placed in the MW oven with a 300 W power. Under these conditions, the temperature reached 95°C in 10 minutes. No stirring was applied. Three extraction times were investigated, namely 5 min (78°C), 10 min (95°C) and 30 min (97°C). After filtration, the extracts were concentrated and finally freeze-dried (see section II.2.3). Each extraction experiment was carried out in triplicate.

II.3.7. Percolation extraction

Percolation extraction was performed using a coffee percolator KRUPS equipment (Model, city, Germany) (Figure SI-II.2C). 2.5 g of dry plant were placed in a filter and 250 mL of water were used. The temperature reached 100°C after a few seconds. No stirring was applied. Two extraction times were investigated, namely 5 min and 10 min. 250 mL of water were percolated in 5 min. For 10 min percolation extraction, the extracted solution obtained after 5 min percolation was passed again in the percolator for again 5 min. After filtration, the extract was concentrated and finally freeze-dried (see section II.2.3). Each extraction experiment was carried out in triplicate.

II.3.8. Optimized infusion extraction

2.5 g grinded material (see section II.3.2) were infused in 250 mL boiling water using 'French press' Bodum® (Bistro model, Triengen, Switzerland). After addition of the boiling water, a good initial mixing of the plant in water was ensured by manually rotating the recipient (with grinded plant, magnetic stirring –which is generally not available at home- was however not required to get optimal extraction). After 10 min, the herbal tea solution was filtrated first with the Bodum® cover to remove the largest particles, then with Whatman filter paper to remove residual solid plant. Finally, the herbal tea solution was concentrated and freeze-dried to get dry extract.

II.3.9. Kinetic monitoring

The kinetic of extraction was monitored by UV absorbance at 198 nm (Perkin-Elmer Model Lambda 20, Wellesley, MA, USA) using UV quartz cells of 1 mL (Hellma GmbH, Müllheim, Germany). 100 µL of solution were taken and added to 4 mL (or 8 mL if the absorbance values were above 1.7) water. The resulting solution was shortly vortexed before UV measurement. The same volume of fresh water (100 µL) was added in the reactor (three-necked flask) to keep constant the total volume. Zero absorbance value was set using 100 µL water instead of herbal tea solution.

II.3.10. Total polyphenols content (TPC)

The total polyphenols content (TPC) in hawthorn extracts was estimated using the Folin-Ciocalteu's reagent as described by Singleton & Rossi [98]. 100 µL of a solution prepared by mixing hawthorn extract (100 µL of 20 mg/mL in water) with 1 mL water, were added to 200 µL Folin-Ciocalteu reagent and 2 mL water. After 3 min, 1 mL of 20% sodium carbonate (20 g/100 mL water) was added. After vortex-mixing for 2 min, followed by incubation at room temperature and in darkness for 90 min, the resulting solution was centrifuged at 8000 rpm for 3 min (Sigma Model 302K, Osterode am Harz,

Germany) and the absorbance at 760 nm was measured using the same equipment as in section II.2.9. Gallic acid (0-250 mg/L) was used for the standard calibration curve. The results were expressed as mg GA equivalent per gram of dry plant, and calculated as mean value \pm one SD ($n=3$). Zero absorbance value was set using 100 μ L water instead of herbal tea solution.

II.3.11. Total flavonoids content (TFC)

The total flavonoids content (TFC) in hawthorn extracts was estimated by the aluminiumchloride method according to Lamaison & Carnet [99]. 200 μ L of hawthorn extract solution (20 mg/mL in water) were firstly added to 200 μ L water and 600 μ L methanol. 200 μ L of the resulting solution were then added to 800 μ L methanol and 1 mL of 2% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ methanolic solution (2 g/100 mL in methanol). After vortex-mixing for 2 min, followed by incubation at room temperature and in darkness for 15 min, the absorbance at 430 nm was measured using the same equipment as in section II.2.9. Quercetin (0-35 mg/L) was used for the standard calibration curve. The results were expressed as mg of equivalent Quercetin per gram of dry plant, and calculated as mean value \pm one SD ($n=3$). Zero absorbance value was set using 200 μ L water instead of herbal tea solution.

II.3.12. Total proanthocyanidin oligomers content (OPC)

The total proanthocyanidin oligomers content (OPC) in hawthorn extracts was estimated using the HCl/n-butanol assay of Porter *et al.* [100]. 200 μ L of hawthorn extract solution (20 mg/mL in water) were firstly added to 200 μ L water and 600 μ L methanol. 250 μ L of the resulting solution were then added to 3 mL of a 95% solution of n-butanol/HCl (95:5 v/v) and 100 μ L of a 2% solution of $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (2 g/100 mL in HCl 2M). After vortex-mixing for 2 min, followed by incubation at 95°C in an oil bath for 40 min and cooling at room temperature, the absorbance at 550 nm was measured using the same equipment as in section II.2.9. Cyanidin chloride (0-30 mg/L) was used for the standard calibration curve. The results were expressed as mg CY equivalent per gram of dry plant, and calculated as mean value \pm SD ($n=3$). Zero absorbance value was set using 200 μ L water instead of herbal tea solution.

II.3.13. UHPLC and UHPLC-ESI-MS analysis

All the samples analysed are given in Table SI-II.1. 20 mg hawthorn dry extract was dissolved in 1 mL MilliQ water, and finally strongly vortexed for 2 min. The resulting solution was diluted 5 times with MilliQ water, vortexed again for 2 min, and analyzed by UHPLC-DAD and UHPLC-ESI-MS.

The UHPLC-DAD system consisted of a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS equipped with a WPS-3000TBRS auto sampler, and a TCC-3000RS column compartment set at 35°C (ThermoFisher Scientific, Waltham MA, USA). The system was operated using Chromeleon 7 software. A Luna® Omega polar C18 column (1.6 µm, 100 × 2.1 mm) combined with a security guard ultra-cartridge was used (Phenomenex Inc., Torrance CA, USA). A binary solvent system was used, consisting of water/formic acid (1%, v/v) as solvent A and acetonitrile/formic acid (1%, v/v) as solvent B. The gradient program started with 5 % B, then B was increased to 100 % in 30 min with a convex increase (curve 5 in Chromeleon 7). The flow rate of the mobile phase was 0.4 mL.min⁻¹, and the injection volume was 4 µL. The peaks were monitored at 273 nm. The UV-Vis spectra of the different compounds were recorded between 200 and 550 nm using the DAD.

UHPLC-ESI-MS analysis was performed using a Synapt G2-S (Waters Corp., Milford MA, USA) equipped with ESI. The UHPLC column, injection volume, flow rate and gradient program were the same as for UHPLC-DAD. Positive mode was used according to [39]. The capillary voltage was set to 3 kV, the cone voltage was set to 30 V and the extractor voltage was set to 3 V. The source temperature was 100°C and the desolvation temperature was 450°C. MS spectra were obtained by scanning ions between $m/z = 100$ and $m/z = 1500$. The system was operated using MassLynx 4.1 software.

II.3.14. (-) ESI FT-ICR-MS analysis

All the analyzed samples are given in Table SI-II.1 and were studied in duplicate. Extraction of the achieved material, depending on the diverse extraction processes, was carried out by 2 mL methanol addition in vial and 5 min ultrasonic bath at room temperature. The methanolic extracts (from light to dark yellow), constituting the stock solutions, were recovered and put in 2.5 mL vials for 2 min centrifugation at 14000 rpm for 2 min. These solutions were then diluted 100 times in methanol. Standardized EPS Phytoprevent® and Crataegisan® Bioforce extracts were diluted to 0.5% in methanol.

Sample analysis was performed with a 12 T FT-ICR mass spectrometer Solarix (BrukerDaltonics) and the parameters were optimized via software FTMS-Control V2.2.0 (BrukerDaltonics). Prior acquisition, the mass spectrometer was externally calibrated with arginine clusters (10 mg/L in methanol). Hawthorn methanolic solutions were infused with a flow rate of 2 µL/min in the ESI source (Apollo II, BrukerDaltonics) used in negative-ion mode with a capillary voltage set at 3.6 kV. The temperature and the flow rate of the drying gas were kept at 180 °C and 4 L/min, respectively, and

the pressure of the nebulizer gas was 2.2 bar. Mass spectra result from the accumulation of 300 scans, over a m/z 122-100 range, and with a 4 megaword time-domain.

The achieved mass spectra were processed in Data Analysis 5.0 (BrukerDaltonics). An internal calibration, with a list of well-known $C_xH_yO_z$ (fatty acids and sugars) anions, was performed with mass accuracy values lower than 200 ppb. Peak lists were generated at signal-to-noise ratio ≥ 4 and exported. Algorithm developed by Kanawati *et al.* was applied to remove signals related to satellite and magnetron peaks [101]. Apart from the standard extracts, the samples were analyzed in duplicate, therefore, only features observed in both replicates were kept. The filtered mass lists of the different samples were finally aligned into a matrix based on their m/z values with a 0.5 ppm tolerance. The achieved matrix was processed for assignment in an in-house software, Netcalc [102], with an annotation tolerance of 0.2 ppm. Eventually, CHO, CHOS, CHON, and CHOCl compound families were assigned.

Perseus software was used to perform Hierarchical Cluster Analysis (HCA) and to generate heatmap from the data achieved by (-) ESI FT-ICR MS analysis of the samples. Close to 400 specific features were retrieved depending on the extraction process, plant status, or physical shape (raw or grinded). They were then represented on histogram according to their heteroatom class and on van Krevelen diagram. Principal Component Analysis (PCA) and Partial least squares-discriminant analysis (PLS-DA) were done by SIMCA-P 9.0 software.

Some of the achieved raw formulae were putatively assigned with compounds previously identified in the fruit, leave, and flower of *Crataegus* [39, 40, 103]. Thus, 56 compounds have been assigned and are referenced, with their exact mass in the $[M-H]^-$ form, in the Table SI-II.3.

II.3.15. Anti-oxidant activities

DPPH (common abbreviation for 2,2-diphenyl-1-picrylhydrazyl) scavenging capacity of the hawthorn extracts was measured using Trolox as a standard [104, 105]. 50 μ L of 0.5 mM methanolic DPPH solution was added to 50 μ L of extracts (or Trolox) in a microplate and the adsorbance was read at 520 nm every 5 min over a period of 60 min. All experiments were carried out in triplicate and for 3 independent extractions. Final results were expressed in milligrams of Trolox equivalent (TE) per gram of hawthorn extract.

II.4. Conclusions

From this thorough study about hawthorn extraction in water, we can conclude that home-made preparation using infusion with simple commercially available equipment and protocol can afford daily intake of TPC, TFC and OPC which is similar to the recommended dose from standardized plant extracts. The optimal home-made conditions are: (i) grinding of 2.5 g of hawthorn flowering tops using a basic commercially available grinder just before the infusion (granulometry < 1 mm); (ii) pouring 250-400 mL of boiling water onto the grinded plant in a French-press coffee maker (no infusion bag, no stirring required!); (iii) waiting for at least 3 min infusion; (iv) pressing the French-press filter before serving. The overall cost for 1 month of daily hawthorn intake (1 infusion per day) is about 2.2 euros to cover the cost of the hawthorn material and if we don't consider the cost of the equipment which can be reused. This is about 10 times lower than the cost of standardized plant extract and this cost can be even reduced if people harvest hawthorn by themselves.

Grinding the plant was found to be the best way to increase the kinetics of extraction and the overall yield of extraction; but it is advisory to grind (granulometry < 1 mm) just before use to avoid undesirable oxidation of the plant. If the plant is grinded, infusion remains the simplest way to extract bioactive components from hawthorn plant, and the other extraction modes (ultrasonic, maceration, microwaves and percolation) did not significantly improve the extraction yield. The UHPLC profiles were also very similar from one extraction mode to the other. As far as the plant is grinded, the automatic stirring of the infusion is not required and simple manual stirring at the beginning and the end of the extraction is enough to get optimal extraction. Similarly, it is not required to wait more than 3 min for the infusion of grinded hawthorn; however, 10 min infusion can be a good option to reach drinkable temperature (i.e. 60°C or lower temperatures), without adding fresh water to the infusion to decrease the temperature. The use of a tea bag is not recommended since it tends to slow down the extraction process/diffusion and to decrease the yields of extraction, either due to pore clogging of the filter constituting the tea bag (this is especially critical for fine/ultrafine granulometry) and/or due to solutes adsorption on the filter. Overall extraction yield for the optimized protocol is about 22% (in wt of the initial dry plant) among which 8% (in wt of the initial dry plant) are non-UV absorbing components. Among the different volumes of water tested (cup = 125 mL, mug = 250 mL and bowl = 405 mL), the higher volume (bowl) was the best to optimize the yields of extraction (10%-15% increase of global yield compared to the cup and up to 50% for TPC, TFC and OPC contents). The global extraction yield remained unchanged for all the five hawthorn lots that were tested; but the repartition between TPC, TFC and OPC may vary from one lot to the other.

Regarding quantitative and qualitative differences according to the nature of the hawthorn plant, we can conclude that: (i) similar UHPLC profiles were obtained between raw and grinded flowering tops but with a higher content in procyanidins B2 and C1 for grinded plant, in good agreement with the higher OPC content (3.93 vs 1.24 mg eq. CY / g of plant); (ii) dry flowers (without leaves) had higher content in hyperoside but lower contents in apigenin-C-hexoside and vitexin-2-O-rhamnoside compared to dry flowering tops; (iii) much higher content of vitexin-2-O-rhamnoside and lower contents in apigenin-C-hexoside, procyanidins and chlorogenic acid were obtained in freshly harvested flowering tops compared to dry raw flowering tops. Interestingly, the differences observed in the UHPLC profiles between the fresh and the dry flowering tops (different lots) tends to vanish after one year drying of the 'fresh' flowering tops, with increasing contents in epicatechin, hyperoside, apigenin-C-hexoside, procyanidins and OPC in general, once the plant is dried. From (-)ESI FT-ICR MS analysis, additional conclusions are that: (i) there is a higher variability in chemical composition in fresh flowering tops samples compared to dry flowering tops, and in dry flowers (without leaves) compared to dry flowering tops; (ii) fresh flowering tops contain higher contents in aminoacids, aminosugars, lipids, carbohydrates and some flavonoids bounded or not to a carbohydrate (such as procyanidin A2); (iii) dry flowers contain more amino acids (e.g. tryptophan and glutamate) or amino sugars and flavonoids linked to sugars such as shaftoside (apigenin 6-C-beta-D-glucopyranosyl-8-C-alpha-L-arabinopyranoside) and quercetin pentoside as compared to dry flowering tops.

We believe that the home-made optimized protocol described in this work, which is based on a simple water-based infusion, is of very general use for those who are interested in medicinal plants. It presents the advantages to be very simple, fast, affordable, repeatable and optimized. These features are some of the key points to address if we want to promote herbal medicine, to favor its acceptance in modern western integrative medicine [109], and to meet the increasing societal demand in that field [110].

Supplementary Materials: The following are available online at www.mdpi.com/. Figure SI-II.1: Picture and localization of fresh hawthorn. Figure SI-II.2: Picture of the experimental set-up used for each extraction mode. Figure SI-II.3: Extraction kinetics of grinded (1 mm) hawthorn followed by UV absorbance at 198 nm for various extraction modes. Figure SI-II.4: UV absorbance values at 198 nm and different extraction times as a function of the extraction yields at the corresponding time for all extraction modes. Figure SI-II.5: UHPLC profiles of hawthorn extracts obtained from different extraction modes for raw hawthorn. Figure SI-II.6: Relative proportions of the main compounds detected by UHPLC in the various hawthorn extracts as a function of the

extraction mode, the granulometry, the nature and the state of the plant. Figure SI-II.7: Chemical structures of all compounds identified by UHPLC-ESI-MS. Figure SI-II.8: Mass spectra achieved by (-)ESI FT-ICR MS analysis of the hawthorn samples according to extraction method, plant parts and state (fresh or dry). Figure SI-II.9: Hierarchical Cluster Analysis (HCA) and heatmap achieved from samples analyzed by (-)ESI FT-ICR MS. Figure SI-II.10: Pictures of cup, mug and bowl with dimensions and weights. Figure SI-II.11: Decrease profile of temperature vs the nature of the container (cup, mug, bowl, Bodum®, three-neck flask). Figure SI-II.12: Pictures of raw and grinded hawthorn materials of various granulometries. Figure SI-II.13: Size distributions of grinded hawthorn materials. Figure SI-II.14: Influence of the lot number of grinded (fine granulometry) hawthorn dry flowering tops and one lot of raw dry flowers on the UHPLC profiles of corresponding hawthorn extracts. Figure SI-II.15: Relative proportions of the main compounds (relative peak area) detected by UHPLC-UV in the various grinded (fine granulometry) hawthorn extracts as a function of the lot number of dry flowering tops and one lot of raw dry flowers. Table SI-II.1: List of the samples analyzed by UHPLC-ESI-MS and (-)ESI FT-ICR-MS. Table SI-II.2: Fitting parameters for the absorbance $A(t)$ trace vs extraction time t for the infusion mode. Table SI-II.3: Compounds identified in hawthorn putatively assigned to raw formulae achieved by (-) ESI FT-ICR MS. Table SI-II.4: Putative compounds obtained from features specifically extracted depending on the plant states (fresh vs dry and grinded vs raw) or parts (flowers vs flowering tops).

Author Contributions: conceptualization, L.L. and H.C.; methodology, all authors; software, P.C.N., L.L., J.C.R., J.H., P.S.K., H.C.; validation, all authors; formal analysis, all authors; investigation, L.L., J.C.R., J.H., P.S.K., H.C.; resources, all authors; data curation, P.C.N., L.L., J.C.R., J.H., P.S.K., H.C.; writing—original draft preparation, P.C.N., L.L., H.C.; writing—review and editing, L.L. and H.C.; visualization, all authors; supervision, L.L. and H.C.; project administration, L.L. and H.C.; funding acquisition, P.C.N., L.L. and H.C.

Funding: P.C.N. PhD fellowship was funded by the Ministry of Education and Training of Vietnam and Campus France.

Acknowledgement: The authors thanks J. Rodriguez (ICGM) for the granulometry experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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SUPPORTING INFORMATION OF CHAPTER II

Article 1: Optimizing Water-based Extraction of Bioactive Principles of Hawthorn: from Experimental Laboratory Research to Homemade Preparations

Molecules, 2019, 24(23), 4420

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Table SI-II.4. Putative compounds obtained from features specifically extracted depending on the plant states (fresh vs dry and grinded vs raw) or parts (flowers vs flowering tops)..... 170



Hawthorn collected on a wild tree located at Le Grand-Village-Plage, F-17370, Oléron Island, France. GPS coordinates: 45°51'57.5"N 1°13'45.3"W



Figure SI-II.1. Picture (A) and localization (B) of fresh hawthorn (marked in red).



Figure SI-II.2. Picture of the experimental set-up used for each extraction mode (A: infusion; B: maceration; C: percolation; D: ultrasonic; E: Microwave; F: infusion using a French-Press Bodum® (with or without stirring)).

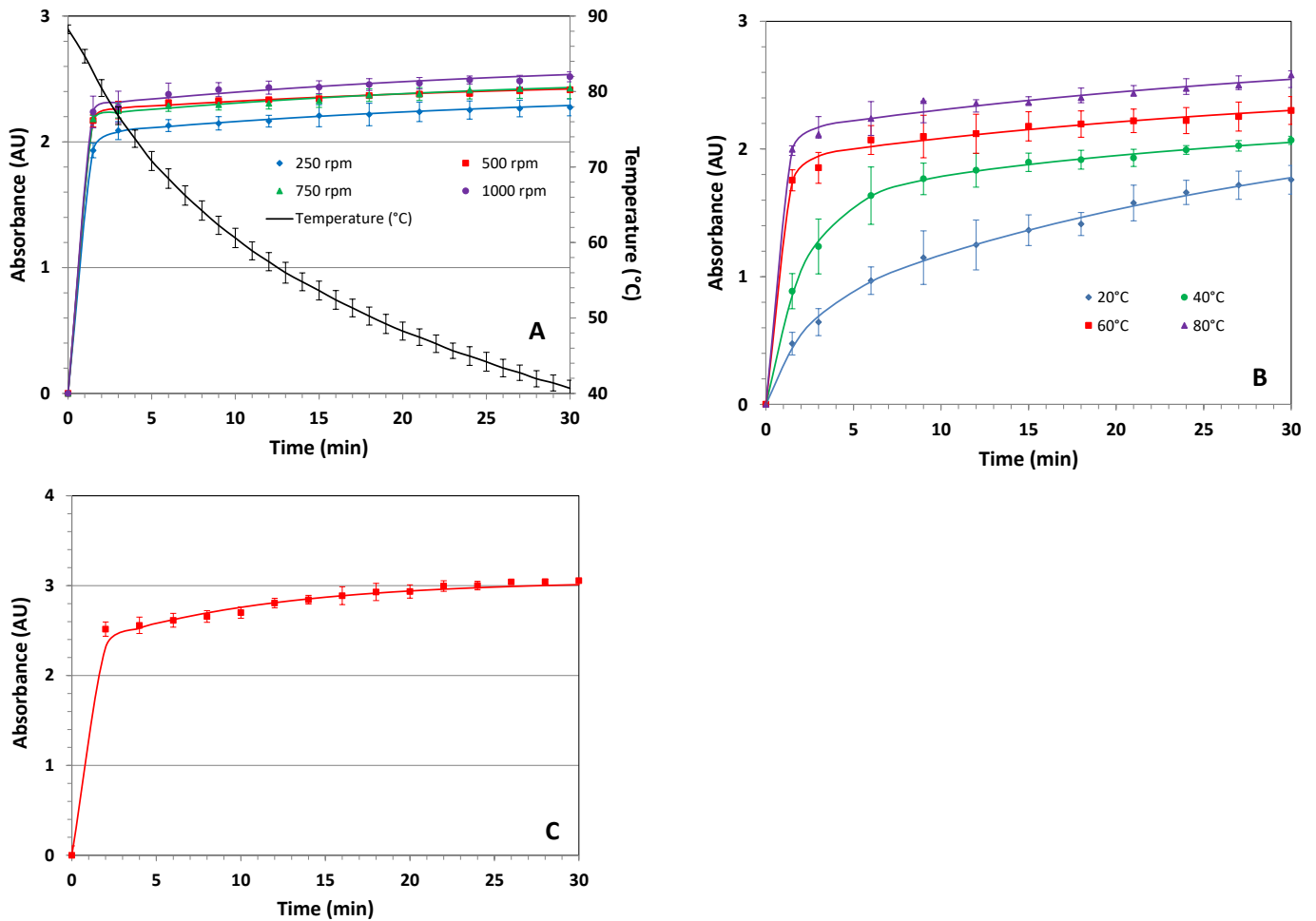
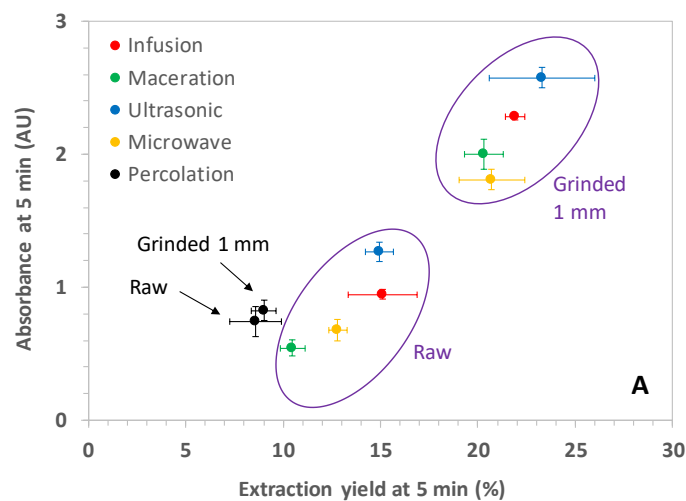


Figure SI-II.3. Extraction kinetics of grinded (1 mm) hawthorn followed by UV absorbance at 198 nm for various extraction modes. A: infusion mode at 250 rpm, 500 rpm, 750 rpm and 1000 rpm stirring speed, including the temperature profile at 500 rpm. B: maceration mode at 20°C, 40°C, 60°C and 80°C and at 500 rpm stirring speed. C: ultrasonic mode at 60°C and at 250 rpm stirring speed. In all cases, 2.5 g of raw hawthorn in 250 mL water was used. 100 μ L of solution were taken and added to 4 mL (or 8 mL if the absorbance values were above 1.7) water before each UV measurement. Error bars are \pm one SD on $n = 3$ repetitions of independent extractions.



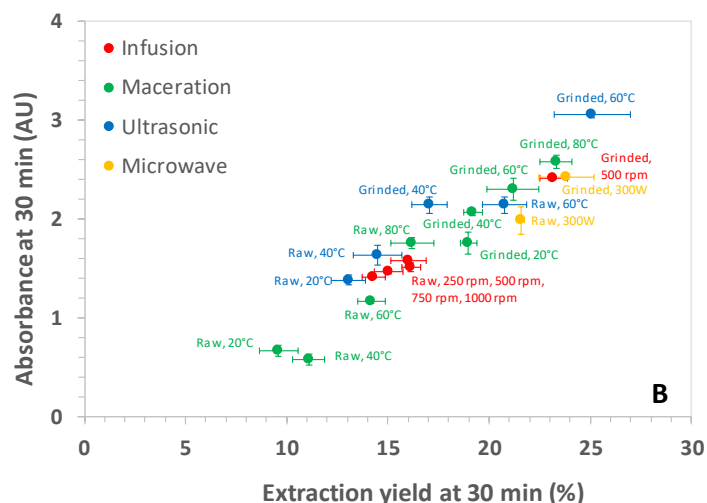


Figure SI-II.4. UV absorbance values at 198 nm and at different extraction times (A: 5 min and B: 30 min) as a function of the extraction yields at the corresponding time for all extraction modes. In all cases, 2.5 g of hawthorn material in 250 mL water was used. Maceration and ultrasonic extractions at 60°C, 100 μ L of solution were taken and added to 4 mL water before UV measurement. For all the absorbance values above 1.6, and to avoid the saturation of the detector, the solutions were diluted twice (the absorbance values were multiplied by 2 for better comparison). Error bars are \pm one SD on $n = 3$ repetitions of independent extractions.

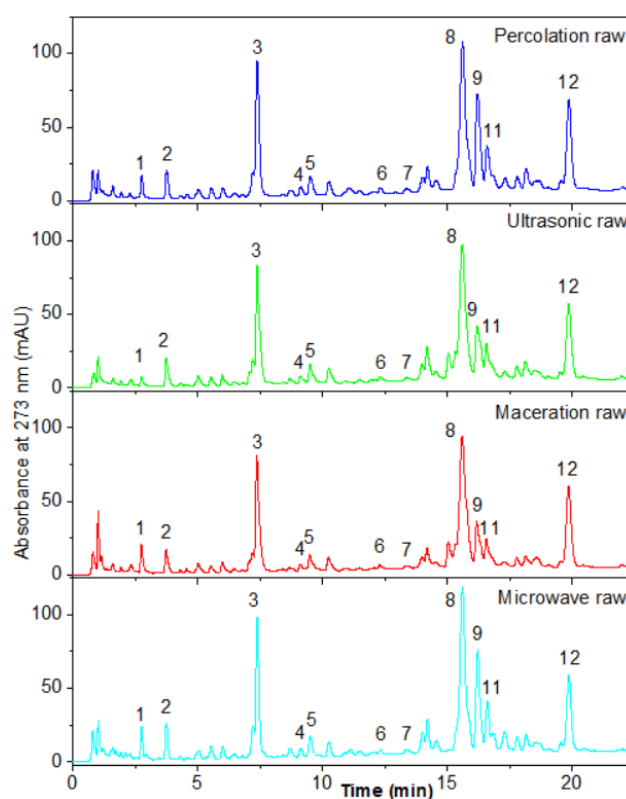


Figure SI-II.5. UPLC profiles of hawthorn extracts obtained from different extraction modes for raw hawthorn. All dry plant extracts are issued from lot n°20335. Experimental conditions: Luna® Omega polar C18 column (1.6 μ m, 100 \times 2.1 mm), binary solvent system: water/formic acid (1%, v/v) as solvent A and acetonitrile/formic acid (1%, v/v) as solvent B. Gradient program: 5 % B, then increase of B to 100 % in 30 min with a convex increase. Flow rate: 0.4 mL.min⁻¹. Injection volume: 4 μ L. Column temperature: 35°C. UV monitoring at 273 nm.

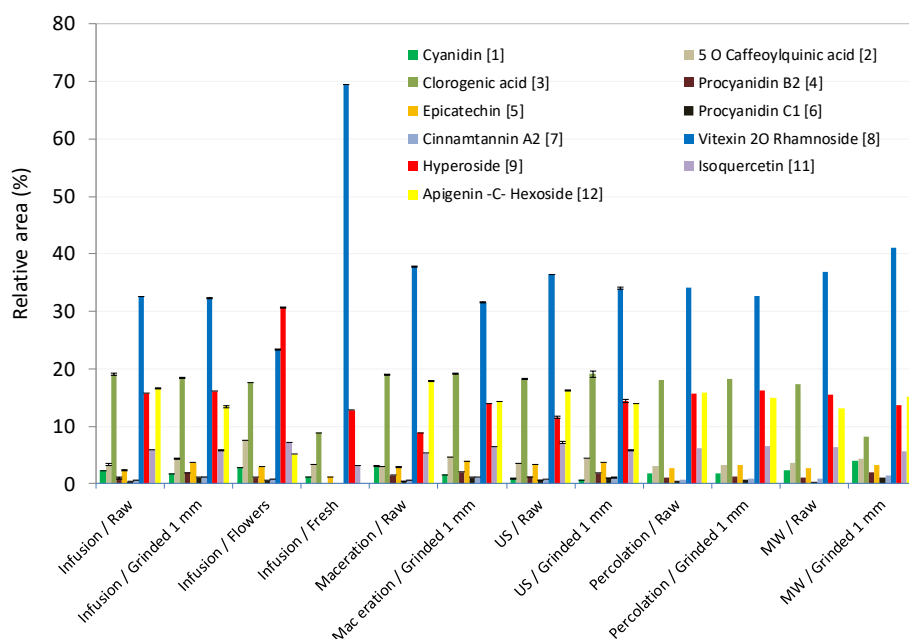
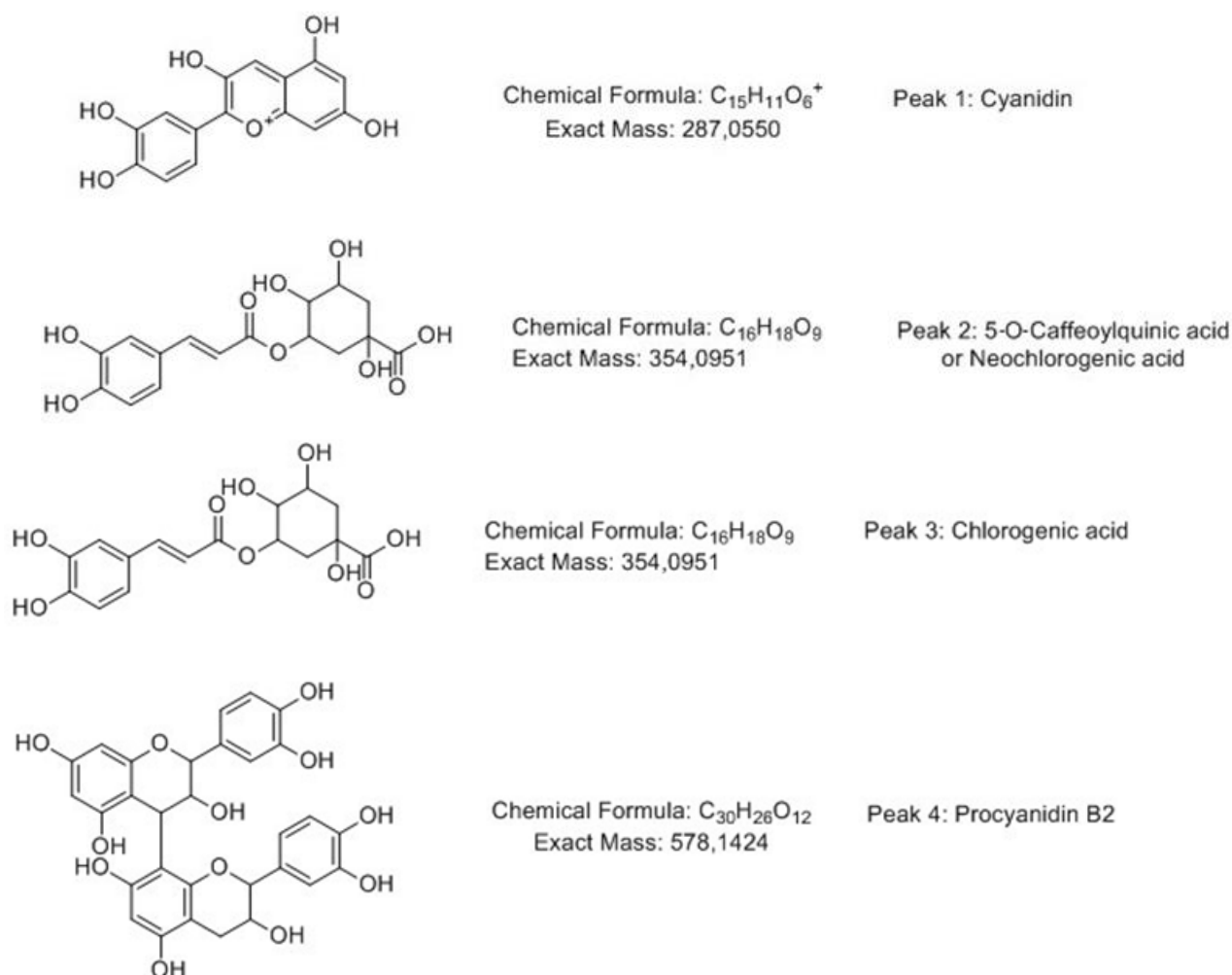
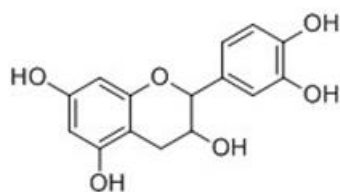


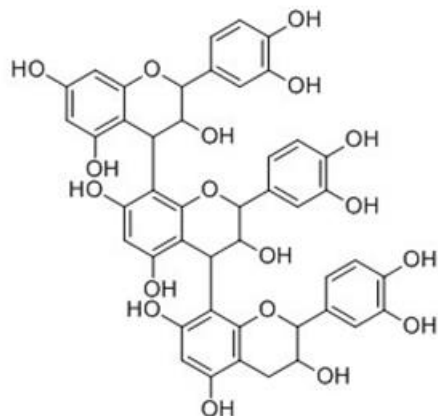
Figure SI-II.6. Relative peak area distributions for the main compounds detected by UHPLC in the various hawthorn extracts as a function of the extraction mode, the granulometry, the nature and the state of the plant. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 12 identified components. Experimental conditions as in Figure SI-II.5.





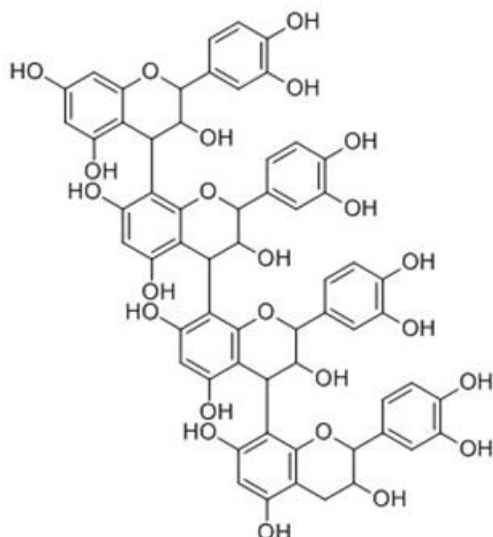
Chemical Formula: $C_{15}H_{14}O_6$
Exact Mass: 290,0790

Peak 5: Epicatechin



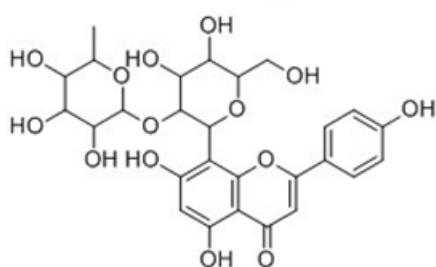
Chemical Formula: $C_{45}H_{38}O_{18}$
Exact Mass: 866,2058

Peak 6: Procyanidin C1



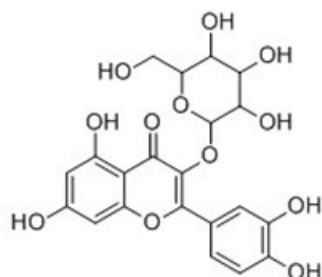
Chemical Formula: $C_{60}H_{50}O_{24}$
Exact Mass: 1154,2692

Peak 7: Cinnamtannin A2



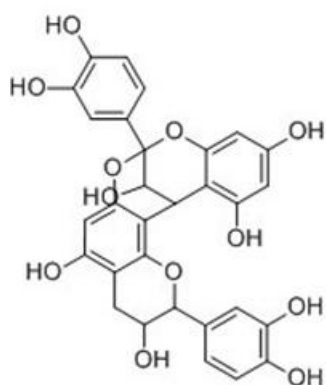
Chemical Formula: $C_{27}H_{30}O_{14}$
Exact Mass: 578,1636

Peak 8: Vitexin 2-O-rhamnoside



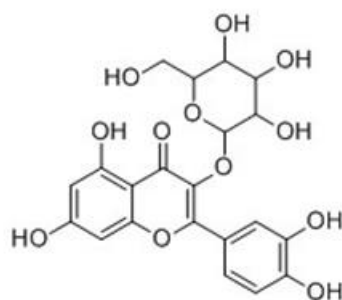
Chemical Formula: $C_{21}H_{20}O_{12}$
Exact Mass: 464,0955

Peak 9: Hyperoside



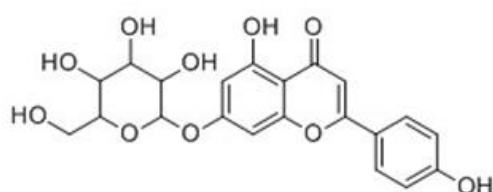
Chemical Formula: $C_{30}H_{24}O_{12}$
Exact Mass: 576,1268

Peak 10: Procyanidin A2



Chemical Formula: $C_{21}H_{20}O_{12}$
Exact Mass: 464,0955

Peak 11: Isoquercetin

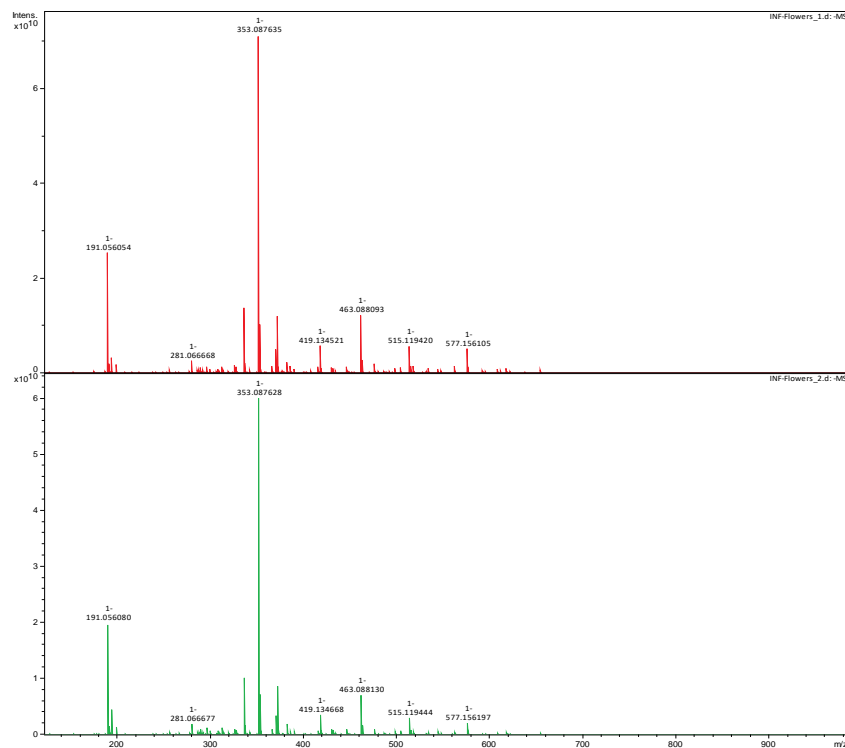


Chemical Formula: $C_{21}H_{20}O_{10}$
Exact Mass: 432,1056

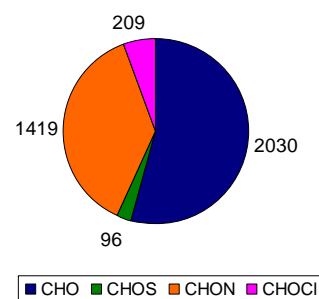
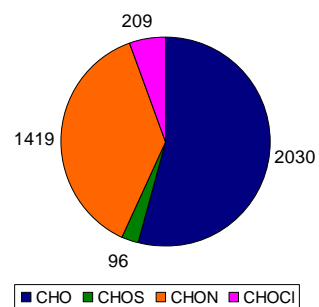
Peak 12: Apigenin-C-hexoside
or Apigenin 7-glucoside

Figure SI-II.7. Chemical structures of all compounds identified by UHPLC-ESI-MS.

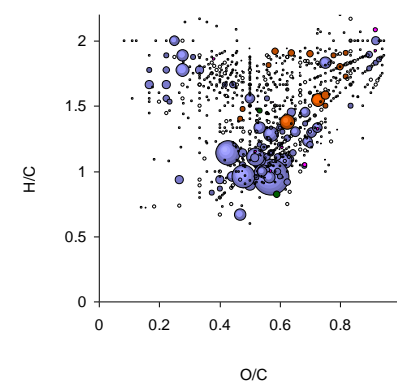
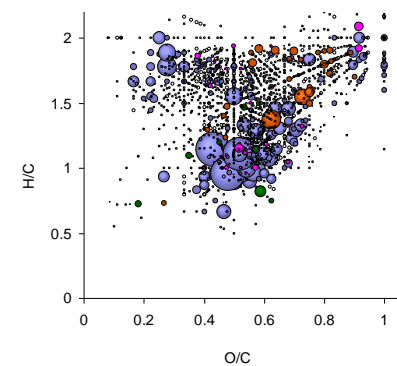
(-) ESI FT-ICR mass spectrum

Dry flowers extracted by infusion

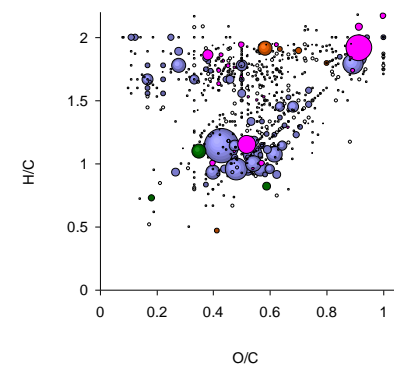
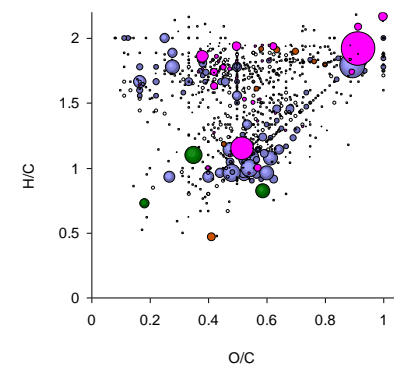
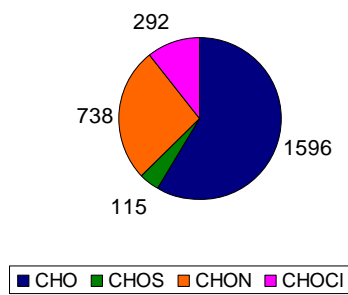
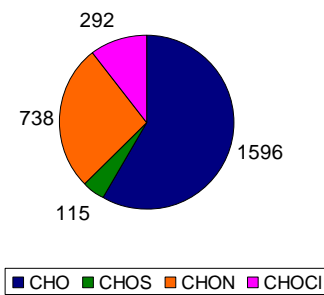
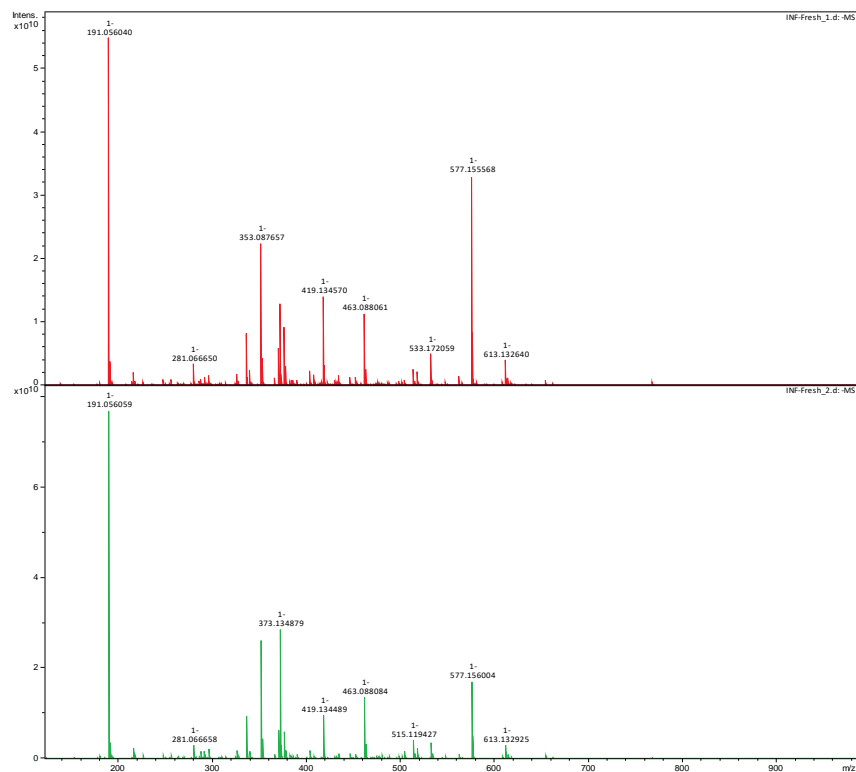
Corresponding heteroatom class distribution



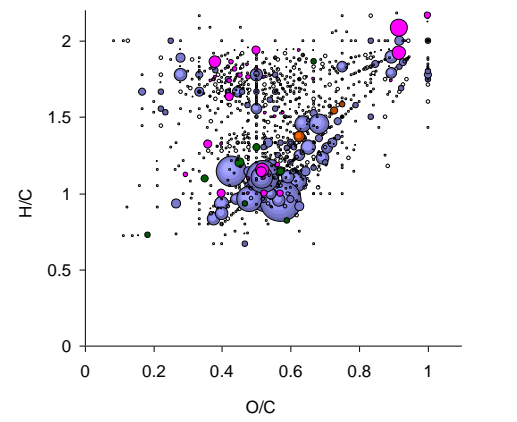
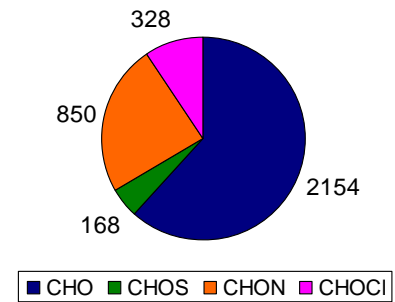
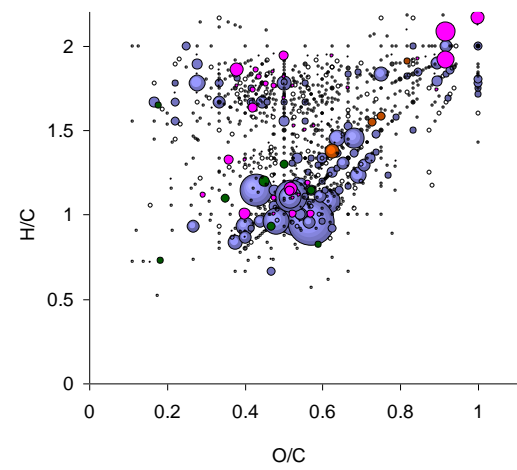
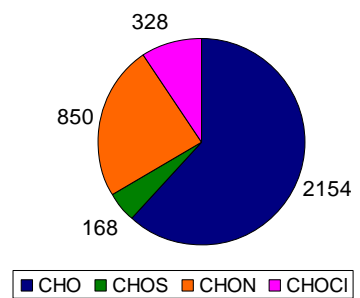
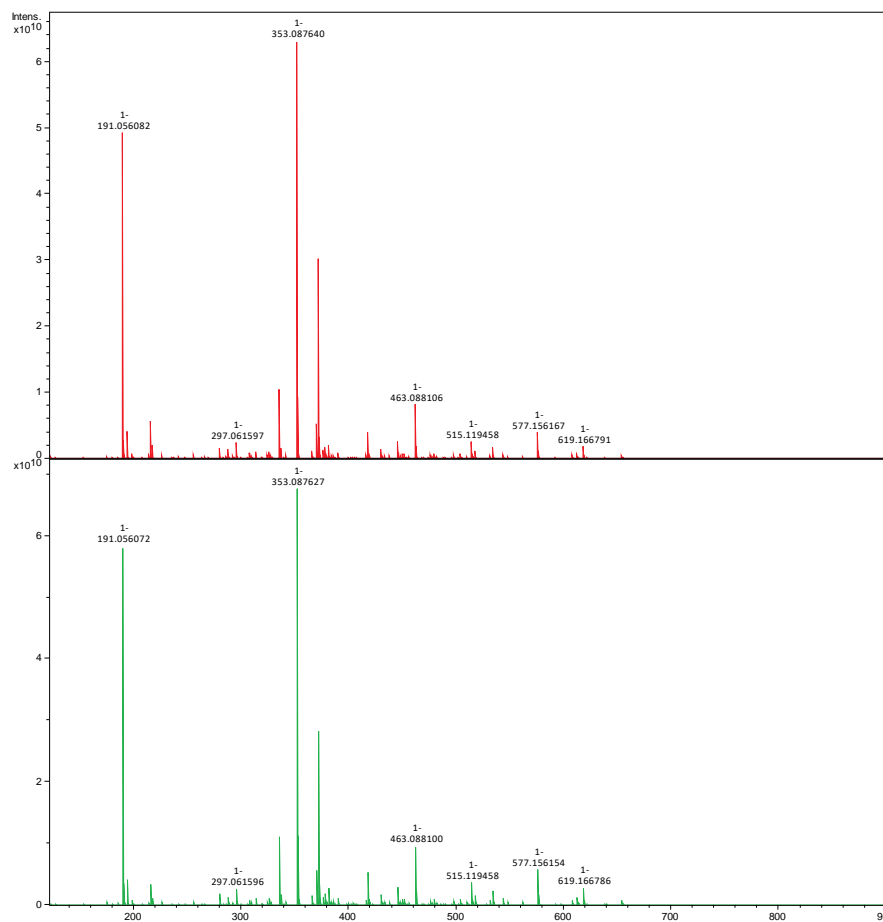
Corresponding van Krevelen diagram



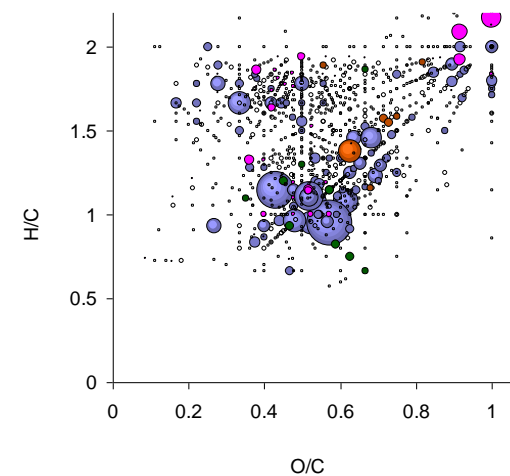
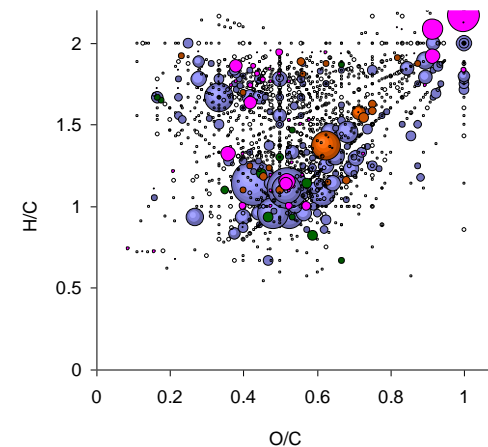
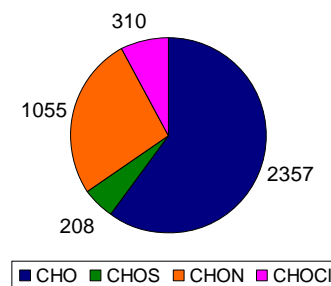
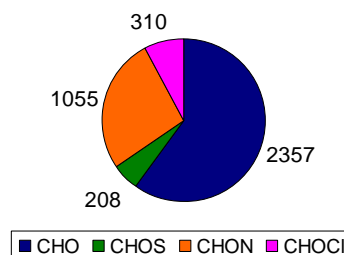
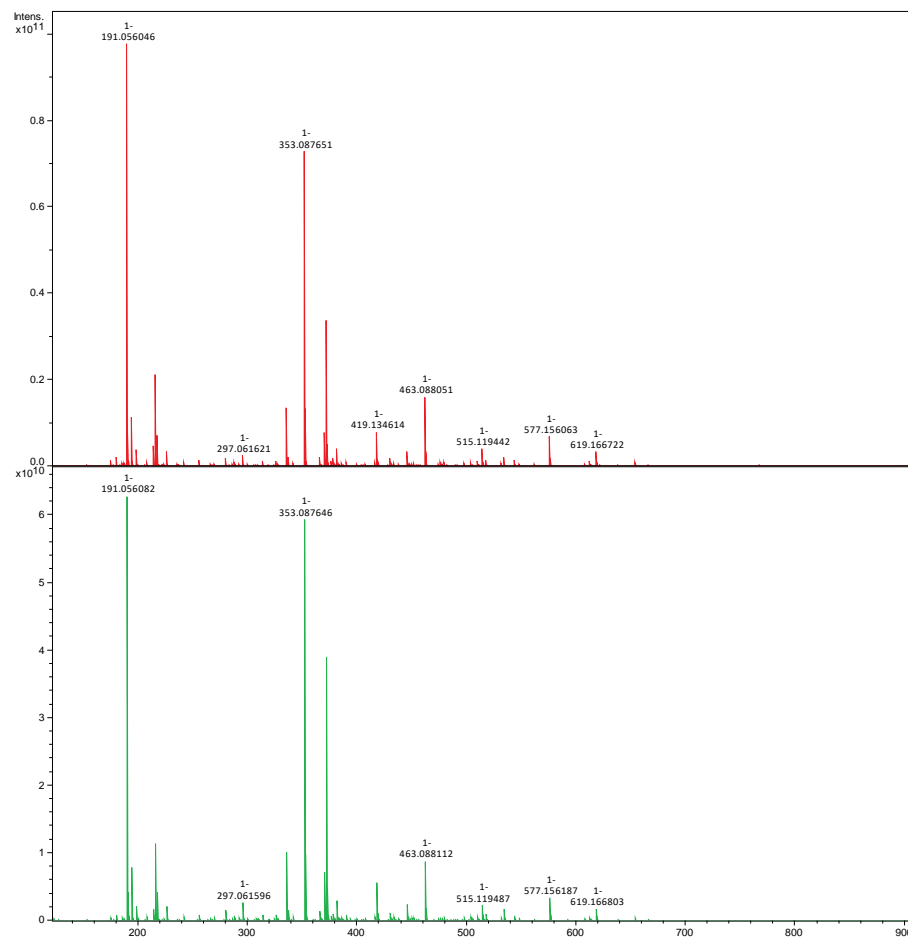
Fresh flowering tops extracted by infusion



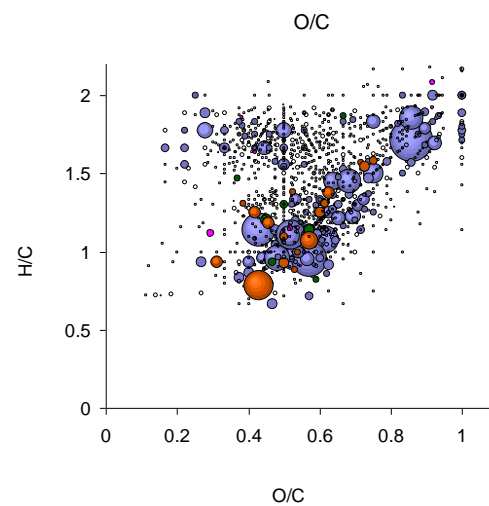
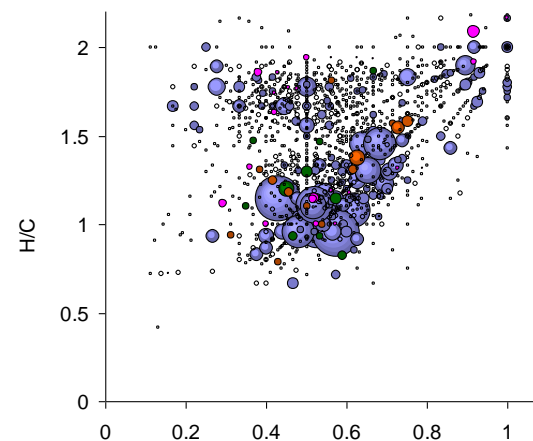
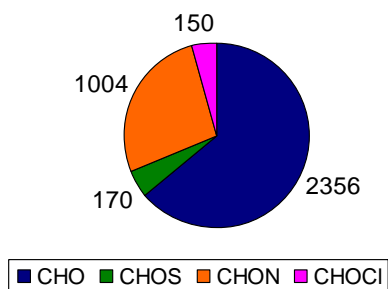
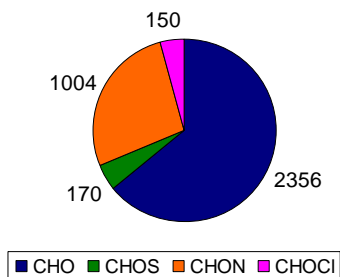
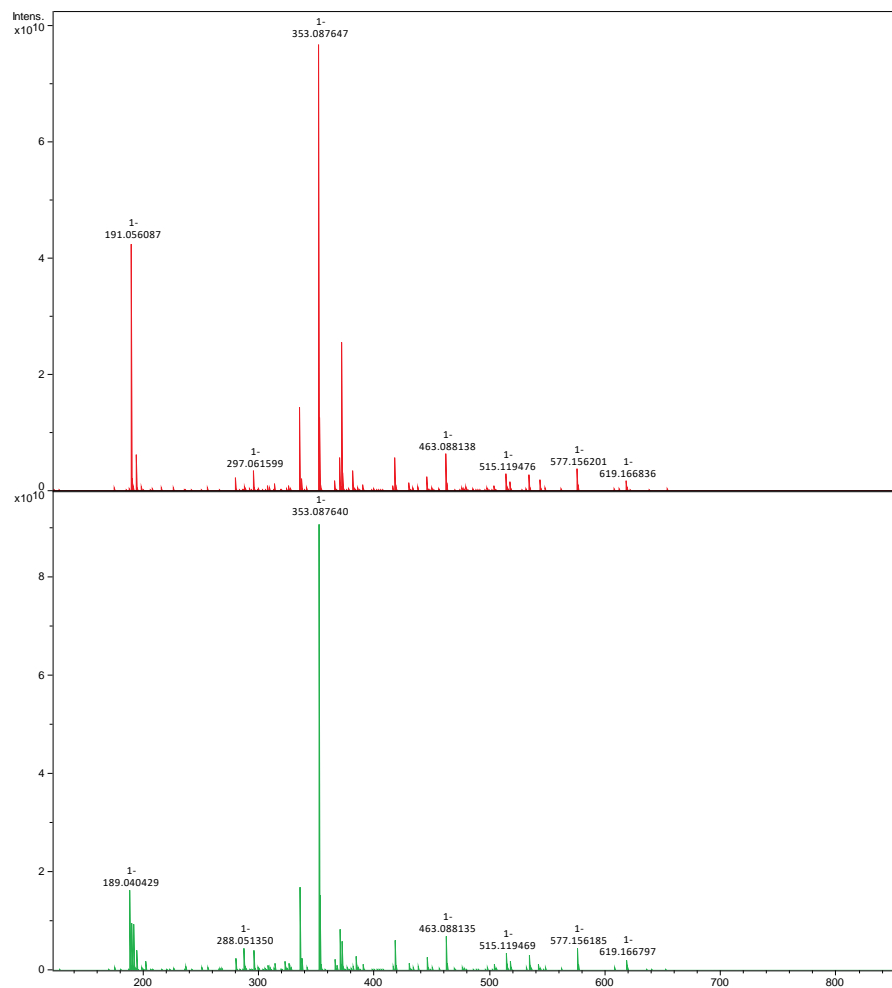
Grinded dry flowering tops extracted by infusion



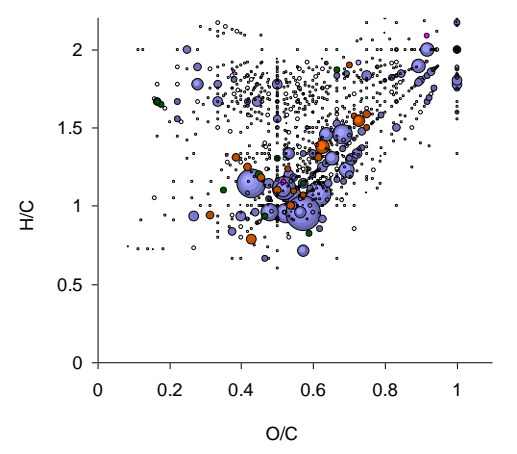
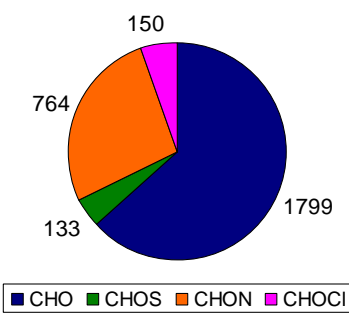
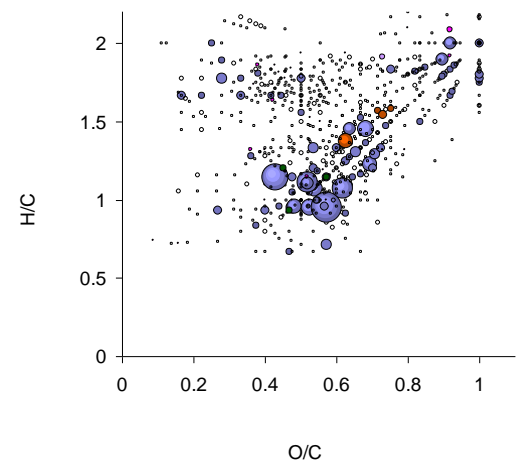
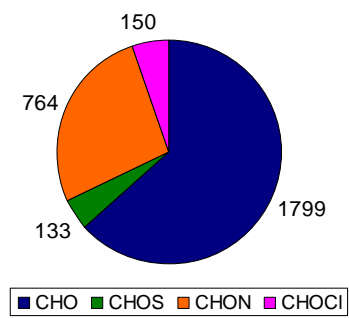
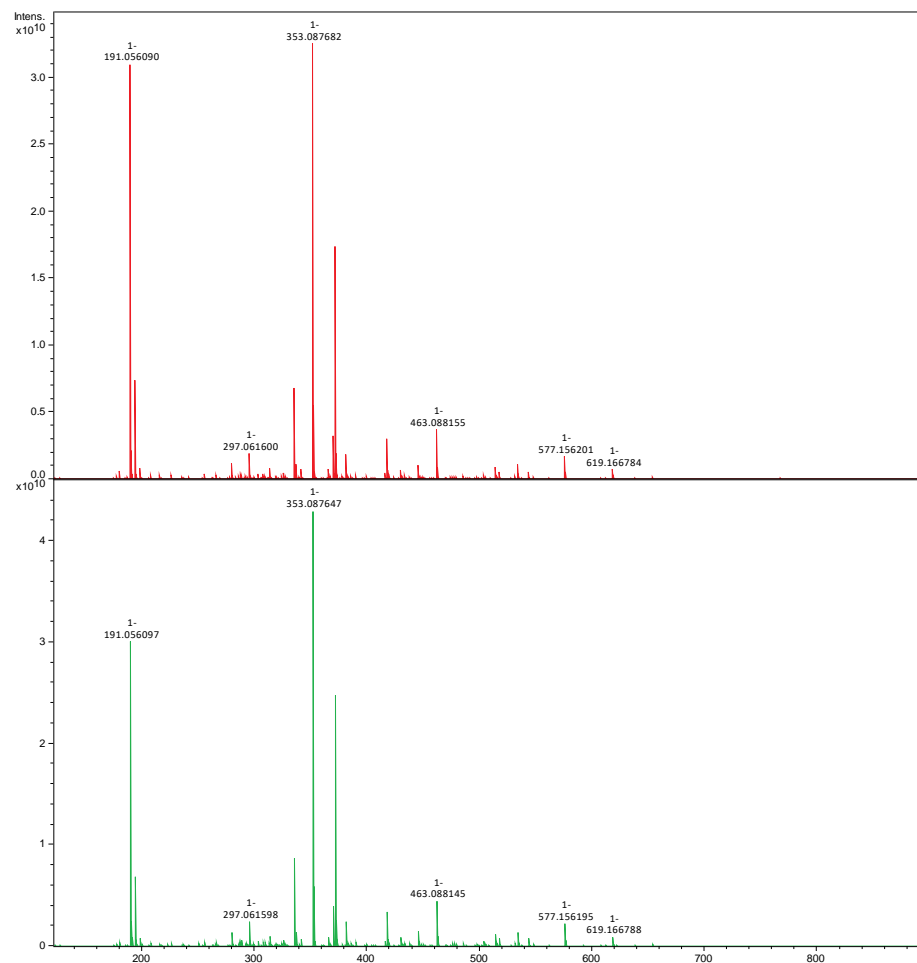
Raw dry flowering tops extracted by infusion



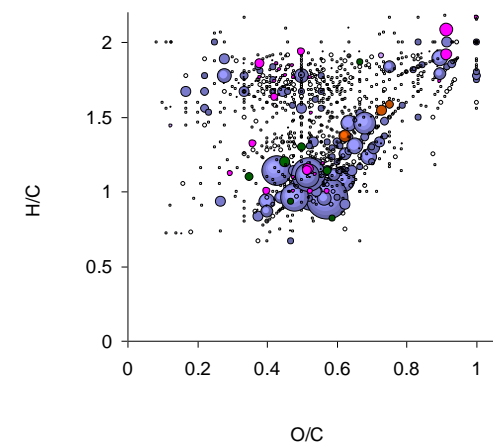
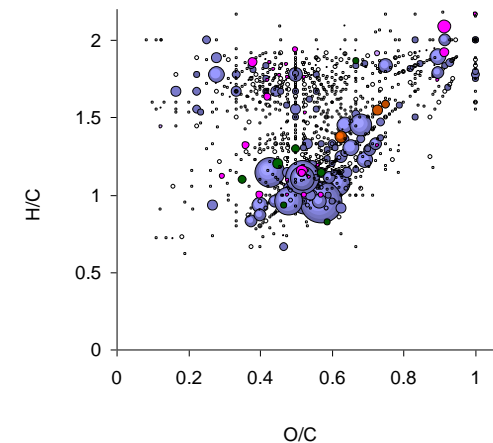
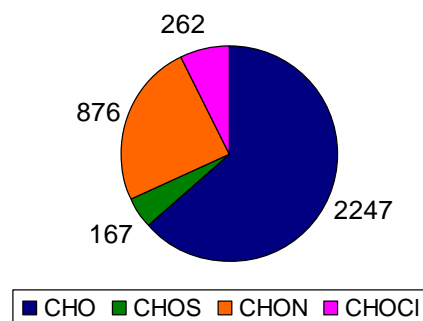
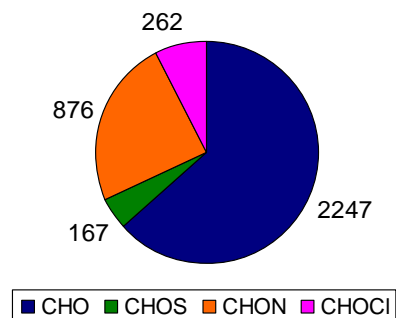
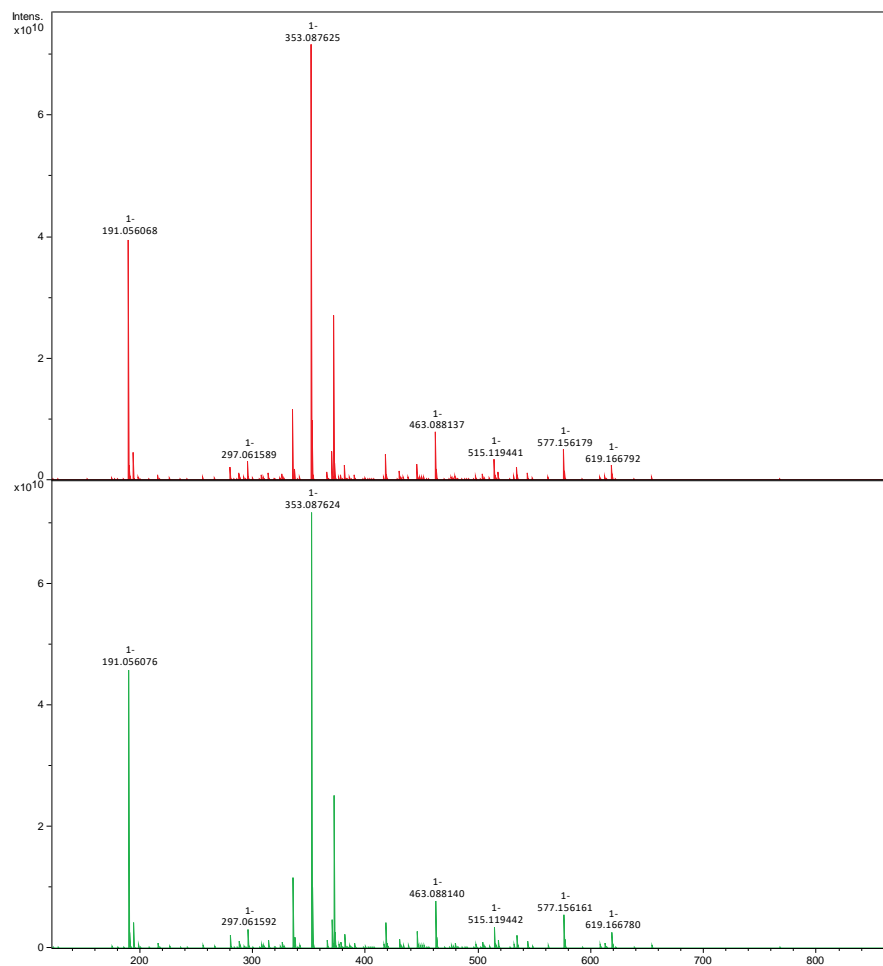
Grinded dry flowering tops extracted by maceration



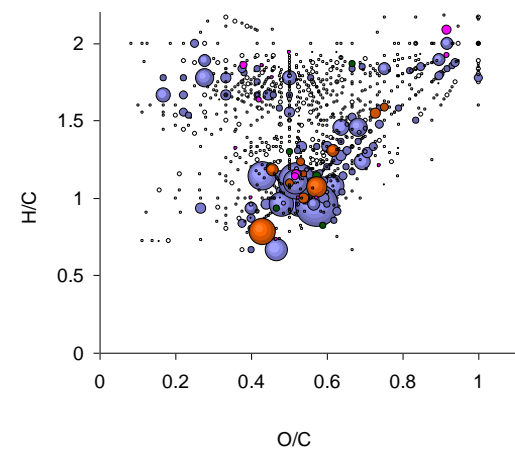
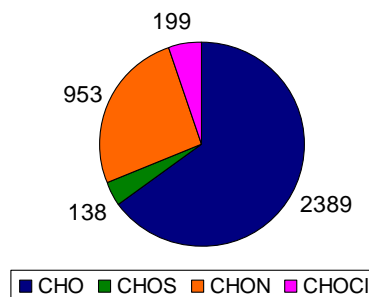
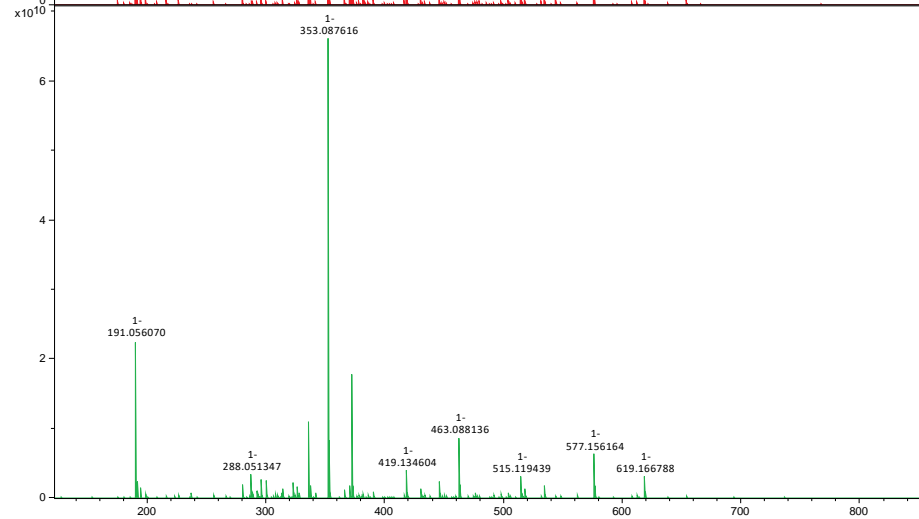
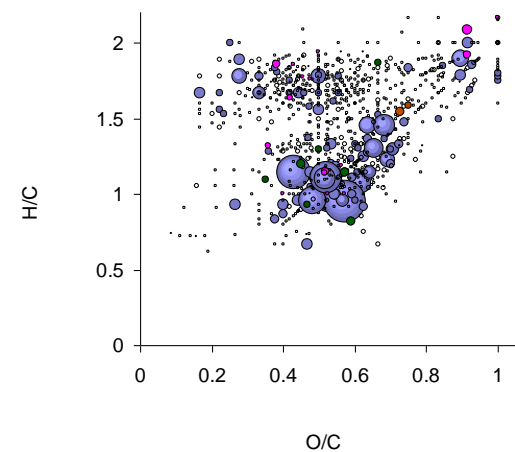
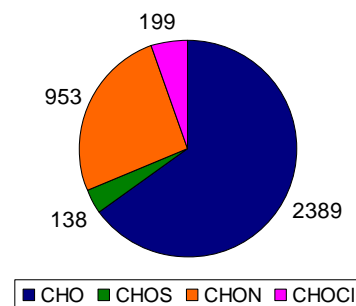
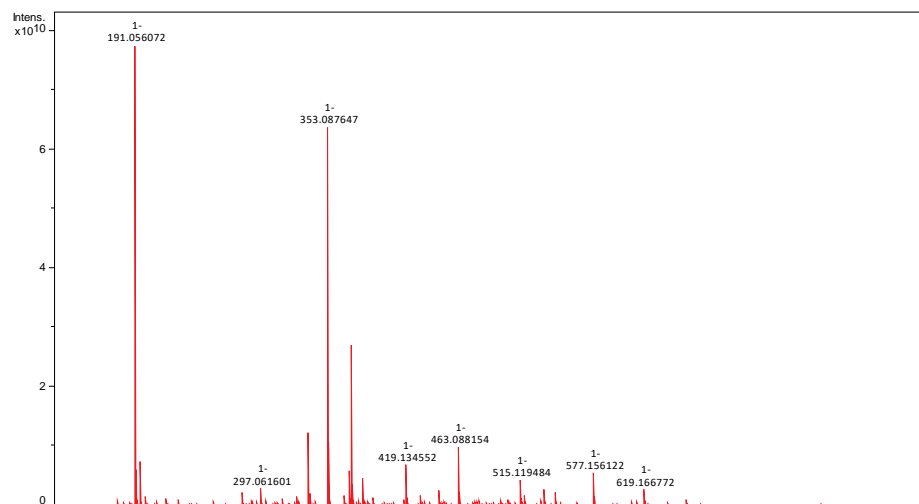
Raw dry flowering tops extracted by maceration



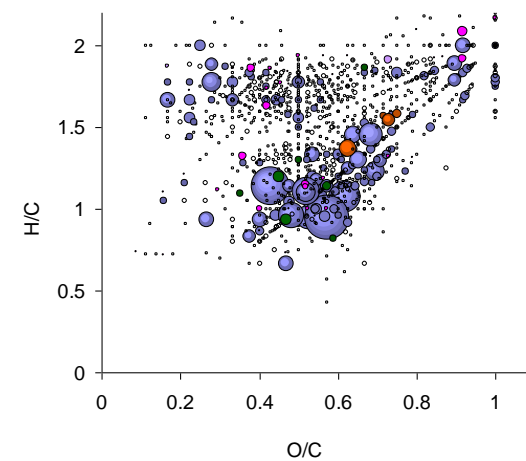
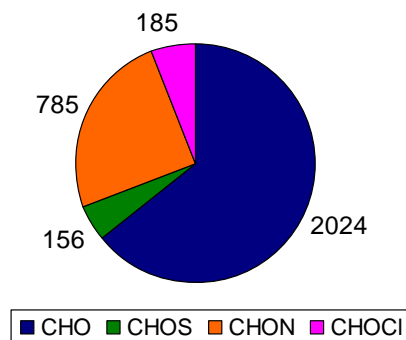
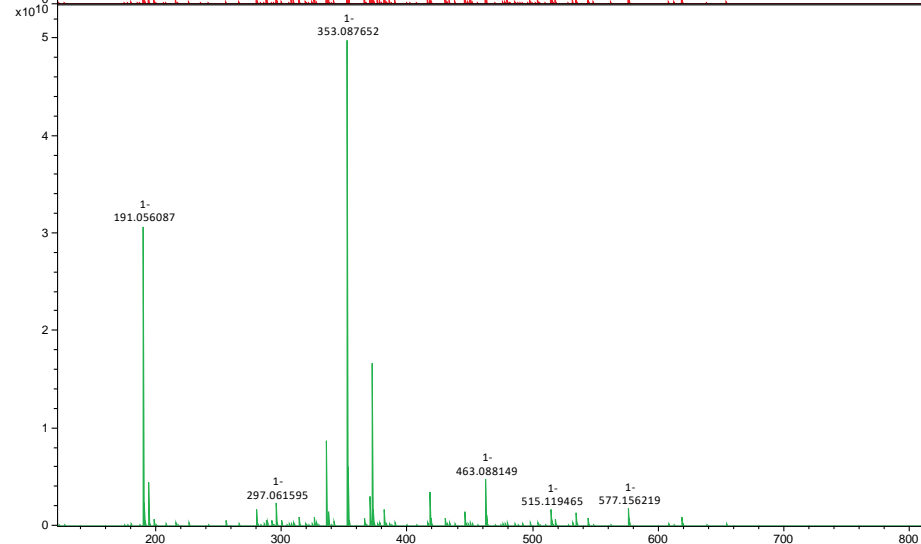
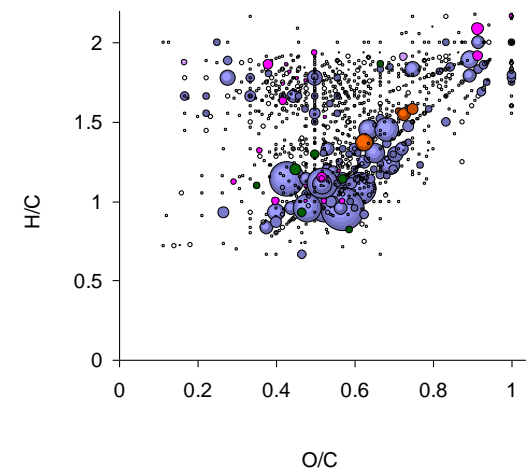
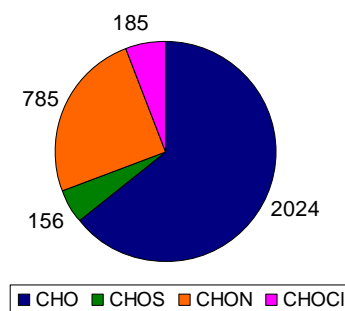
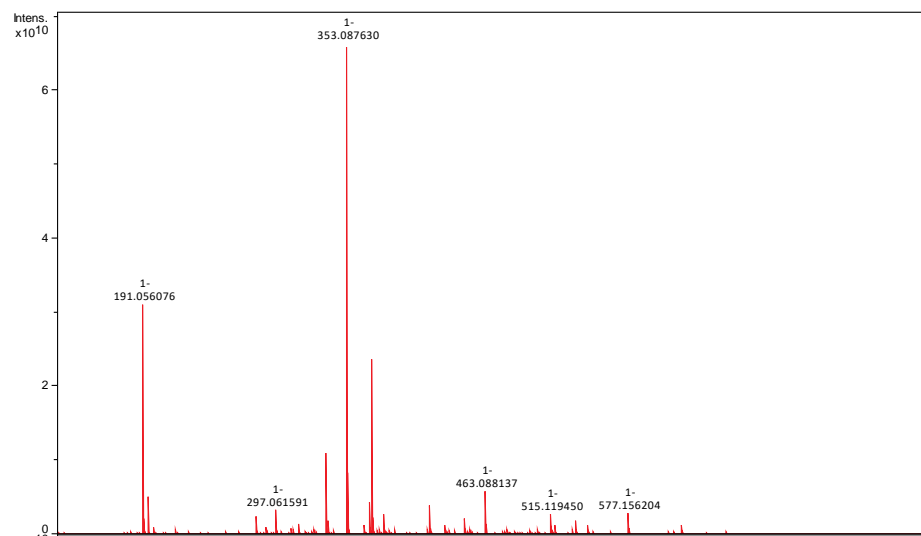
Grinded dry flowering tops extracted by microwave



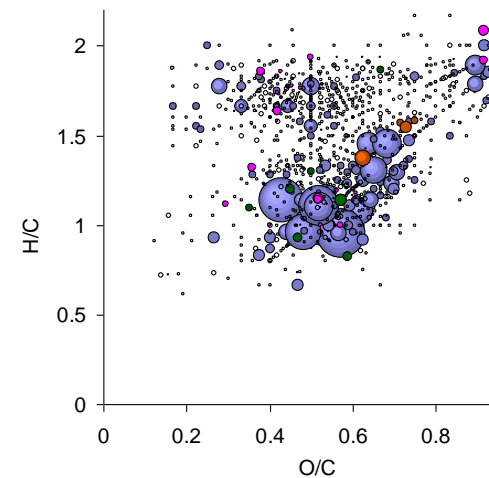
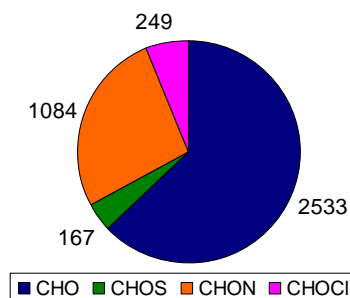
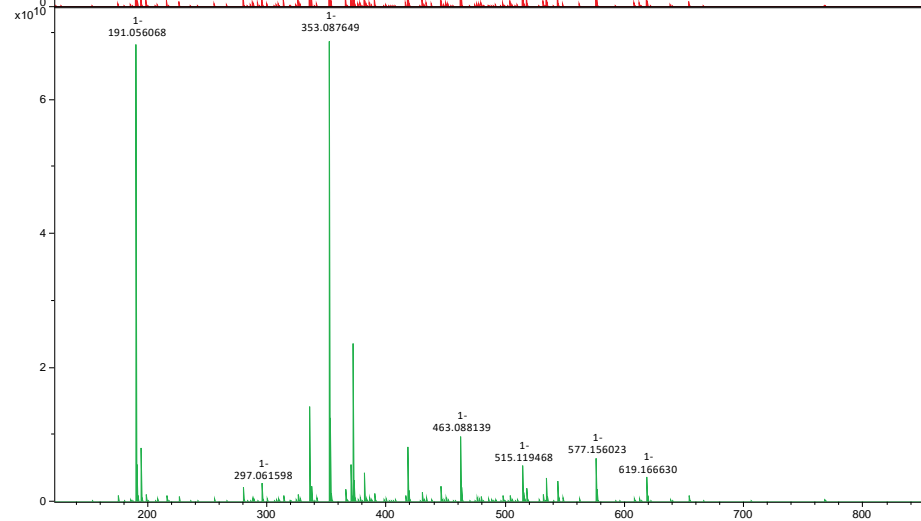
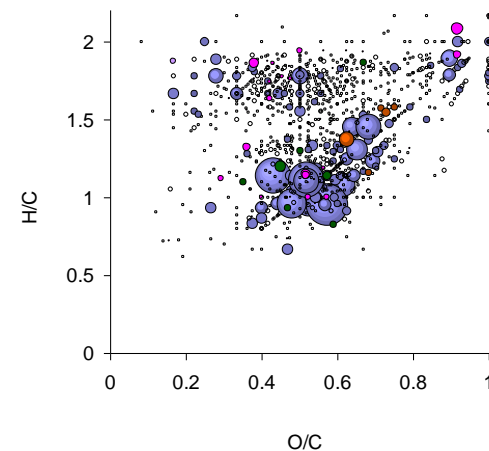
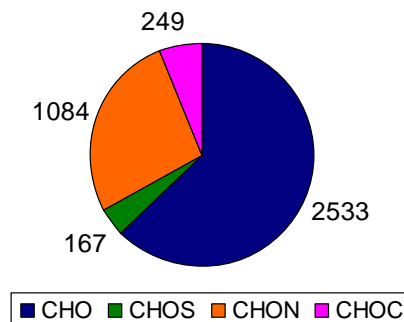
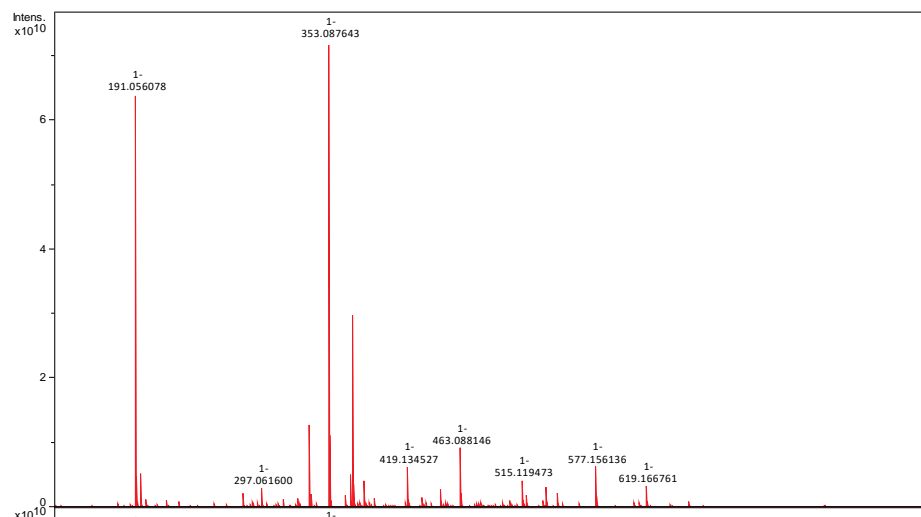
Raw dry flowering tops extracted by microwave



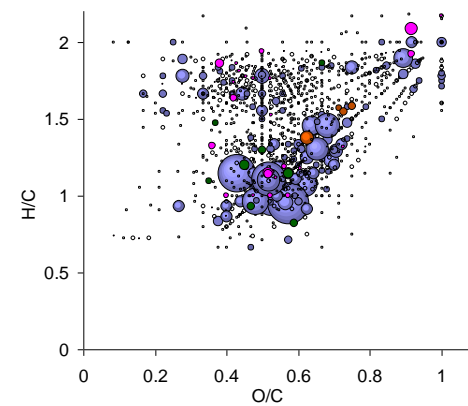
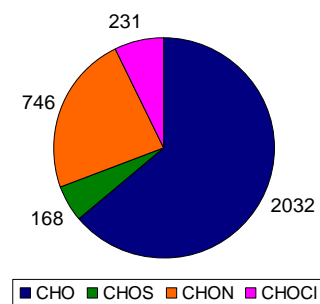
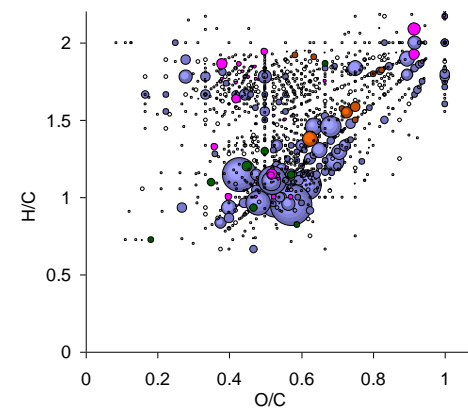
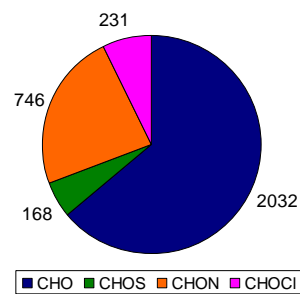
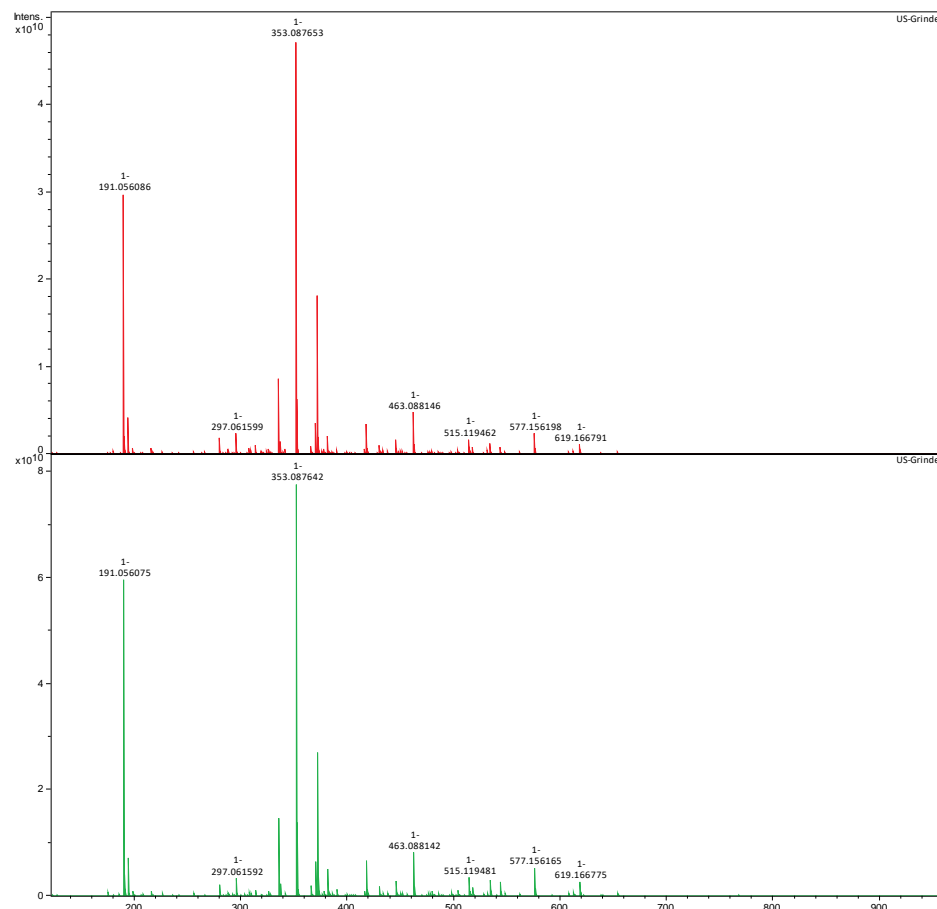
Grinded dry flowering tops extracted by percolation



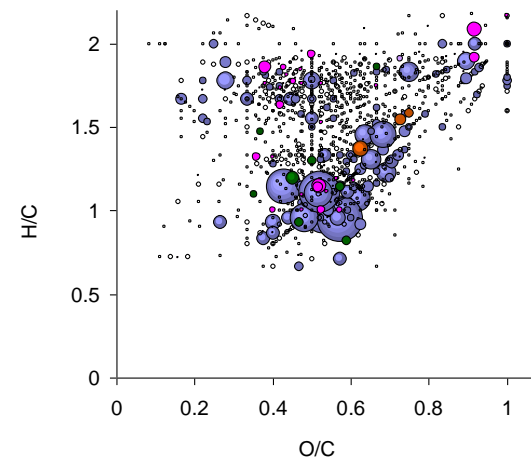
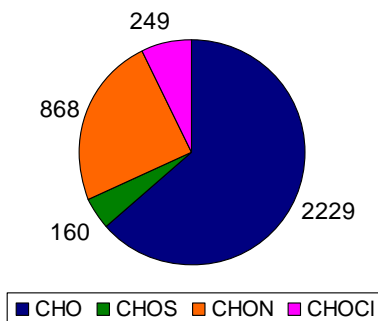
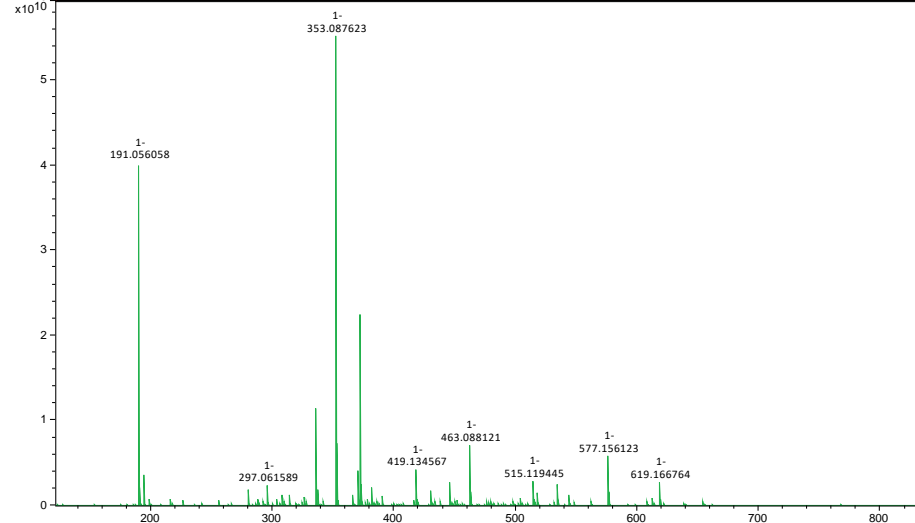
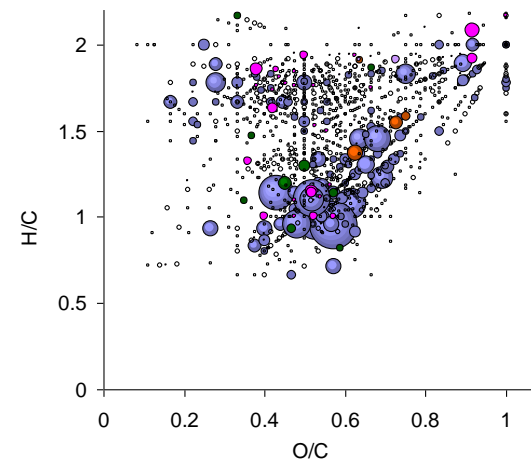
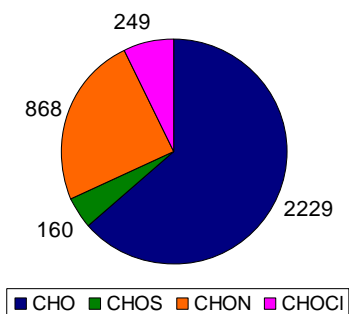
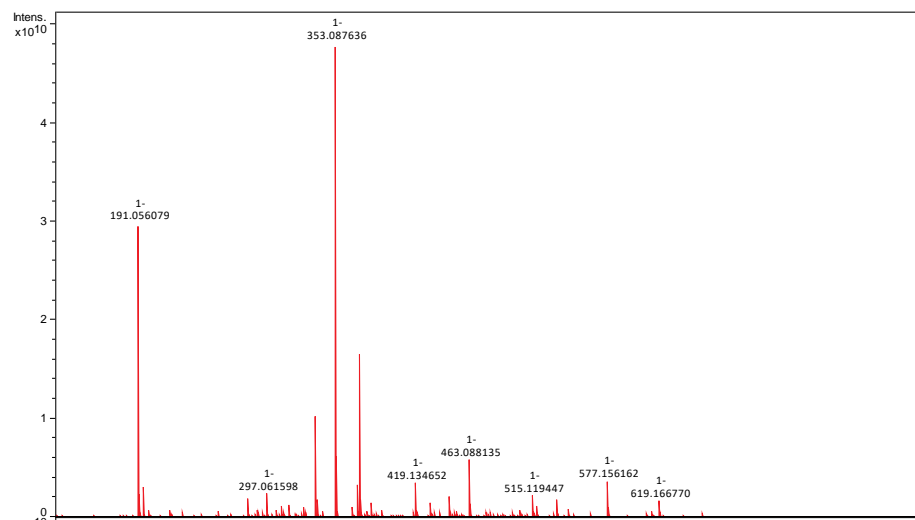
Raw dry flowering tops extracted by percolation



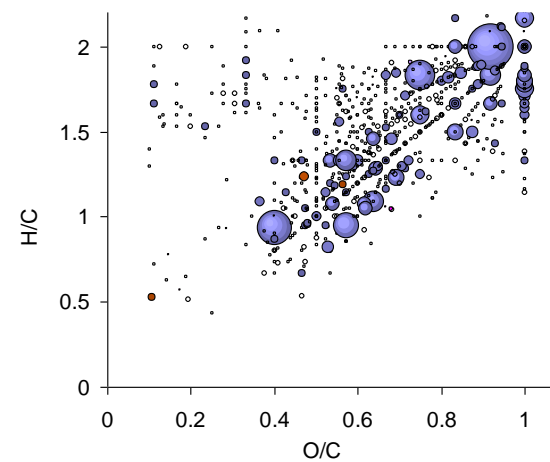
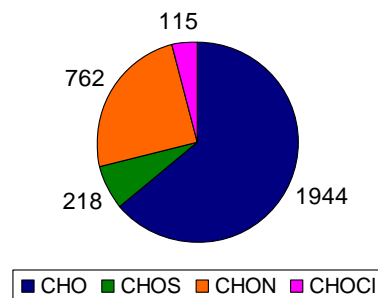
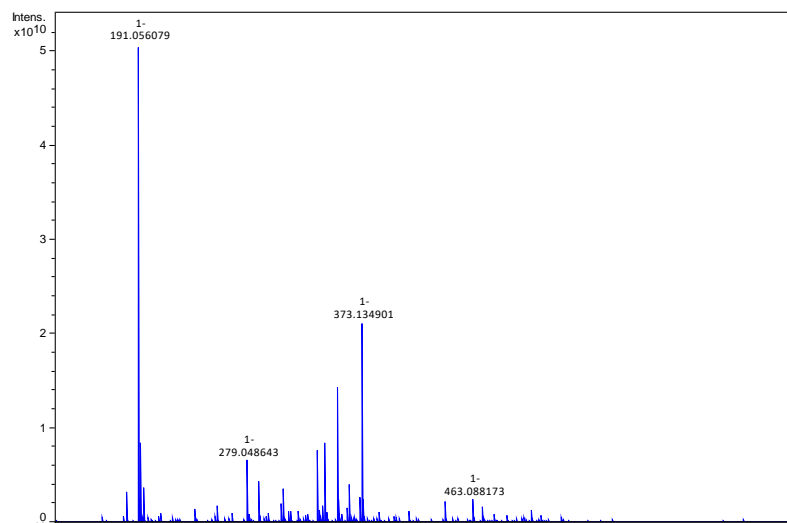
Grinded dry flowering tops extracted by ultrasonication



Raw dry flowering tops extracted by ultrasonication



Hydroalcolic plant extract



Standard plant extract

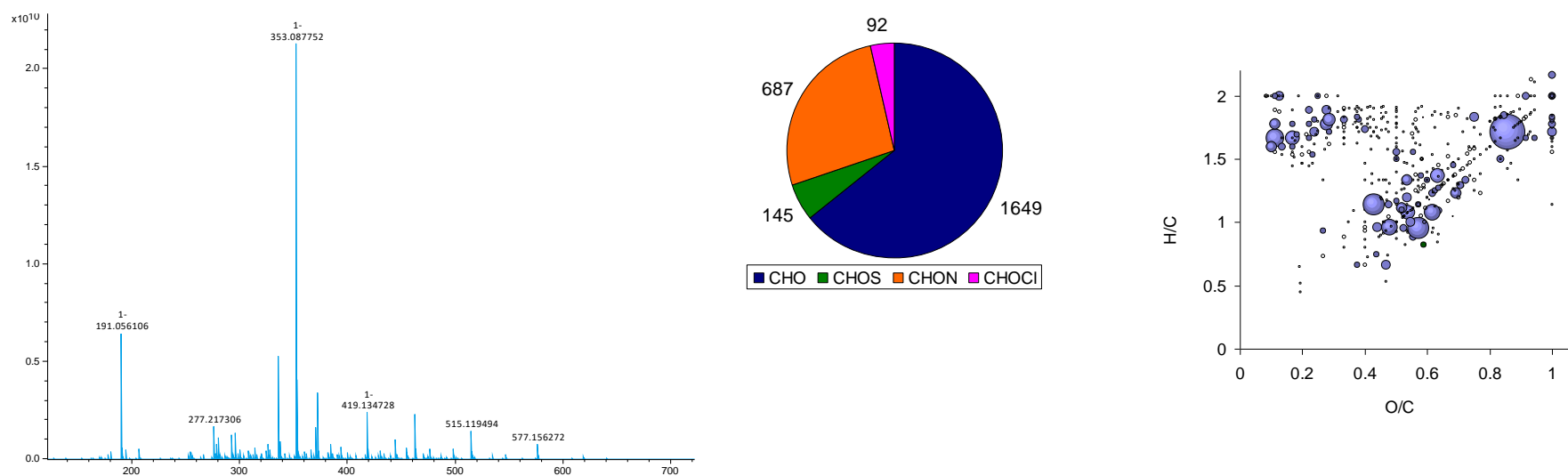


Figure SI-II.8. Mass spectra achieved by (-) ESI FT-ICR MS analysis of the hawthorn samples, in duplicate (green and red mass spectra), according to the extraction method, plant parts and state (fresh or dry). The pie charts show the heteroatom class distribution and the achieved corresponding feature numbers. Van Krevelen diagram represents all the assigned features coloured by chemical class. The size of the bubble is relative to the peak intensity.

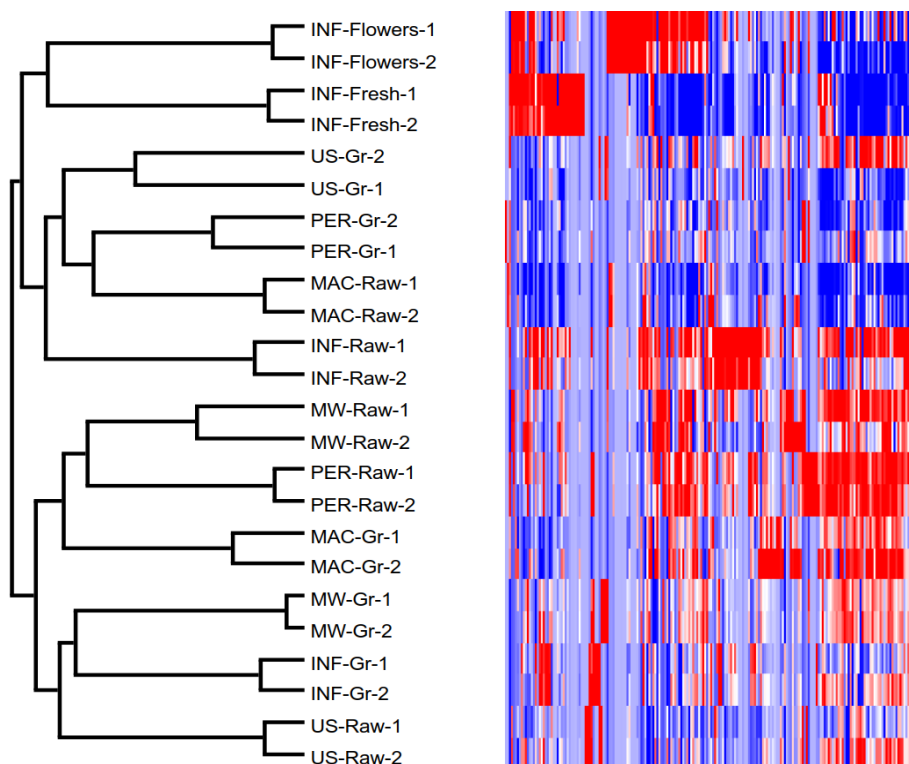


Figure SI-II.9. Hierarchical Cluster Analysis (HCA) and heatmap achieved from samples analyzed by (-) ESI FT-ICR MS. INF = Infusion. MAC = Maceration (at 60°C). PER = Percolation. US = Ultrasonic (at 60°C). MW = Microwave. Raw = raw dry flowering tops. Gr = Grinded (1 mm) flowering tops. Lot number: 20335 (flowering tops) and 20334 (flowers). 1 and 2 numbers correspond to two independent extractions of the same sample (2 repetitions).

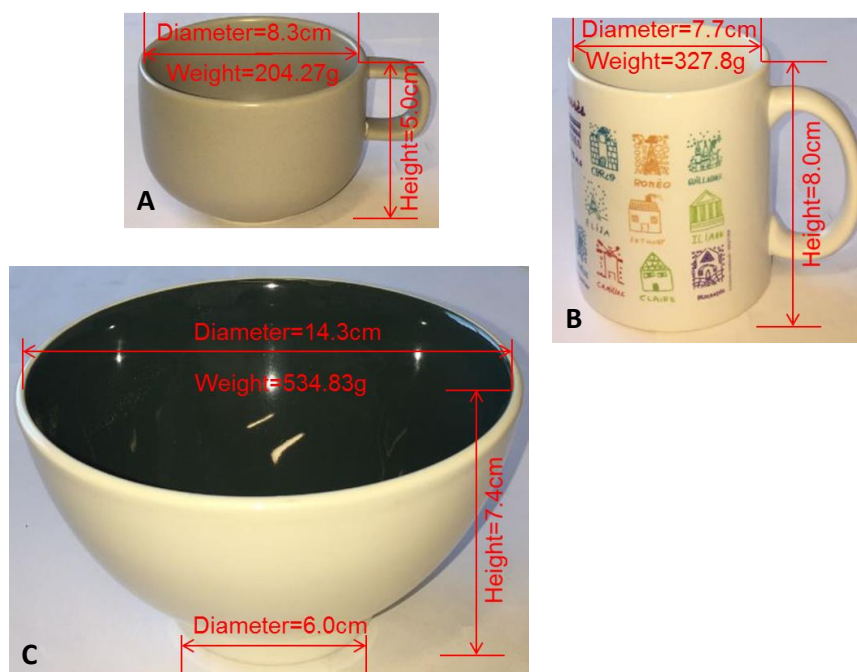


Figure SI-II.10. Pictures of cup (A), mug (B) and bowl (C) with dimensions and weights.

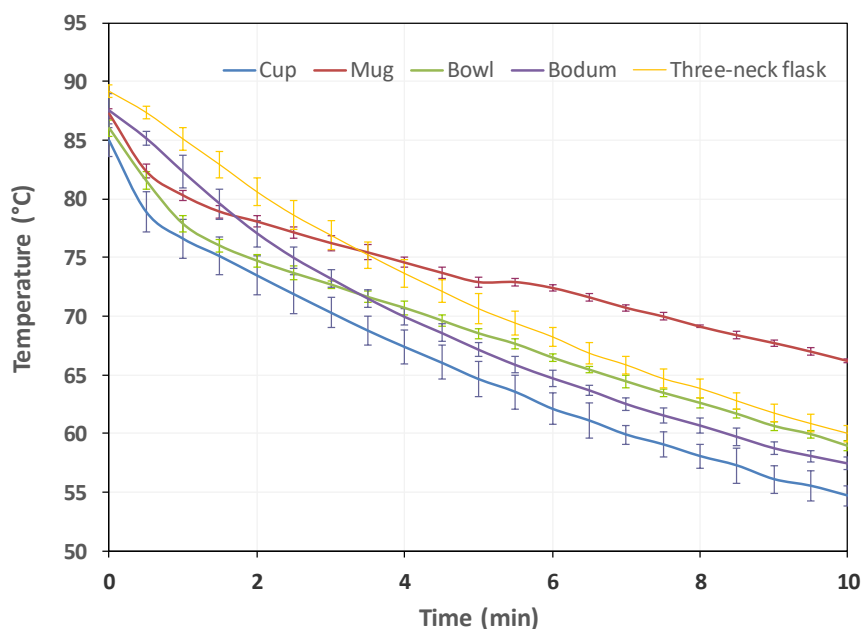


Figure SI-II.11. Decrease profile of temperature (without stirring) vs the nature of the container (cup, mug, bowl, Bodum®, three-neck flask). Volume used: 125 mL (cup), 250 mL (mug, Bodum® and three-neck flask), 405 mL (bowl). Lines are guides for better reading. Error bars are \pm one SD on $n = 3$ repetitions of independent extractions.

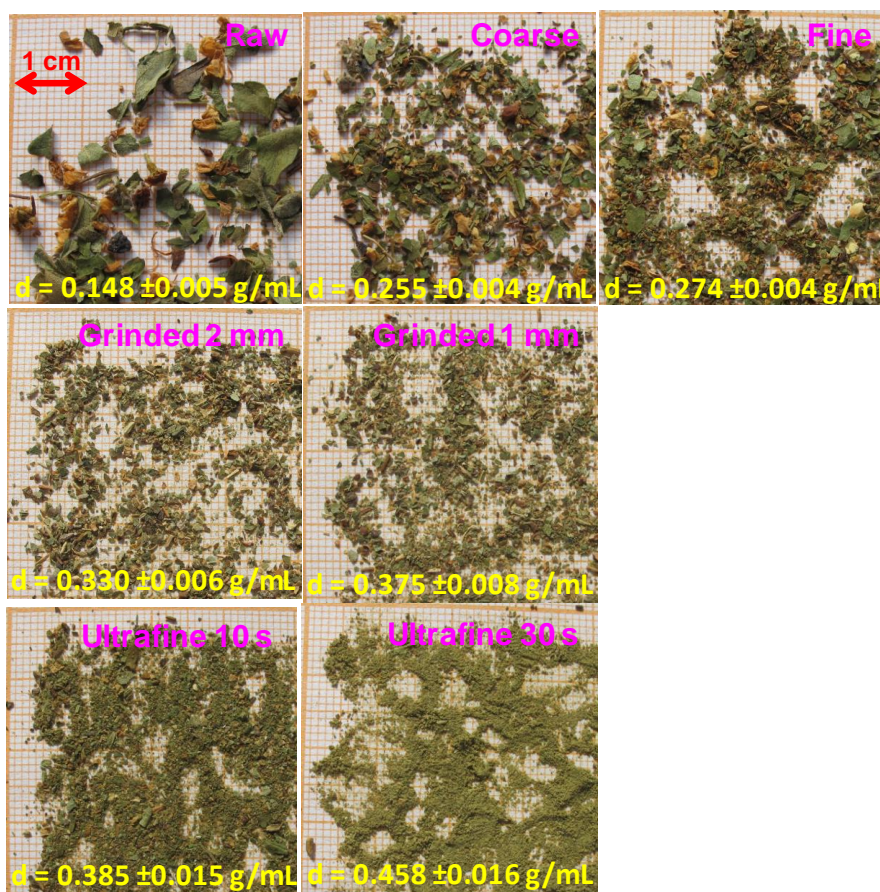
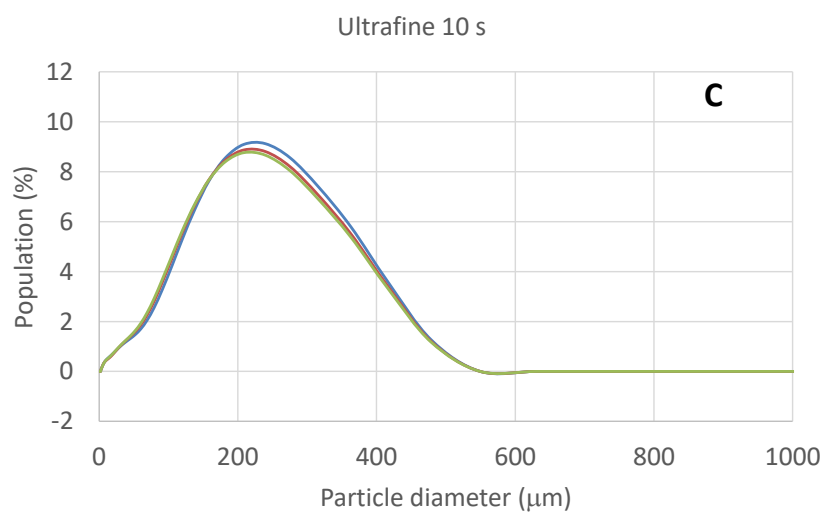
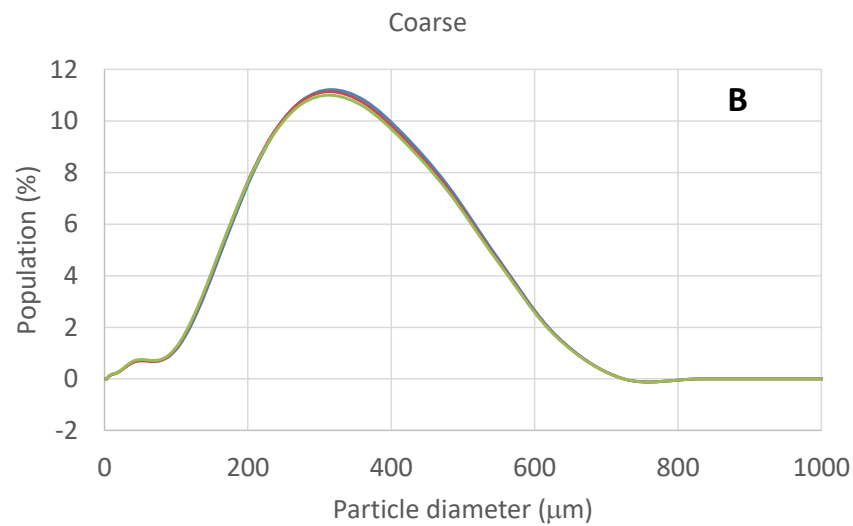
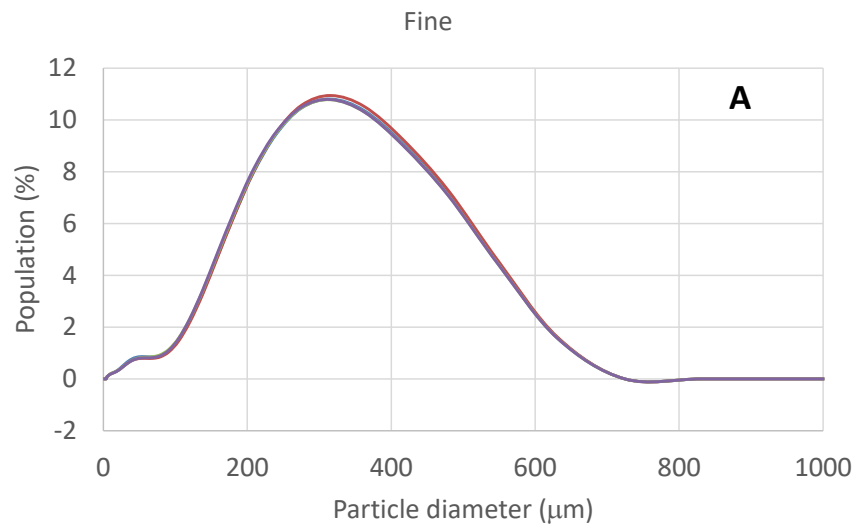


Figure SI-II.12. Pictures of raw and grinded hawthorn materials of various granulometries.



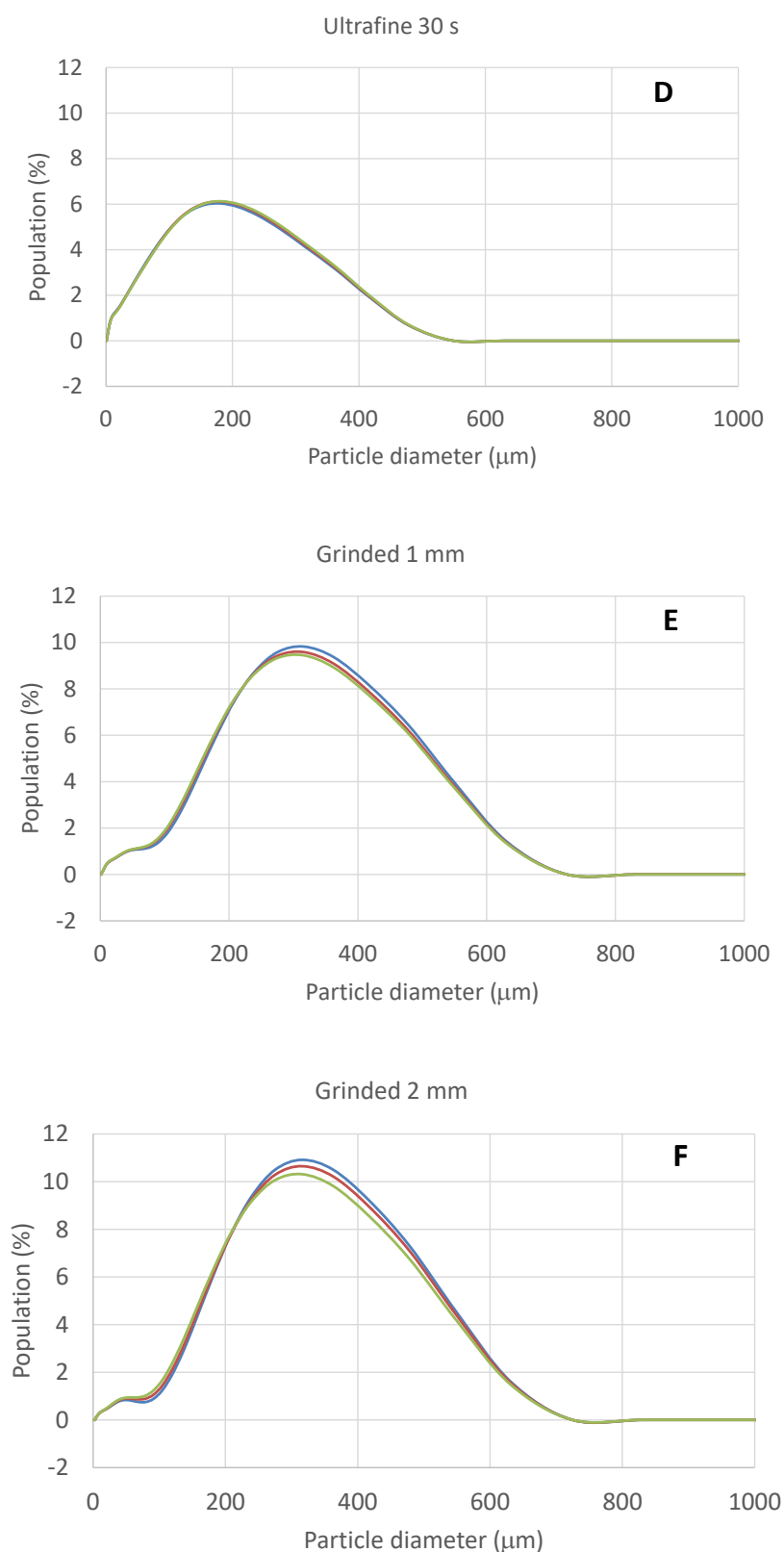


Figure SI-II.13. Size distributions of grinded hawthorn materials. (A) Fine; (B) Coarse; (C) Ultrafine 10''; (D) Ultrafine 30''; (E) Grinded 1mm; (F) Grinded 2 mm. Lot N°CB58120. Experimental condition: see section II.3.2.

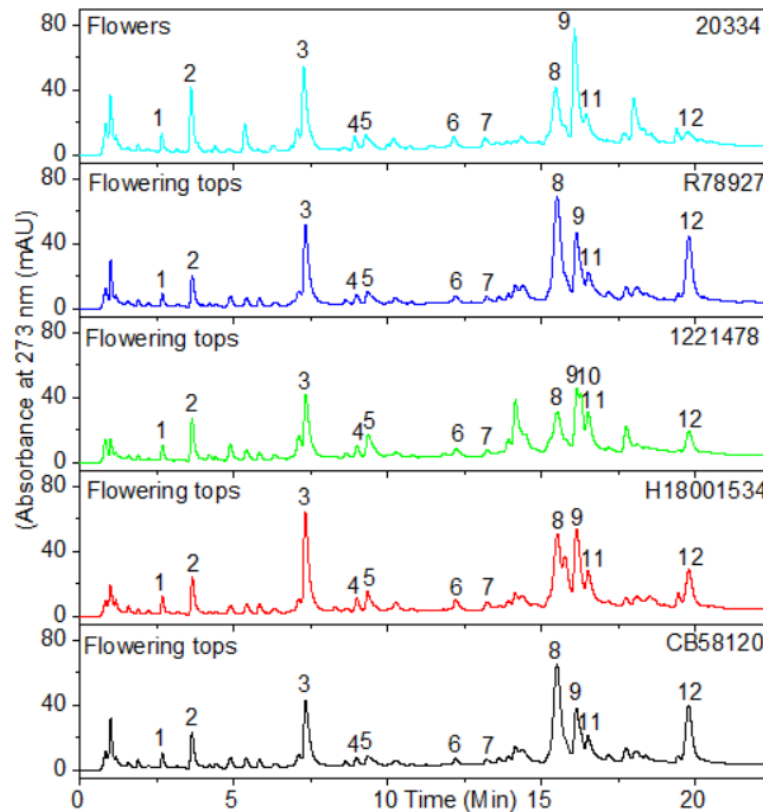


Figure SI-II.14. Influence of the lot number of grinded (fine granulometry) hawthorn dry flowering tops (R78927, 1221478, H18001534, CB58120) and one lot of raw dry flowers (20334) on the UHPLC profiles of the corresponding hawthorn extracts. Infusion extraction using the optimized Bodum® set-up (see section II.3.8) using 2.5 g plant infused in 250 mL water. Experimental conditions of UHPLC as in Figure SI-II.5.

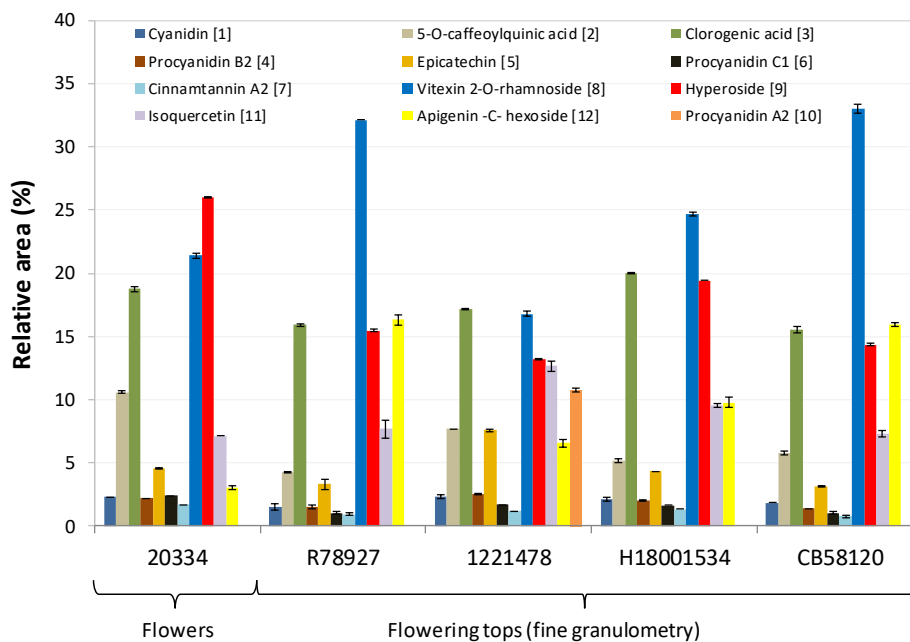


Figure SI-II.15. Relative peak area distributions for the main compounds detected by UHPLC-UV in the various grinded (fine granulometry) hawthorn extracts as a function of the lot number of dry flowering tops (R78927, 1221478, H18001534, CB58120) and one lot of raw dry flowers (20334). Same experimental conditions as in Figure SI-II.14.

Table SI-II.1. List of the samples analyzed by UHPLC-ESI-MS and (-)ESI FT-ICR-MS. All the samples correspond to 10 min extraction time (see section II.3.14 for experimental details) and were duplicated (two independent extractions).

Infusion	Granulometry/nature
Lot n°20335	Flowering tops, raw
Lot n°20335	Flowering tops, grinded 1 mm
Fresh	Flowering tops, fresh
Lot n°20334	Flowers
Maceration at 60°C	Granulometry/nature
Lot n°20335	Flowering tops, raw
Lot n°20335	Flowering tops, grinded 1 mm
US at 60°C	Granulometry/nature
Lot n°20335	Flowering tops, raw
Lot n°20335	Flowering tops, grinded 1 mm
Percolation at 60 °C	Granulometry/nature
Lot n°20335	Flowering tops, raw
Lot n°20335	Flowering tops, grinded 1 mm
MW at 300W	Granulometry/nature
Lot n°20335	Flowering tops, raw
Lot n°20335	Flowering tops, grinded 1 mm

Table SI-II.2. Fitting parameters for the absorbance $A(t)$ trace vs extraction time t for the infusion mode. Extraction kinetic curves are presented Figure II.1 and Figure SI-II.3. *: Temperature at 30 min extraction time.

Extraction mode	Plant	T (°C)	Stirring speed (rpm)	Stirring type	T_1 (min)	T_2 (min)	$A_{30\text{min}}$	A_∞	A_1	
Infusion	Raw dry	41.7	250	Magnetic	1.55	21	1.407	1.627	0.640	
		41.4	500		1.95	21	1.512	1.713	0.821	
		40.0	750		2.3	25	1.466	1.685	0.995	
		39.6	1000		1.9	30.5	1.579	1.847	1.183	
	Grinded (1 mm)	41.7	250	Magnetic	0.55	26	2.274	2.400	2.050	
		41.4	500		0.45	26	2.413	2.506	2.237	
		40.0	750		0.4	24	2.426	2.528	2.194	
		39.6	1000		0.4	26	2.517	2.658	2.272	
	Fresh	41.4	500	Magnetic	2.35	32.5	0.721	1.105	0.122	
	Maceration	Raw dry	20			2.1	34	0.663	0.910	0.292
			40	250	Mecanic	1.95	31.5	0.803	1.100	0.320
			60			1.9	29	1.165	1.596	0.378

		80			2.0	30	1.755	2.404	0.642
	Fresh	60	500	Magnetic	1.2	36.5	0.58	0.798	0.244
		20			2.1	30	1.759	2.409	0.685
	Grinded (1 mm)	40		Magnetic	2.1	26	2.068	2.275	1.573
		60	500		0.65	30	2.301	2.531	1.904
		80			0.6	33.5	2.579	2.837	2.121
US		20			2.1	32	1.381	1.892	0.637
	Raw dry	40	250	Mecanic	2.2	38	1.63	2.233	0.967
		60			2,0	38	2.14	2.932	1.140
		Grinded (1 mm)	60	250	Mecanic	0.65	10.5	3.055	3.100

Table SI-II.3. Compounds identified in hawthorn putatively assigned to raw formulae achieved by (-) ESI FT-ICR MS.

Theoretical mass [M-H] ⁻	Putative compounds M
131.046217	Asparagine
132.030233	Aspartate
133.014249	Malic acid
137.024419	Protocatechuic aldehyde/Hydroxybenzoic acid
146.045883	Glutamate
153.019334	Protocatechuic acid
163.040069	Coumaric acid
164.071703	Phenylalanine
169.014249	Gallic acid
179.034984	Caffeic acid
179.056114	Glucose/fructose/Inositol
180.066618	Tyrosine
181.071764	Sorbitol
188.035318	alpha-cyano-4-hydroxycinnamic acid (HCCA)
191.019729	Citric acid
191.056114	Quinic acid
193.050634	Ferulic acid
203.082602	Tryptophan
223.061199	Sinapinic acid
285.040464	Kaempferol/cyanidin (-2H ⁺)
289.071764	Catechin/epicatechin
300.998994	Ellagic acid
301.035379	Quercetin
315.051029	Sexangularetin
331.067074	Galloylglucose
341.108939	Sucrose
353.087809	Chlorogenic acid / 5-O-Caffeoylquinic acid
385.092894	Diferulic acid
413.087809	Pinnatifida A/C
417.082724	Kaempferol-O-arabinoside (crataegide)
431.098374	Vitexin / Isovitexin / Apigenin-C-hexoside
433.077639	Quercetin pentoside
447.093289	Orientin / Luteolin-7-O-glucuronide/ Ideain / Methoxykaempferol-pentoside / Luteolin-C-hexoside
455.098374	Pinnatifida B/D
455.353069	Oleanolic or ursonic acid
461.072554	Luteolin-7-O-glucuronide

461.108939	Methyl luteolin-C-hexoside
463.088204	Hyperoside/Isoquercitin/Spiraeoside
473.072554	Chicoric acid
473.108939	Acetyl vitexin
477.103854	Sexangularetin-3-O-glucoside
483.078034	Digalloylglucose
489.103854	Acetylorientin
505.098769	Quercetin acetyl hexoside
563.104249	Sexangularetin-3-O-(malonyl) glucoside
563.140634	Schaftoside
575.119504	Procyanidin A2
577.135154	Procyanidin B2
577.156284	Iso/Vitexin 2-O-rhamnoside
593.151199	Vincenin / Keampferol-3-O-neopheridoside / Iso/Orientin-O- rhamnoside
609.146114	Rutin / Quercetin-3-O-rhamnosylgalactoside
619.166849	Vitexin acetyl rhamnoside
623.161764	Sexangularetin-3-O-neohesperidoside / metoxykaempferol methylpentosylhexoside
755.204024	Quercetin di rhamnosyl hexoside/ Rhamnosyl rutin
771.198939	Vitexin-di-O-glucoside
865.198544	Procyanidin C1

Table SI-II.4. Putative compounds obtained from features specifically extracted depending on the plant states (fresh vs dry and grinded vs raw) or parts (flowers vs flowering tops). See Table SI-II.1 for the lot numbers.

Order*	Fresh vs. Dry flowering tops		Dry Flower vs. dry Flowering tops		Grinded vs. Raw Flowering tops	
	Fresh	Dry	Flowers	Flowering tops	Grinded	Raw
1	Sucrose	Chlorogenic acid / 5-O-Caffeoylquinic acid	Sexangularetin-3-O-glucoside	Orientin / Luteolin-7-O-glucuronide/ Ideain / Methoxykaempferol-pentoside / Luteolin-C-hexoside	Chlorogenic acid / 5-O-Caffeoylquinic acid	Ferulic acid
2	Sexangularetin-3-O-(malonyl) glucoside	Orientin / Luteolin-7-O-glucuronide/ Ideain / Methoxykaempferol-pentoside / Luteolin-C-hexoside	Kaempferol-O-arabinoside (crataegide)	Vitexin acetyl rhamnoside	Catechin/Epicatechin	
3	Acetylorientin	Vitexin acetyl rhamnoside	Schaftoside	Galloylglucose	Procyanidin B2	
4	Quercetin pentoside	Vitexin/Isovitexin/Apigenin-C-hexoside	Quercetin pentoside	Luteolin-7-O-glucuronide	Coumaric acid	
5	Oleanolic/ursonic acid	Schaftoside	Vincenin / Keampferol-3-O-neopheridoside / Iso/Orientin-O-rhamnoside	Ellagic acid	Procyanidin A2	
6	Pinnatifida A/C	Galloylglucose	Coumaric acid		Diferulic acid	
7	Procyanidin A2	Malic acid	Malic acid		Pinnatifida B/D	
8			Tryptophan		Protocatechuic aldehyde/hydroxybenzoic acid	
9			Glutamate		Glutamate	

**CHAPTER III:
WATER-BASED EXTRACTION OF BIOACTIVES
PRINCIPLES FROM BLACKCURRANT LEAVES
(BC) AND CHRYSANTHELLYM AMERICANUM
(CA): A COMPARATIVE STUDY WITH
HAWTHORN**

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Foods, 2020, 9, 1978

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Received: date; Accepted: date; Published: date

Abstract:

The water-based extraction of bioactive components from flavonoid-rich medicinal plants is a key step that should be better investigated. This is especially true when dealing with easy-to-use home-made conditions of extractions, which are known to be a bottleneck in the course for a better control and optimization of the daily uptake of active components from medicinal plants. In this work, the water-based extraction of Blackcurrant (*Ribes nigrum*) leaves and *Chrysanthellum americanum*, known to have complementary pharmacological properties, was studied and compared with a previous work performed on the extraction of Hawthorn (*Crataegus*). Various extraction modes in water (infusion, percolation, maceration, ultrasounds, microwaves) were compared for the extraction of bioactive principles contained in blackcurrant leaves (BC) and *Chrysanthellum americanum* (CA) in terms of extraction yield, of amount of flavonoids, phenolic compounds, and proanthocyanidin oligomers, and of UHPLC profiles of the extracted compounds. The qualitative and quantitative aspects of the extraction, in addition to the kinetic of extraction, were studied, leading to an optimized easy-to-use home-made extraction

protocol by infusion. UHPLC-ESI-MS and high-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) were also implemented to get more qualitative information on the specific chemical compositions allowing discriminating the three plants (including Hawthorn, HAW). Their hyaluronidase, antioxidant and anti-hypertensive activities were also determined and compared, demonstrating similar activities as the reference compound for some of these plants.

Keywords: blackcurrant; chrysanthellum americanum; hawthorn; water-based extraction; procyanidin; polyphenol; flavonoid; infusion; granulometry; enzymatic activity.

III.1. Introduction

The extraction of bioactive principles from medicinal plants depends on a large number of factors, such as the extraction temperature, the extraction time, the granulometry of the dry plant, the relative proportion of plant and solvent used for the extraction, to cite only some of them [1]. To promote herbal medicine and favor its acceptance in modern Western integrative medicine [2] and to meet the increasing societal demand in that field [3], it is crucial to investigate and optimize the extraction protocol, so that a daily uptake of active components can be obtained in a repeatable way.

In a previous study [4] the extraction of Hawthorn (*Crataegus*, abbreviated HAW in the following) in water was thoroughly investigated leading to a simple, fast and optimized protocol that can be used by anyone at home. If the plant is ground (typically with a granulometry < 1 mm using a commercially grinder), infusion with simple manual stirring is the easiest way to extract bioactive components from HAW, and the other extraction modes (ultrasounds, microwaves, percolation, maceration) did not significantly improve the extraction yield. The optimized protocol (3 min infusion of 2.5 g hawthorn ground flowering tops in 250 mL boiling water) using a French-press coffee maker (no infusion bags) can afford a daily intake of polyphenols, flavonoids, and proanthocyanidin oligomers similar to the recommended dose from standardized hawthorn extracts [4] Extraction yields about 22

% in mass can be reached in a repeatable and controlled way, with a quantified daily uptake of active components.

HAW is used for its cardiogenic, hypotensive, vasodilative, sedative, antiatherosclerotic, and antihyperlipidemic properties. In this work, we aim at pursuing our study initiated with HAW by investigating two other plants of complementary known pharmacological activities, namely blackcurrant leaves (*Ribes nigrum*) (BC) and *Chrysanthellum americanum* (CA). Blackcurrant (*Ribes nigrum*) is a woody shrub that is widely cultivated across temperate Europe, Russia, New Zealand, parts of Asia and to a lesser extent North America [5,6]. BC contain a valuable source of bioactive compounds especially anthocyanins, proanthocyanidins, phenolic acids, flavonoids and also vitamin C [7]. Since ancient times, BC have been generally used in European folk medicines to treat rheumatism, arthritis and respiratory problems [8] due to its anti-oxidant, anti-inflammatory, and anti-microbial activities, as well as its vasomodulatory, anti-haemostatic and muscle-relaxing effects, and even some neuroprotective and cancer-preventive activities [6,9-14]. *Chrysanthellum americanum*, a genus of yellow flowering plants in the *Chrysanthemum* family [15,16], grows mainly in the mountainous regions or moderate altitude areas in Africa from Senegal to Nigeria and South America from southern Mexico to northern Brazil [17,18]. CA has been traditionally used for its significant wound healing properties in the African and American folk medicine and in the treatment of fever, hepatitis, jaundice and dysentery [19]. In Cuba traditional medicine, it has been used for gastro-intestinal pains, rheumatism and kidney diseases [18]. More recently, CA was reported for its hepatoprotective properties (mainly evidenced against ethylic alcohol and CCl₄), lipid lowering actions and its positive effects against vascular diseases by promoting blood microcirculation [17-23]. The pharmacological properties of CA are generally attributed to saponosides (such as chrysanthellins A and B) and flavonoids compounds. However, CA was much less studied in the literature compared to HAW and BC.

In this work, various extraction modes in water (infusion, maceration, percolation, ultrasounds, microwaves) were compared for the extraction of bioactive principles

contained in BC and CA in terms of extraction yield, of amount of phenolic compounds, flavonoids, and proanthocyanidin oligomers, and of UHPLC profiles of the extracted compounds. The quantitative and qualitative aspects of the extraction, as well as the kinetic of extraction were studied, with an emphasis on the easy-to-use home-made extraction by infusion. High-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) was also implemented to get more qualitative information on the specific chemical compositions allowing discriminating the three plants (including HAW). Their potential hyaluronidase, antioxidant and anti-hypertensive activities were also determined and compared.

III.2. Results and Discussion

The aim of the first part of this work was to investigate if the optimized extraction protocol that was previously developed for HAW, also stands for the extraction of BC and CA bioactive components. Therefore, five different modes of extraction (infusion, maceration, ultrasonic, microwave, and percolation) were compared. For reasons of simplicity and to get a final optimized protocol that can be used by anyone, this study was voluntarily restricted to water as extracting solvent. The protocols of extraction according to each extraction mode are described in the experimental part (see sections III.3.3 to III.3.8). The kinetics of extraction have been studied for both infusion and maceration extraction modes (see section III.3.9). The global mass extraction yields together with the specific contents in polyphenols (TPC), flavonoids (TFC) and proanthocyanidin oligomers (OPC) were determined for all extraction modes and for two plant granulometry (see sections III.3.10 to III.3.12). All extractions were carried out in triplicate (3 independent extractions) to ensure the reproducibility of the measurements. UHPLC-ESI-MS, (-)ESI-FT-ICR-MS and enzymatic activity analyses were also performed to get a better insight in the differences of chemical compositions and pharmacological properties between the three plants. Table III.1 summarizes all the experiments realized on the plants with the corresponding lot numbers, the used granulometry and extraction mode.

Table III.1. List of samples (and lot numbers) studied in this work with the corresponding experimental investigation. For each lot number, three plant extract samples coming from independent extractions were tested. ^a: on raw and grinded 1 mm granulometry; ^b: on raw, grinded 1 mm and grinded 'fine' granulometry; ^c: on grinded 'fine' granulometry; ^d: all extraction modes were tested; ^e: optimized infusion protocol only; ^f: grinded 1 mm granulometry. For the granulometry, see section III.3.2. All extraction modes are described in sections III.3.3 to section III.3.8.

	Kinetics of extraction ^a	TPC ^b , TFC ^b , OPC ^b	UHPLC-MS ^c	(-) ESI FT-ICR-MS ^c	Enzymatic activity ^c
<i>Chrysanthellum americanum</i> (CA)	559980 (infusion and maceration)	559980 ^d	559980 ^e , CP44120 ^e , NH558088 ^e	559980 ^e , CP44120 ^e , NH558088 ^e	559980 ^e
Blackcurrant (<i>Ribes nigrum</i>) leaves (BC)	55870 (infusion and maceration)	55870 ^d	55870 ^e , NH558024 ^e	55870 ^e	55870 ^e
Hawthorn (<i>Crataegus</i>) flowering tops (HAW)	20335 (infusion, maceration and ultrasonic) [4]	20335 ^d [4]	20335 ^d / R78927 ^e , 1221478 ^e , H18001534 ^e , CB58120 ^e [4]	CB58120 ^e , APC27031904 ^e	20335 (infusion) ^f CB58120 ^e

III.2.1. Influence of the extraction mode and of the plant grinding on the kinetics of extraction and on the global extraction yields

The kinetics of extraction were investigated for infusion and maceration of CA and BC, both on raw and ground plants, by monitoring the UV absorbance at 198 nm for 30 min (Figure III.1). This simple analytical method provides interesting information about the kinetics of extraction of the water-soluble components from the plants. Low UV wavelength is used to detect the largest number of extracted chemical compounds. In parallel to the UV monitoring, but on independent experiments, extraction yields (expressed in mass % of the solid extract compared to the initial mass of dry plant) were determined by evaporation and freeze-drying of the whole extract at 10 min (or 30 min) extraction times (see numerical results in Table III.2).

For raw materials, the kinetics of extraction is much faster for infusion mode (see Figure III.1A, open symbols) compared to maceration at 60°C (see Figure III.1B), as already

observed for HAW. This can be quantitatively assessed by the decrease of the time $t_{70\%}$ to get 70 % of the absorbance value at highest extraction time at 30 min (see Table III.2): from 7 min (resp. 15.5 min) for maceration at 60°C of CA (resp. BC) to 2 min (resp. 8.5 min) for infusion of CA (resp. BC). As for the extraction yield at 10 min (see Table III.2), the increasing order of extraction yield for raw CA and BC was: percolation < maceration at 60°C < infusion < US < MW. The same order was found for raw HAW extraction, except for the percolation mode that was the most efficient in that latter case. For the three plants, infusion was always more performant compared to maceration at 60°C, and this was especially true at 10 min extraction for CA (8.4 % extraction yield for maceration vs 15.2 % for infusion).

In a second set of experiments, similar extractions were performed on grinded (using 1 mm mesh size grinder) BC and CA, using the same lots as the previous experiments. As already shown before for grinded HAW [4], grinded BC and CA led to much faster kinetics of extraction (see Figure III.1A for infusion and Figure III.1B for maceration, plain symbols) with $t_{70\%}$ lower than 1.5 min for both extraction modes (see Table III.2). Extraction yields were similar at 10 min and 30 min extraction times (30-33 % for CA and BC, and 20-25 % for HAW [4] for all extraction modes, due to fast extractions. These yields were much higher compared to those obtained with raw materials (+40 % for CA and HAW infusion and +100 % for BC infusion, at 10 min extraction time, see Table III.2 and [4]. This gain on the extraction yield is especially remarkable for BC, most likely because of the large size of the raw leaves for which the grinding ensures a considerable increase of the specific surface compared to raw material. The impact of grinding on the extraction yields tends to decrease with the extraction time but still remains substantial at 30 min (+28 % for CA infusion, +60 % for BC infusion and +40 % for HAW infusion [4].

As a conclusion of this part, for all the plants considered in this work and in [4] (i.e. CA, BC and HAW), the extraction by infusion was found to be an efficient and the easiest way to extract the water-soluble components, in less than 2 min provided that the plant was grinded. It is worth noting that between the lowest extraction yield at 10 min (percolation

on raw BC, 7.4 %) and the highest value (MW on grinded BC, 33.1 %), a factor of ~ 4.5 was found on the extraction yield, demonstrating the importance of the protocol of extraction.

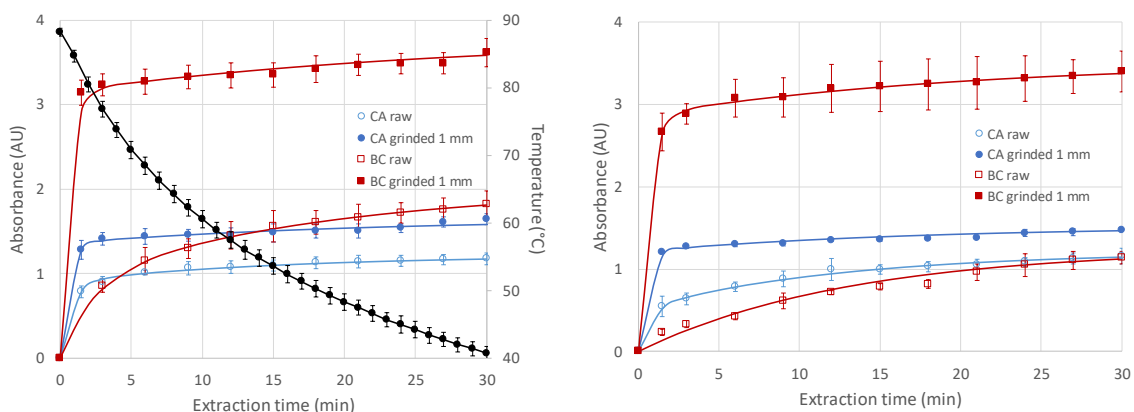


Figure III.1. Kinetics of extraction of CA (lot no. 559980) and BC (lot no. 55870) monitored by UV absorbance at 198 nm. (A) Infusion mode at 500 rpm stirring speed, with the corresponding temperature profile. (B) Maceration mode at 60 °C and at 500 rpm stirring speed. In all cases, 2.5 g of material in 250 mL water was used. A 100 μ L sample of the solution was taken and added to 4 mL ultrapure water before each UV measurement. Error bars are ± 1 SD on $n = 3$ repetitions of independent extractions. If the absorbance values were above 1.7, dilution in 8 mL (instead of 4 mL) was used, but the experimental values were then multiplied by two to allow for a comparison with dilutions in 4 mL. For grinded materials, 1 mm grinder was used (see section III.3.2).

Table III.2. Physicochemical characteristics of the extracts of CA and BC depending on the extraction mode, the extraction time, and the plant granulometry. In all cases, 2.5 g of plant material in 250 mL water was used. For kinetic UV monitoring, 100 μ L of solution was taken and added to 4 mL water before UV measurement, except for ^a, where 100 μ L was added to 8 mL of water to avoid spectrometer saturation (values reported in the table are multiplied by a factor of 2 for better comparison). ^b: ± 1 standard deviation calculated on $n = 3$ repetitions. ^c: in mg eq. GA/g dry plant, ± 1 standard deviation calculated on $n = 3$ repetitions. ^d: in mg eq. Q/g dry plant, ± 1 standard deviation calculated on $n = 3$ repetitions. ^e: in mg eq. CY/g dry plant, ± 1 standard deviation calculated on $n = 3$ repetitions. ^f: in mg / g dry plant, ± 1 standard deviation calculated on $n = 3$ repetitions. Lot number for CA: 559980. Lot number for BC: 55870.

Plant nature & granulometry	Extraction mode	Experimental conditions	Extraction time to get 70% of the Abs at 30 min (min)	Absorbance at 30 min	10 min extraction time				30 min extraction time			
					Extraction yield (%) ^b	TPC ^c	TFC ^d	OPC ^e	Extraction yield (%) ^b	TPC ^c	TFC ^d	OPC ^e
CA (raw)	Infusion	500 rpm	2	1.177	22.70 \pm 0.94	11.29 \pm 0.30	2.64 \pm 0.06	0.36 \pm 0.03	25.12 \pm 1.96	14.79 \pm 0.60	3.12 \pm 0.07	0.43 \pm 0.03
	Maceration	60°C	7	1.161	22.41 \pm 0.45	8.37 \pm 0.60	2.23 \pm 0.18	0.34 \pm 0.03	24.86 \pm 1.48	11.48 \pm 0.93	2.64 \pm 0.22	0.37 \pm 0.02
	Ultrasonic	60°C	-	-	24.12 \pm 1.03	8.91 \pm 0.44	3.21 \pm 0.15	0.39 \pm 0.03	-	-	-	-
	Microwave	300W	-	-	27.61 \pm 1.62	13.86 \pm 0.60	3.29 \pm 0.12	0.48 \pm 0.02	-	-	-	-
	Percolation	-	-	-	16.60 \pm 0.01	8.29 \pm 0.05	2.08 \pm 0.10	0.26 \pm 0.02	-	-	-	-
CA (grinded 1 mm)	Infusion	500 rpm	< 1.5	1.647	31.81 \pm 0.18	17.08 \pm 0.31	4.42 \pm 0.28	0.66 \pm 0.04	32.34 \pm 0.80	18.37 \pm 0.51	3.62 \pm 0.16	0.67 \pm 0.06
	Maceration	60°C	< 1.5	1.478	30.85 \pm 0.41	12.87 \pm 0.35	3.76 \pm 0.05	0.50 \pm 0.02	31.50 \pm 0.47	14.59 \pm 0.80	3.76 \pm 0.16	0.54 \pm 0.02
	Ultrasonic	60°C	-	-	31.65 \pm 1.42	12.74 \pm 0.94	4.21 \pm 0.07	0.63 \pm 0.05	-	-	-	-
	Microwave	300W	-	-	31.29 \pm 1.05	17.38 \pm 0.83	4.21 \pm 0.08	0.63 \pm 0.02	-	-	-	-
	Percolation	-	-	-	31.09 \pm 0.89	13.04 \pm 0.48	4.32 \pm 0.05	0.67 \pm 0.04	-	-	-	-

Chapter III: Water-based extraction of bioactives principles from BA and CA

BC (raw)	Infusion	500 rpm	8.5	1.824	15.16 ±0.39	15.84 ±0.45	1.72 ±0.08	0.55 ±0.04	20.69 ±0.72	23.92 ±1.38	2.48 ±0.04	0.83 ±0.07
	Maceration	60°C	15.5	1.137	8.44 ±0.42	6.99 ±0.35	0.81 ±0.06	0.24 ±0.02	18.49 ±1.19	17.09 ±0.28	1.98 ±0.11	0.64 ±0.01
	Ultrasonic	60°C	-	-	17.61 ±1.48	18.82 ±0.30	2.28 ±0.17	1.02 ±0.02	-	-	-	-
	Microwave	300W	-	-	20.45 ±0.21	23.08 ±1.09	2.74 ±0.21	1.02 ±0.10	-	-	-	-
	Percolation	-	-	-	7.40 ±0.38	6.50 ±0.26	0.88 ±0.01	0.30 ±0.05	-	-	-	-
BC (grinded 1 mm)	Infusion	500 rpm	< 1.5	3.615 ^a	30.49 ±1.22	47.28 ±0.57	4.75 ±0.09	2.35 ±0.17	32.41 ±0.52	45.39 ±1.25	4.82 ±0.13	2.66 ±0.16
	Maceration	60°C	< 1.5	3.399 ^a	30.39 ±0.36	45.75 ±2.34	4.73 ±0.10	2.07 ±0.05	30.51 ±0.44	44.94 ±1.35	4.70 ±0.14	2.74 ±0.16
	Ultrasonic	60°C	-	-	29.54 ±0.35	42.42 ±1.67	4.45 ±0.32	2.55 ±0.01	-	-	-	-
	Microwave	300W	-	-	33.13 ±0.27	50.62 ±1.30	5.25 ±0.09	3.60 ±0.32	-	-	-	-
	Percolation	-	-	-	31.80 ±0.96	46.05 ±0.18	4.77 ±0.01	2.42 ±0.07	-	-	-	-

III.2.2. Quantification of total polyphenol, flavonoid and proanthocyanidin contents

After their extraction in water, the plant extracts were evaporated, lyophilized and kept in the dark at -18°C for better conservation prior to analysis. The total amounts of polyphenol (TPC), flavonoid (TFC) and proanthocyanidin oligomers (OPC) contents in the dry plant extracts were determined by colorimetric methods (see sections III.3.10 to III.3.12) and expressed as equivalent content in gallic acid (GA) for TPC, quercetin (Q) for TFC and cyanidin (CY) for OPC. The numerical values are reported in Table III.2 for 10 min and 30 min extraction times ($n=3$ repetitions, on 3 independent extractions).

As expected, and as observed for the global extraction yield, the extraction yields of TPC, TFC and OPC were much higher for grinded material than for raw material. The gain in extraction due to the grinding is even more important for the phenolic components than for the global yield: TPC (+50 % CA, +200 % BC and +70 % HAW), TFC (+60 % CA, +180 % BC and +50 % HAW) and OPC (+80 % CA, +330 % BC and +210 % HAW).

Regarding the different extraction modes, the MW extraction mode generally gives the best extraction yields, either for raw and grinded plants, in good agreement with the literature [4,22-25]. On raw plants, the extraction mode can have a great impact, with an enhanced extraction factor (EF) at 10 min extraction of about 1.7 for CA (resp. 2.8 for BC) between the worst (percolation) and the best (MW) modes. However, the differences between the extraction modes are very limited for grinded materials (only 5 to 10 % differences on TPC, TFC and OPC extraction yields, see Table III.2). Therefore, and as already observed for HAW [4], the grinding of the dry plant is the most important parameter to increase the extraction yields for all the quantified components, and the infusion mode is the simplest and most accessible extraction mode to be selected.

The dry extract content and the TPC, TFC and OPC contents obtained from a single 10 min infusion (at 500 rpm) of 2.5 g of 1 mm grinded plant in 250 mL water were determined. One infusion brings about 795 mg / 760 mg / 555 mg of dry extract for CA, BC and HAW respectively. These extracts contain 43 mg / 118 mg / 82 mg equivalent GA (TPC), 11.1 mg

/ 11.9 mg / 8.6 mg equivalent Q (TFC) and 1.6 mg / 5.9 mg / 9.8 mg equivalent CY (OPC) (Table III.2 and [4]). It is worth noting that BC extracts contain much more phenolic compounds than the two other plants, while HAW extracts contain much more proanthocyanidin oligomers than the two other plants. CA and BC extracts contain similar amounts of flavonoids.

III.2.3. Characterization of the plant granulometry and optimized easy-to-use infusion protocol.

As the plant granulometry is the main factor influencing the kinetics and the yield of extraction, a simple way was used to grind the plants in a reproducible and affordable manner. Two electric coffee grinders were used, i.e. the Delonghi (Model KG79) grinder (used at the two extremes positions, namely 'coarse' and 'fine' positions) and a Bosch electric (Model MK6003) grinder (used with 10 s and 30 s grinding times by shaking the grinder simultaneously). A grinding laboratory equipment (Ika, Model MF10 basic) was also used with 1 mm grid size. Figure SI-III.1 shows the pictures of the CA and BC materials on a millimeter paper, before (raw) and after the previously described different grinding protocols. The plant density of the grinded material is also given for each picture and was simply measured using a graduated test tube (see section III.3.2 for more details). To get better quantitative data, the size distribution of the plant particle was determined by laser granulometry in dry phase (see section III.3.2) and the corresponding distributions are presented in Figure III.2A (CA) and Figure III.2B (BC). Broad volume size distributions (given in diameter) were obtained with typically a bimodal curve having sizes ranging between 10-80 μm for the first mode (or as a peak shoulder), with a main mode between 200-250 μm , and maximum size up to 500-700 μm . The correlation between the grinded dry plant density and the average particle diameters are presented in Figure III.2C and Figure III.2D for different deciles of the distribution (D_{10} is the first decile, D_{50} is the median value, and D_{90} is the 9th decile). From these correlations, we can roughly estimate the minimum density required to get an adequate granulometry to optimize the plant extraction. This could be

very useful in practice (for instance at home) when granulometry measurement is not available. To get a granulometry corresponding to the 'fine' position of the Delonghi (Model KG79) grinder, or even lower granulometry, a plant density equal or higher than 0.22 g mL⁻¹ for CA, 0.26 g mL⁻¹ for BC and 0.27 g mL⁻¹ for HAW is required. As a first approximation, the value of 0.25 g mL⁻¹ can be retained for the three plants.

Since the infusion mode was found to be the easiest way to perform the water-based extraction on grinded materials, the optimized protocol already used for HAW [4] was also implemented for the extraction of CA and BC. This easy-to-use-at-home protocol is based on the following steps: (1) the grinding of 2.5 g of raw dry plant using a basic commercially available grinder just before the infusion ('fine' granulometry, density of grinded plant about 0.25 g mL⁻¹ or higher); (2) pouring 250 mL of boiling water onto the ground plant in a French-press coffee maker (no infusion bag, the piston permits to push the residual solid parts of the plant to the bottom of the recipient); (3) vigorous manual stirring of the mixture; (4) waiting for at least 3 min of infusion; and (4) pressing the French-press filter to retain the remaining solid part of the plant before serving. Using this protocol, the global extraction yields are optimized and repeatable (25.9 % for CA; 28.6 % for BC and 21-22 % for HAW, see Table III.3). The same stands for the quantities of TPC, TFC and OPC that were extracted (Table III.3). The impact of the plant granulometry on the extraction yields was not investigated in this work for CA and BC; however, this study was previously performed for HAW showing that 'fine' position granulometry was sufficient to get optimal extraction yields [4].

In the laboratory conditions of infusion (section III.3.3), the temperature decreased in the reactor from about 90°C to 40°C after 30 min extraction at 500 rpm and for a volume of water of 250 mL. The drinkable temperature (60°C) to avoid any side effect on the health [28,29] was reached after 10 min extraction. In practice, we find it interesting to study the

temperature profile that we would encounter at home by infusion in the French press Bodum® (see section III.3.8), for two volumes of water (250 mL corresponding to a mug; or 405 mL for a bowl, see Figure SI-III.7). Cooling down the infusion at drinkable temperature (60°C) requires much longer times (8 min for 250 mL, and 12 min for 405 mL) than the time required for the extraction (about 3 min). To speed-up the cooling process after the 3 min extraction, and drink safely without waiting too long, it is possible to add ice cube(s) in the French press Bodum®, as shown in Figure SI-III.7. For 250 mL of water, only one ice cube (~23.4 g) is necessary to reach 60°C; with a full melting of the ice cube within 1 min, leading to a total preparation time of about 4 min (at home). For 405 mL of water, two ice cubes (~23.4 g each) are required to reach 60°C; with a total preparation time of about 5 min.

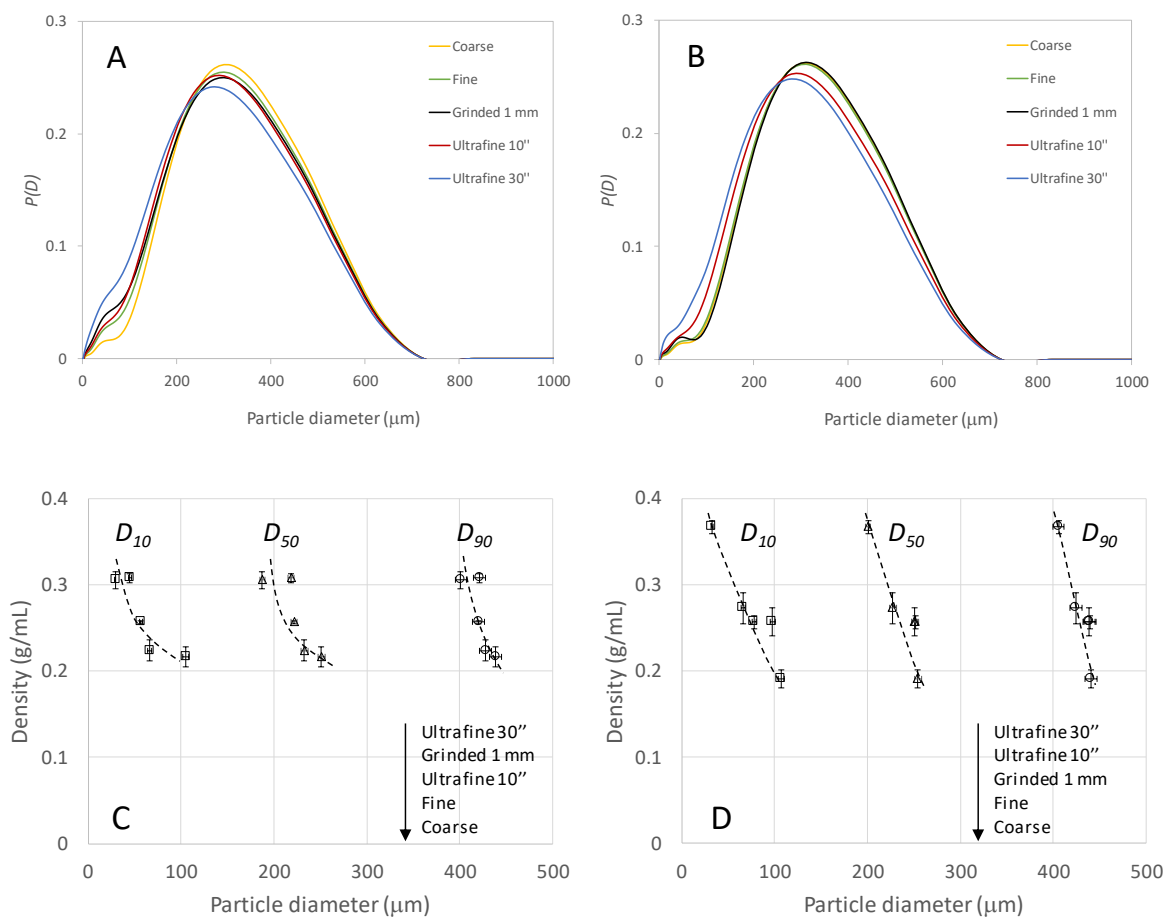


Figure III.2. Relative size distributions of the raw and ground CA (A) and BC (B) materials obtained by laser granulometry in dry mode and variation of the density of CA (C) and BC (D) materials as a function of the particle diameter. D_{10} (\square), D_{50} (Δ), and D_{90} (\circ) with D_x being the 10th decile of the distribution. See Section III.3.2 for more details on the experimental conditions. Lot number for CA: 559980. Lot number for BC: 55870.

Table III.3. Extraction yield, TPC, TFC, and OPC values in CA and BC extracts ('fine' grinded) issued from infusion mode at 10 min extraction time using easy-to-use infusion extraction (2.5 g of plant material in 250 mL water introduced in Bodum® French press recipient, see section III.3.8). Manual stirring was done by manually rotating the recipient at the beginning of the extraction and 10' later before filtration. *: data extracted from [4].

Nature	Lot number	Extraction yield (%)	TPC	TFC	OPC
<i>Chrysanthellum americanum</i> (CA)	559980	25.9 ±0.9	15.51 ±0.05	3.31 ±0.04	0.42 ±0.01
Blackcurrant leaves (<i>Ribes nigrum</i>) (BC)	55870	28.6 ±0.5	39.60 ±0.49	4.44 ±0.17	1.67 ±0.10
Hawthorn (<i>Crataegus</i>) (HAW)*	CB58120	21.7 ±0.1	20.1 ±0.4	2.86 ±0.02	1.81 ±0.05

III.2.4. Chemical composition of the CA and BC plant extracts investigated by UHPLC and its coupling with MS.

To get a better insight into the differences between CA and BC (as well as with previously published HAW results), reversed phase UHPLC and positive mode UHPLC-ESI-MS analysis were performed (see section III.3.13 for more details) according to [30]. A detailed list of the samples studied in this work by UHPLC-ESI-MS is given in Table III.1. The UV chromatographic profiles obtained for the infusion extracts (issued from the infusion of 'fine' grinded materials using the optimized easy-to-use infusion protocol, see section III.3.8) of BC and CA, are presented in Figures III.3 and III.4 respectively, at three different wavelengths, 280 nm for flavanones and flavan-3-ols, 320 nm for hydroxycinnamic acids, and 360 nm for flavonols (see also Figure SI-III.3 for the chemical structure of all identified compounds and Figures SI-III.4 and S5 for the profiles of the other lots). Tables III.4 and III.5 contain the list with names and molar masses of the identified components of BC and CA, respectively.

Among 9 major peaks detected in BC (see Figure III.3), two of them (peak 1 = Chlorogenic acid, and peak 3^b = Quercetin 3-O-glucoside (isoquercetin)) were unambiguously identified by comparing the retention times, UV spectrum and MS or MS/MS data with the reference standards used in our previous study [4]. The peaks 2, 3^a, 4^a, 4^b, 5, 6 and 7 were tentatively assigned, respectively to: Quercetin 3-rutinoside (2), Quercetin 3-O-galactoside (3^a), Quercetin-3-6-malonyl-glucoside (4^a), Kaempferol-3-O-rutinoside (4^b), Kaempferol-3-O-hexoside (5), Kaempferol-malonylglucoside (6), Kaempferol-malonylglucoside isomer (7); based on the data from literature review [9,29-31], UV spectra maximum values and their fragmentation patterns from ESI-MS (+/-) and MS/MS data, as presented in Table III.4.

Chemical composition of CA extracts has been much less investigated in the literature than for BC or HAW. Among 10 major peaks detected in CA (see Figure III.4 and Table III.5), one of them (peak 1, Chlorogenic acid) was unambiguously identified by comparing the retention times, UV spectra maximum values and ESI-MS(-), MS/MS data with the reference standards used in our previous study [4]. The peaks 8^a, 8^b, 9, 10, 11, 12, 13, 14 and 15 were tentatively identified, respectively to: Eriodicyol-7-O-glucoside (8^a), 6,8-C,C-Diglucosylapigenin (8^b), Isookanin-7-O-glucoside (Flavanomarein) (9), Maritimetin-6-O-

glucoside (Maritimein) (10), Luteolin-7-O-glucuronide (11), Di-caffeoylquinic acid (12), Apigenin-7-glucuronide (14), Di-caffeoylquinic acid isomers (13, 15); based on the data from literature [17,32-36] and their fragmentation patterns from ESI-MS(-), MS/MS data as presented in Table III.5. Compound peak 8^a, 9 and 10 have been described by Shimokoriyama & Honore-Thorez [17,34] and those peaks showed UV maximum absorption band at 282 nm, 284 nm and 414 nm, respectively, very similar to Eriodicyol-7-O-glucoside, Flavanomarein and Martitimein, respectively. The MS/MS spectrum of peak 8^a, 9 revealed three fragments corresponding to Eriodicyol-7-O-glucoside (m/z, 287.0532, 151.0001, 135.048) and Flavanomarein (m/z, 287.0532, 269.0437, 135.0055, 135.0429). Thus, peak 8^a, 9 were tentatively identified as Eriodicyol-7-O-glucoside, Flavanomarein. Additionally, the MS/MS spectrum of m/z 447 ([M-H]⁻) presents the fragments corresponding to m/z 285 (Maritimetin) and another m/z 151, m/z 135 and the molecule ion at m/z 447 ([M-H]⁻) in agreement with Maritimein glucoside. Thus, peak 10 was tentatively identified as Maritimein. 6,8-C,C-diglucosylapigenin, Luteolin-7-O-glucuronide, Apigenin-7-glucuronide were described for the first time in CA. Based on the literature on *Chrysanthemum* species [35-37], peak 8^b presented the UV maximum absorption band at 267nm, the molecule ion at m/z 593 ([M-H]⁻) and other fragment ion at m/z 473, 353, 191; peak 11 revealed the UV maximum absorption band at 280 nm, 335 nm, molecule ion at m/z 461 ([M-H]⁻); peak 14 showed the UV maximum absorption band at 266 nm, 334 nm, the molecular ion at m/z 445 ([M-H]⁻) in agreement with 6,8-C,C-Diglucosylapigenin, Luteolin-7-O-glucuronide, and Apigenin-7-glucuronide respectively. The MS spectra of peak 12, 13 and 15 showed the same molecular ion at m/z 515 ([M-H]⁻), and another fragment ion at m/z 191, 179, 173, 135, based on the literature [36-38] those were described as a characteristic of a Dicaffeoylquinic acid. There are six isomers of Dicaffeoylquinic acid in nature, thus, those were tentatively identified as Dicaffeoylquinic acid isomers.

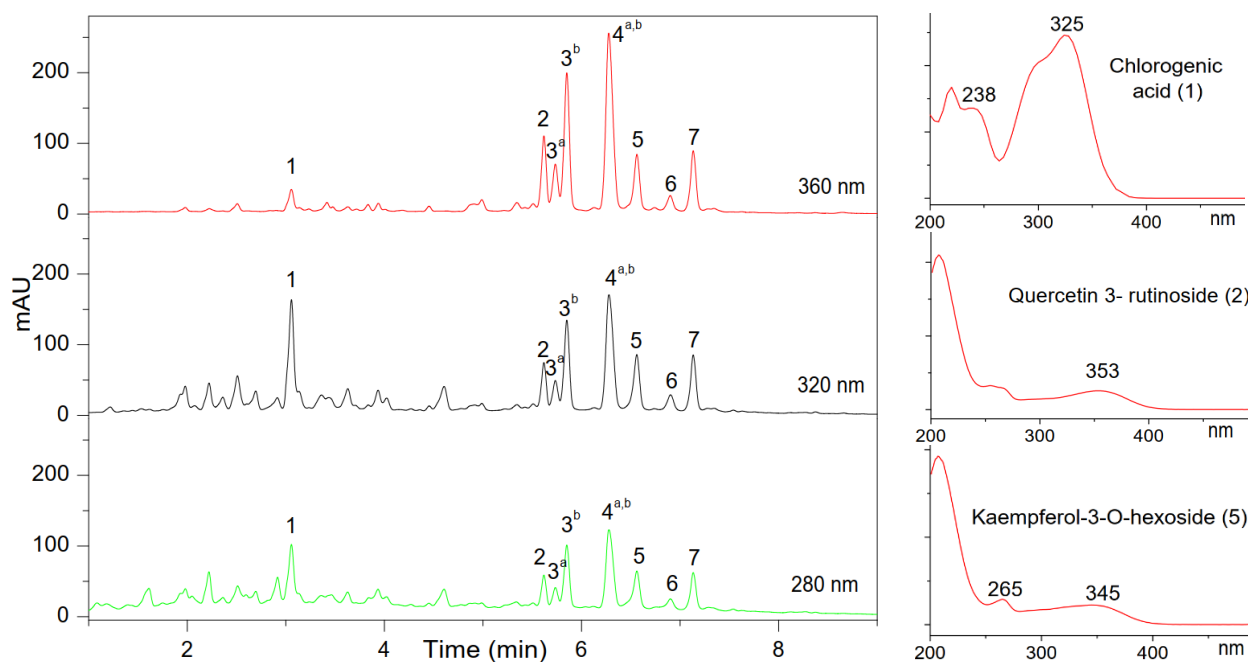


Figure III.3. UHPLC profiles of BC extracts obtained from infusion Bodum® extraction mode for grinded 'fine' BC material monitored at 280 nm, 320 nm and 360 nm. Typical UV spectra of major compounds are given on the right. UV spectrum of Chlorogenic acid represents hydroxycinnamic acid derivatives, UV spectrum of Quercetin 3- rutinoside (2) represents Quercetin 3-O-galactoside (3^a), Quercetin 3-O-glucoside (3^b) and Quercetin-3-6-malonyl-glucoside (4^{a,b}), UV spectrum of Kaempferol-3-O-hexoside (5) represents Kaempferol-3-O-rutinoside (4^b), Kaempferol-malonylglucoside (6), and Kaempferol-malonylglucoside isomer (7). See Table III.4 for peak ID. Lot number: 55870. See Figure SI-III.4 for UHPLC profiles of other lots.

Table III.4. Putative peak identification of the main compounds detected by UHPLC-DAD in the BC extracts. λ_{max} are the local maximum of absorbance on the UV spectrum. [M+H]⁺ and [M-H]⁻ columns provides the m/z value of the precursor ion, other ions column gives the m/z value of fragments detected in the MS spectra, identification method using UV spectrum and ESI(+) and ESI(-) spectrum. Lot number: 55870.

Peak	Retention time (min)	λ_{max} (nm)	[M+H] ⁺ / [M-H] ⁻	Other ions in the spectrum (positive/negative mode)	Family (Subclass)	Identified compound
1	3.25	219, 238, 325	355/353	377, 163/191, 179	Phenolic acid (hydroxycinnamic acid)	Chlorogenic acid
2	5.64	254, 353	611/609.2	303/301, 179	Flavonoid (Flavonol)	Quercetin 3- rutinoside
3 ^a	5.75	254, 353	465/463.1	303/301	Flavonoid (Flavonol)	Quercetin 3-O-galactoside (hyperoside)

3 ^b	5.85	254, 353	465/463.1	303/301	Flavonoid (Flavonol)	Quercetin 3-O-glucoside (isoquercetin)
4 ^a	6.24	255, 353	551/549	303/505.1, 301	Flavonoid (Flavonol)	Quercetin-3-6-malonyl-glucoside
4 ^b	6.24	263, 347	287/593	287/285	Flavonoid (Flavonol)	Kaempferol-3-O-rutinoside
5	6.51	265, 345	287/447.1	287/285	Flavonoid (Flavonol)	Kaempferol-3-O-hexoside
6	6.84	265, 344	535/533	287/489, 285	Flavonoid (Flavonol)	Kaempferol-malonylglucoside
7	7.05	265, 345	535/533	535, 287/489, 285	Flavonoid (Flavonol)	Kaempferol-malonyl-glucoside isomer

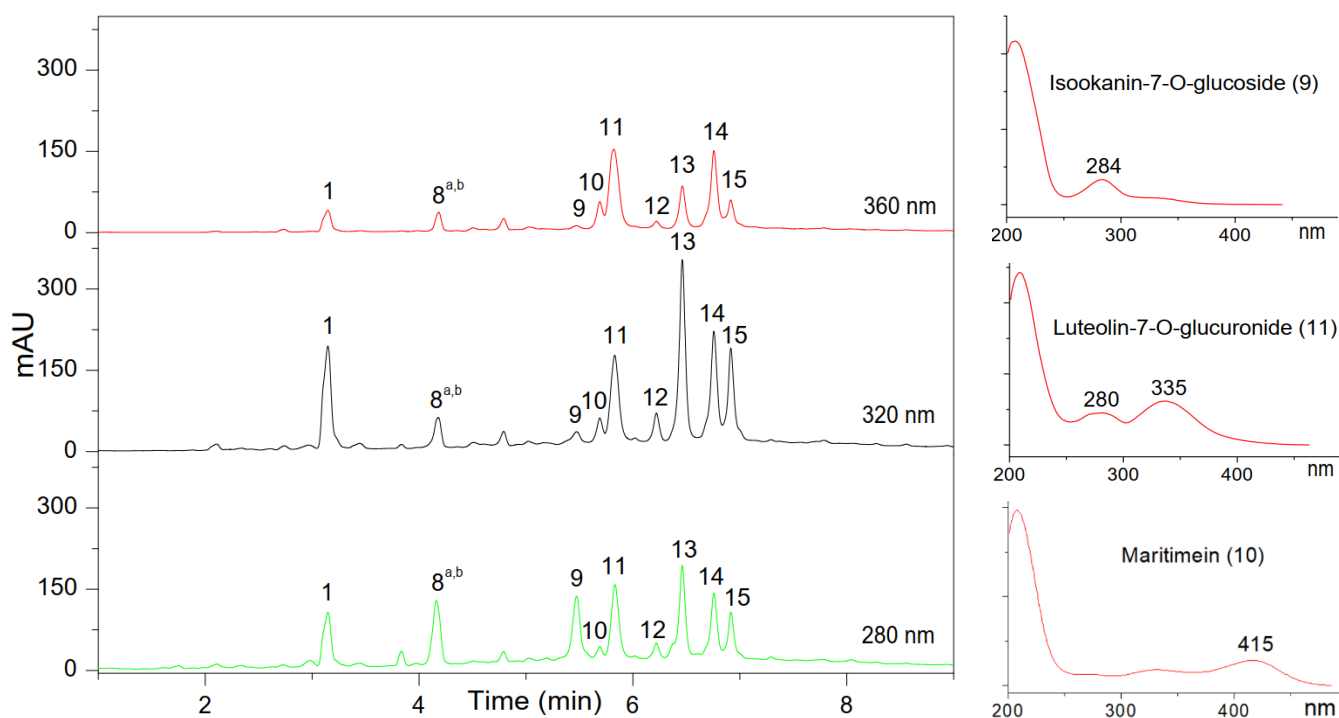


Figure III.4. UHPLC profiles of CA extracts obtained from infusion Bodum[®] extraction mode for grinded 'fine' CA material monitored at 273 nm. Typical UV spectra of major compounds are given on the right. UV spectrum of Chlorogenic acid (1) represents hydrocinnamic acid derivatives (12, 13, 15), UV spectrum of Isookanin-7-O-glucoside (9) represents Eriodicyol-7-O-glucoside (8^a), UV spectrum of Luteolin-7-O-glucuronide (11) represents 6,8-C-diglucosyl-apigenin (8^b), Apigenin-7-glucuronide (14) and UV spectrum of Maritmein (10). See Table III.5 for peak ID. Lot number: 559980. See Figure SI-III.5 for UHPLC profiles of other lots.

Table III.5. Putatively peak identification of the major compounds detected by UHPLC-DAD in the CA extracts. λ_{max} are the local maximum of absorbance on the UV spectrum. [M-H]⁻ column provides the m/z value of the precursor ion. Other ions column gives the m/z value of fragments detected in the MS spectra. Identification method using UV spectrum and ESI (-) spectrum. Lot number: 559980.

Peak	Retention time (min)	λ_{max} (nm)	[M-H] ⁻	Other ions in the spectrum	Family (Subclass)	Identified compound
1	3.24	219, 238, 325	353	191, 179	Phenolic acid (Hydroxycinnamic acid)	Chlorogenic acid
8 ^a	4.25	282	449	287, 151, 135	Flavonoid (Flavanone)	Eriodicyol-7-O-glucoside
8 ^b	4.25	267	593	473, 353, 191	Flavonoid (Flavone)	6,8-C,C-diglucosylapigenin
9	5.51	284	449	287, 269, 151, 135	Flavonoid (Flavanone)	Isookanin-7-O-glucoside (Flavanomarein)
10	5.71	415	447	285, 151, 135, 133	Flavonoid (Aurone)	Maritimetin-6-O-glucoside (Maritimein)
11	5.83	280, 335	461	285	Flavonoid (Flavone)	Luteolin-7-O-glucuronide
12	6.24	208, 323	515	353, 191, 179, 173, 135	Phenolic acid (Hydroxycinnamic acid)	di-caffeoylquinic acid
13	6.45	211, 327	515	353, 191, 179, 173, 135	Phenolic acid (Hydroxycinnamic acid)	di-caffeoylquinic acid isomer
14	6.74	207, 266, 334	445	269	Flavonoid (Flavone)	Apigenin-7-glucuronide
15	6.90	209, 326	515	353, 191, 179, 173, 135	Phenolic acid (Hydroxycinnamic acid)	di-caffeoylquinic acid isomer

III.2.5. Global composition and differences in chemical composition between plants achieved by ESI FT-ICR-MS in negative mode.

In this part, the discussion is essentially based on the grinded 'fine' samples obtained with the infusion extraction mode using the optimized infusion protocol (see section III.3.8). A detailed list of the samples studied by ESI FT-ICR-MS is given Table III.1. Mass spectra obtained for these samples are given in Figure SI-III.2 in duplicate (two independent

extractions), with the corresponding global composition description in heteroatom classes and van Krevelen diagram. The van Krevelen diagram is obtained by plotting the achieved raw formulae according to their H/C and O/C ratio. It helps to distinguish between different biochemical families such as lipids, polyphenols, amino acids, carbohydrates depending on the plot location [39,40].

Heteroatom class distributions and van Krevelen diagrams obtained for BC and CA show similar fingerprints (Figure SI-III.2), as observed in the previous study with HAW samples [4]. CHO molecular series is predominant irrespective of the sample type. Its representation on the van Krevelen indicates some carbohydrates, polyphenols, hydrolysable tannins, and lipids. Concerning the CHON species, they correspond to amino acids, small peptides, and likely amino sugars. All the (-) ESI FT-ICR mass spectra present intense peaks at m/z 191.0561 and m/z 353.0878 that could respectively correspond to the deprotonated form of Quinic acid and Chlorogenic acid or 5-O-Caffeoylquinic acid.

Nevertheless, as illustrated by the PCA (Figure III.5), there are some compositional differences between the samples depending on the plant. Therefore, a Venn diagram was performed to highlight differences in the chemical composition of the samples depending on the plant type (Figure III.6). For each plant, about 2500 hints were obtained among which about 1100 hints (25 % of all the features) are common to all 3 plants; about 350 hints are common to any group of two plants; and about 700 hints (about 15 % of all the features) are specific to each plant.

The van Krevelen diagrams of the features specific to BC samples evidences CHO species relative to carbohydrates, polyphenols, terpenoids, and fatty acid-like species. CHOCl components cover the same areas. CHON and CHONS components are also well represented with some carbohydrates, polyphenols, and possibly some amino sugars. The most intensively detected species in these samples are mainly flavonoids, diterpenes, and, to a lesser extent, fatty acids and carbohydrate (Table SI-III.1). The features specifically

observed in CA are mainly from CHON and CHO classes. Concerning the CHO species, the corresponding plots on the van Krevelen diagram suggest some polyphenols or flavonoid species (such as e.g. maritinein derivatives). Moreover, some fatty acid-like species, which likely possess some hydroxyl or carbonyl functions due to their higher O/C values, and terpenoid components are also observed. The most represented putative features are relative to terpenes, flavonoids, and other minor classes (see Table SI-III.1). For the HAW samples, several specific CHO species are highlighted, which are relative to carbohydrates and polyphenols according to the corresponding van Krevelen diagram. Within the CHON class, a few amino sugars are observed. Among the yielded putative compounds, of highest intensity, there are mainly some amino acids or derivatives, and some flavonoids (Table SI-III.1). Eventually, the van Krevelen diagram achieved for all common species shows a chemical fingerprint, very close to those achieved in the different samples (Figure SI-III.2) and in our previous work [4]. This chemical composition is dominated by CHO series, followed by the CHON one. The CHO compounds refer to lipids, carbohydrates, hydrosoluble tannins, and polyphenols. Regarding the CHON class, it involves amino acids, small peptides, and amino sugar-like species. The putative species attributed via MasSTRIX are gathered in Table SI-III.1.

Additionally, the contributions specific to each plant sample were compared to formula of compounds identified in HAW for putative assignments [30,41,42]. Thus, no hints were obtained for BC sample whereas it seems that Oleanolic or Ursolic acid is specifically observed in CA samples (Table SI-III.1). For the HAW samples, two putative amino acids and three organic acids matched. However, 38 hints were found in the common features, which correspond to carbohydrates and organic acids but mostly to flavonoid-based components. Apigenin-7-glucuronide was putatively found in both HAW and CA samples whereas formula of Quercetin-3-6-malonyl-glucoside was observed in BC and HAW samples.

This approach ensured highlighting specific compounds in regards to their plant origin. Even if 25 % of the features are commonly observed in all the samples, some differences

can be evidenced. The focus was put on these compositional variations by means of graphical representations and putative assignments of the corresponding features.

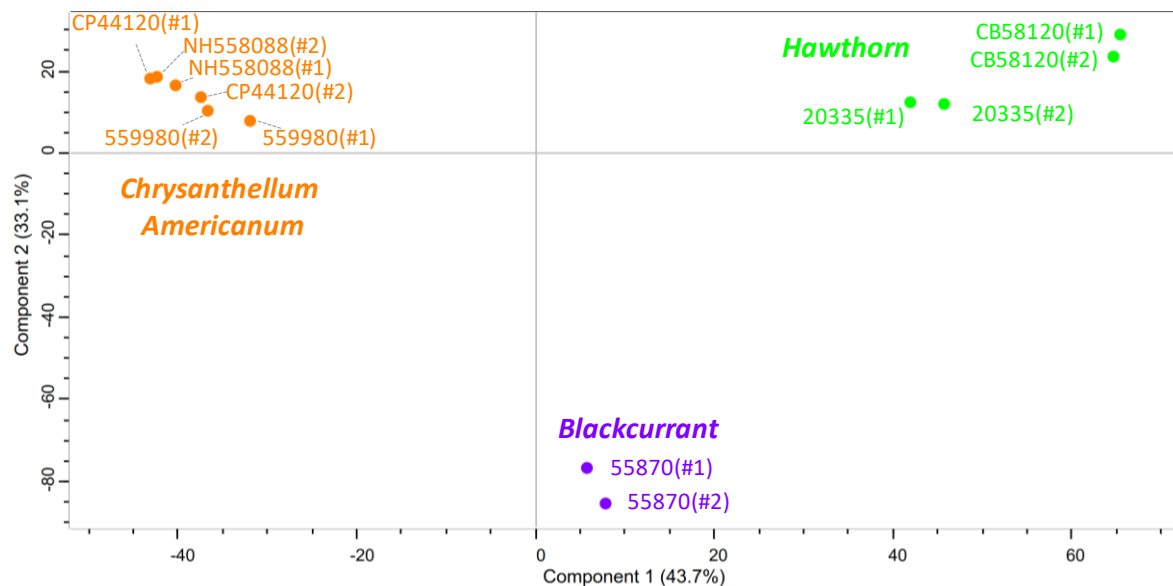


Figure III.5. Principal component analysis (PCA) score plot of all mass features from extracts measured by (-) ESI FT-ICR MS obtained from HAW (green, lot numbers: CB58120 and APC27031904), BC (purple, lot number: 55870), and CA (orange, lot numbers: 559980, CP44120 and NH558088).

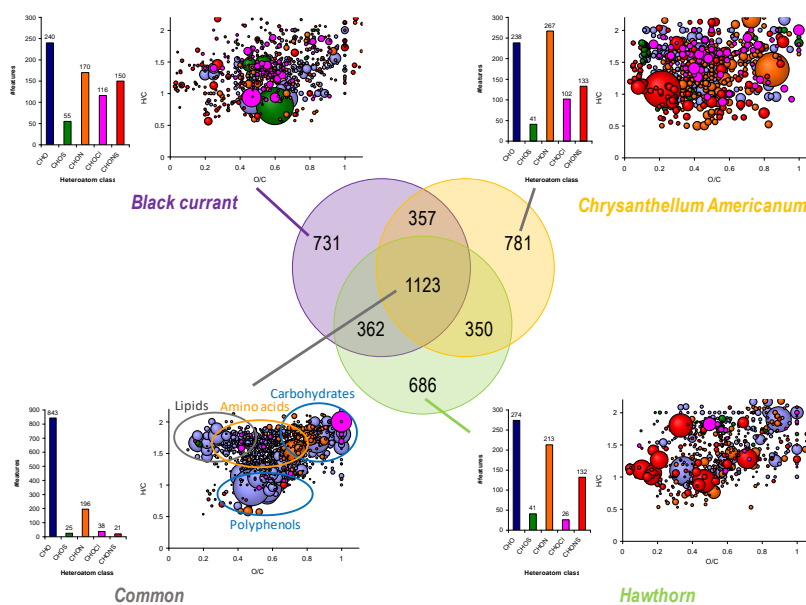


Figure III.6. Venn diagram achieved from BC, CA, and HAW samples analyzed in ESI(-) FT-ICR MS. Heteroatom class distribution and van Krevelen diagram concern features specifically and commonly observed in all samples. Lot numbers: see Figure III.5.

III.2.6. Enzymatic activities

The effects of the extracts obtained from three different plants on hyaluronidase and Angiotensin-converting enzyme (ACE) activities were assessed. For all assays, the extract concentration in the reaction medium was 1 mg mL⁻¹. All plant extracts were obtained by the optimized easy-to-use infusion protocol (see Section III.3.8). Briefly, 2.5 g of dry plant was infused for 3 min in 250 mL boiling water before lyophilization. The obtained dry extracts were denoted as follow: (i) HAW extracts (Lot n°20335, grinded 1 mm, #1/#2 and Lot n°CB58120, grinded 'fine', #1/#2), (ii) BC extracts (Lot n°55870, grinded 'fine', #1/#2) and (iii) CA extracts (Lot n°559980, grinded 'fine', #1/#2).

III.2.6.1. Hyaluronidase CE inhibition assay

Hyaluronidase is well known for its involvement in numerous physiological and pathological processes such as skin aging, cancer progression and inflammation by catalyzing the hydrolysis of hyaluronic acid (HA), a major compound of extracellular matrix [43,44]. The modulation of hyaluronidase activity in the presence of different plant extracts at a final concentration of 1 mg mL⁻¹ was investigated by following the formation of the tetrasaccharide product and the percentage of inhibition was calculated according to Eq. 2 (see Section III.3.15). Results obtained with different extracts are reported in Figure III.7, where, for each extract, the average inhibition percentage of three enzymatic reactions ($n=3$) was calculated as well as their standard deviation. The absence of any interferents with the tetrasaccharide peak was also verified by injecting the raw aqueous extracts at 1 mg mL⁻¹ using the same Capillary Electrophoresis (CE) method described in Section III.3.15.1.

Results reported in Figure III.7 showed an overall reduction in hyaluronidase activity by all of the tested extracts obtained from the three different plants. An interesting inhibition, superior to 90 %, was reported for HAW extracts similar to the inhibition observed with the referenced hyaluronidase's inhibitor, epigallocatechine gallate (EGCG), at the same concentration in the reaction media. The inhibition by BC and CA extracts was lower; 64 %

and 60 % respectively. These differences in inhibition effect towards hyaluronidase are mainly correlated to the subclasses of polyphenols and flavonoids identified in each extract [45-47] and not to the sum of TPC and TFC extracted mass. This is clearly observed with BC leaves which demonstrated the lowest hyaluronidase inhibition despite having the highest TPC and TFC amongst all extracts. In fact, BC extracts are rich in Quercetin and Kaempferol isomers (as reported in Table III.4) both of which are flavonoids known to have moderate to low inhibitory effect towards hyaluronidase. Indeed, Gonzalez-Pena et al. [46] reported only 27 % hyaluronidase inhibition by Quercetin at 0.23 mg mL^{-1} ($750 \text{ }\mu\text{M}$) and Mohamed et al. [45] showed only 8 % hyaluronidase inhibition by Kaempferol at 1 mg mL^{-1} .

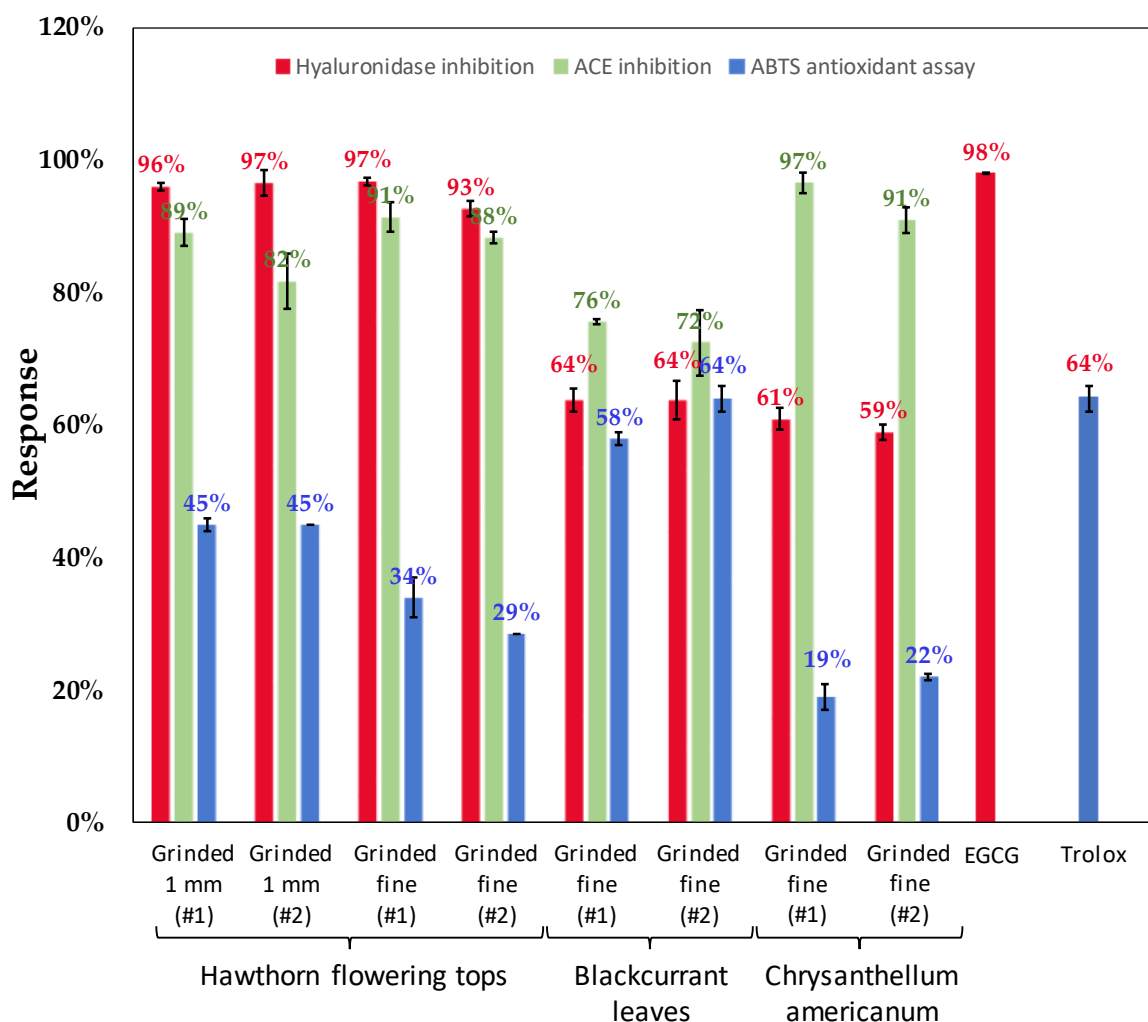


Figure III.7. Percentage of inhibition of tested enzymes: hyaluronidase (in red) and ACE (in green) and ABTS antioxidant assay (in blue) in the presence of different extracts obtained from HAW, BC and CA. For the enzyme's inhibition assays, the plant extracts were screened at 1 mg mL^{-1} and the inhibition percentages of hyaluronidase and ACE were calculated according to Eq. 2 and Eq. 3,

respectively. The antioxidant capacities of the plant extracts were determined at 0.01 mg mL^{-1} and calculated according to Eq. 4. The absorbance of the multi-well plates was read twice for both ACE inhibition and ABTS antioxidant capacity assays and the average of obtained results were plotted. All assays were carried out in triplicates ($n=3$). Plant extracts were obtained from HAW (#1/#2, grinded 1 mm, lot n°20335 and #1/#2, grinded 'fine', lot n°CB58120), BC (#1/#2, grinded 'fine', lot n°55870) and CA (#1/#2, grinded 'fine', lot n°559980). EGCG, hyaluronidase referenced inhibitor, showed 98 % hyaluronidase inhibition at 1 mg mL^{-1} , and Trolox, an antioxidant reference, demonstrated 64 % antioxidant capacity at 0.01 mg mL^{-1} . Both references were used to validate the methods (more details in Table SI-III.2).

III.2.6.2. ACE inhibition assay

Angiotensin-converting enzyme (ACE) catalyzes the cleavage of angiotensin I to angiotensin II resulting in elevation of blood pressure through vasoconstriction [48,49]. ACE inhibitors block the production of angiotensin II and thus help in the regulation of blood pressure [50]. Certain naturally occurring molecules such as flavonoid and non-flavonoid polyphenols are abundant in plants and have been described for their ACE inhibitory potential [51].

The results reported in Figure III.7 demonstrated an interesting ACE inhibitory trend for all of the tested plant extracts. The percentage of ACE inhibition ranged roughly between 72 % and 97 %. HAW extracts demonstrated strong ACE inhibition (81-92 %). HAW extracts have been described as an effective treatment of mild hypertension [52] and moderate heart failure [53-59]. The extracts of leaves and flowers (flowering tops) were shown to induce cardiovascular effects such as vasodilation and endothelial protection [52,60]. The plant lot did not appear to influence ACE inhibition as both extracts of flowering tops demonstrated a similar range of inhibition. BC extracts demonstrated the lowest percentage of ACE inhibition (72-76 %) compared to HAW and CA extracts. Indeed, blackcurrant leaves have been shown to induce blood vessel dilation through the activation of endothelial nitric oxide synthase and not through the inhibition of ACE [61]. CA extracts demonstrated the highest ACE inhibition (91-97 %). To the best of our knowledge, CA extracts have not been previously described as ACE inhibitors. *Chrysanthellum* plants,

however, have been reported to have a hypotensive effect through their intravenous use [23,62].

The baseline inhibition of ACE by all three plant extracts may be linked to the presence of certain compounds in all of these extracts. For example, Chlorogenic acid demonstrated 88 % ACE inhibition at 0.167 mg mL^{-1} ($\text{IC}_{50} = 310.5 \text{ }\mu\text{M}$) [63] and Galloylglucoses 1,2,3,6-Tetra-O-galloyl- β -D-glucose and 1,2,3,4,6-Penta-O-galloyl-B-D-glucose demonstrated 42 % ACE inhibition at 0.1 mg mL^{-1} ($\text{IC}_{50} = 101 \text{ }\mu\text{M}$) and 49 % ACE inhibition at 0.075 mg mL^{-1} ($\text{IC}_{50} = 73 \text{ }\mu\text{M}$), respectively) [64]. The differential inhibition between CA (~ 95 %) and BC (~ 75 %) may be explained by the specific phytochemical contents determined by ESI(-)FT-ICR-MS of each individual extract. Comparing the heteroatom class distribution and van Krevelen diagrams of all three extracts (Figure III.6) shows that blackcurrant leaves had the lowest number and levels of CHON features (170) compared to CA which had the highest number of these features (267) as well as the largest bubble sizes. The sizes of the bubbles in the van Krevelen diagrams represent peak intensities of species detected by ESI(-) FT-ICR. The levels and numbers of amino acids, small peptides, amino sugar like-species (CHON) in CA was the highest compared to the other two extracts. This may suggest that the ACE inhibition potential of the extracts is related to their quantity of CHON species.

III.2.7. ABTS antioxidant assay

Antioxidant activities of both HAW [65] and BC leaves [66] have been previously reported. As far as we know, this is the first report of CA antioxidant capacity. The extracts' antioxidant capacities were assessed based on their ABTS radical scavenging abilities (see section III.3.16) and Trolox a known antioxidant compound was used as positive control and results were compared to its antioxidant capacity. As for hyaluronidase and ACE assays, the antioxidant capacities of extracts and Trolox were investigated first at 1 mg mL^{-1} for comparison. At this concentration, all of the tested extracts demonstrated high antioxidant capacities equal to Trolox's (~100 %). For this, new assays were conducted at two lower concentrations 0.1 and 0.01 mg mL^{-1} . At 0.1 mg mL^{-1} , HAW extracts (lot n°20335, #1/#2 and

lot n°CB58120, #1/#2) and BC extracts (lot n°55870, #1/#2, grinded 'fine') showed high antioxidant capacities equal to Trolox's once again (95 %), while CA extracts (lot n°559980, #1/#2) had only 64 % of antioxidant capacity.

At 0.01 mg mL⁻¹, obtained results are reported in Figure III.7 where Trolox showed an antioxidant capacity equal to 64 % at this low concentration. BC extracts demonstrated the highest antioxidant capacities (58 % and 64 %, respectively) compared to the other extracts. More interestingly, the antioxidant activity of BC turns out to be equal to the antioxidant capacity of the referenced compound Trolox. On the other hand, the lowest antioxidant capacity was observed again with the extracts of CA (19 % and 22 %, respectively). Finally, HAW extracts demonstrated a range of antioxidant capacities between 28 % - 45 %. This difference in antioxidant capacities of the three plant extracts can be correlated to the TPC and TFC extracted contents. In fact, BC extracts had the highest antioxidant capacity and highest contents of TPC and TFC while CA extracts presented the lowest contents of TPC and TFC and thus the lowest antioxidant capacity.

III.3. Materials and Methods

III.3.1. Chemicals

Dry *Chrysanthellum americanum* (lot n° 559980, n° CP44120, n° 558088, origin Ivory Coast), dry blackcurrant leaves (*Ribes nigrum*, lot n° 55870, lot n° 558024, origin Poland) and dry hawthorn flowering tops (*Crataegus oxyacantha*, lot n°20335, lot n° CB58120, lot n° APC27031904, origin France) raw materials were purchased from France Herboristerie (Noidans-Lès-Vesoul, France). All reagents were of analytical grade and used as received without any further purification. Folin-Ciocalteu reagent, sodium carbonate (Na₂CO₃), aluminium chloride hexahydrate (AlCl₃.6H₂O), methanol (CH₃OH), hydrochloric acid (HCl), n-butanol (CH₃-(CH₂)₃-OH), ammonium iron(III) sulfate dodecahydrate (NH₄Fe(SO₄)₂.12 H₂O), gallic acid (GA), quercetin (Q), and cyanidin chloride (CY), ammonium acetate (CH₃COONH₄, purity ≥ 98 %), epigallocatechin gallate (EGCG, purity ≥ 95 %), hyaluronidase type I-S from bovine testes (BTH, 400-1000 units mg⁻¹ solid, CAS 37326-33-3, Sigma-Aldrich),

sodium acetate (CH_3COONa , purity $\geq 99\%$), sodium hydroxide (NaOH , purity $\geq 98\%$) oligohyaluronic acid (oligo-HA4 or tetrasaccharide (Tet), $\text{C}_{28}\text{H}_{44}\text{N}_2\text{O}_{23}$) and trolox (purity $\geq 98\%$) were purchased from Merck (Saint-Quentin Fallavier, France). Hyaluronic acid, sodium salt, *Streptococcus pyrogenes* (HA, CAS 9067-32-7 – Calbiochem) was purchased from Merck Millipore (Molsheim, France). ACE kit-WST was purchased from Dojindo Laboratories (Kumamoto, Japan). 2,2'-azino-bis-(3-ethylbenzathiazoline-6-sulfonic acid) (ABTS, purity = 98 %) reagent was purchased from ThermoFisher-Alfa Aesar (Kandel, Germany). Glacial acetic acid (CH_3COOH) and ammonia (NH_4OH , 28 %) were purchased from VWR International (Fontenay-sous-Bois, France). Syringes and hydrophilic polyvinylidenedifluoride (PVDF) Econo Syringe Filters, pore size 0.2 μm , were purchased from Agilent, USA. Ultrapure water was obtained using a MilliQ system from Millipore (Molsheim, France).

III.3.2. Grinded plant, density and granulometry

Dry plants were grinded using three different grinders. 'Coarse' and 'fine' plant materials were obtained by grinding 2 g of raw material using Delonghi (Model KG79, Treviso, Italy) grinder at the position named 'coarse' and 'fine', respectively. 'Ultrafine 10 s' or 'Ultrafine 30 s' plant materials were obtained by grinding 2 g of raw material using the Bosch grinder (Model MKM6003, Munich, Germany) at different manual shaking times (10 s and 30 s, respectively). '1 mm' plant materials were obtained by grinding required amount of raw material on a laboratory Ika grinder (Ika-Werke GmbH, Model MF10 basic, Staufen, Germany). The density of each plant material was simply determined by measuring the volume occupied by 2 g plant material in a 10 mL (or 25 mL) graduated test tube ($n=3$ determinations). Distribution in size of each plant material was determined by dry laser Malvern granulometer (Malvern Panalytical, Royston, United Kingdom).

III.3.3. Infusion extraction

Infusion extraction was performed using a 500 mL three-necked flask equipped with an olive magnetic stirrer [4]. 2.5 g of dry plant were placed and 250 mL of boiled ultrapure

water were added. The mixture was stirred at 500 rpm. The decrease in temperature was measured upon time using temperature sensor (Ebro EBI20-IF, Ingolstadt, Germany). Two extraction times were investigated, namely 10 min and 30 min. After filtration of the plant residues using Whatman filter paper placed on a Büchner funnel and a vacuum pump (KNF Model N820FT.18, Freiburg, Germany), the extract was concentrated using a rotary evaporator (until 10 mL volume) and finally freeze-dried (Cryotec Model CRIOS-80, Saint-Gély-du-Fesc, France). Lyophilized dry extracts were stored at 4°C. Each extraction experiment was carried out in triplicate.

III.3.4. Maceration extraction

Maceration extraction was performed using a 500 mL three-necked flask equipped with an olive magnetic stirrer, an oil bath, and a heating magnetic stirrer with a digital thermostat (Fisher Scientific Model FB15002, Illkirch, France) [4]. 2.5 g of dry plant were placed and 250 mL of water were added. The temperature was set at 60°C. The mixture was stirred at 500 rpm. Two extraction times were investigated, namely 10 min and 30 min. After filtration, the extract was concentrated and finally freeze-dried (as described in section III.3.3). Each extraction experiment was carried out in triplicate.

III.3.5. Ultrasound-assisted extraction

Ultrasound-assisted (US) extraction was conducted with ultrasonic homogenizer (UIP 1000 hdT, 1kW, Hielscher Ultrasonics GmbH, Germany) [4]. Experiments have been performed in a double jacket reactor of 1 L volume connected with a mechanical stirrer (IKA RSC classic, Germany) and a temperature sensor. Temperature was maintained constant with a cooling system connected to the double-jacket reactor. 2.5 g of dry plant were placed in the double jacket reactor and 250 mL of water were added. The temperature was set at 60°C. The mixture was mechanically stirred at 250 rpm. Extraction time was 10 min. After filtration, the extract was concentrated and finally freeze-dried (see section III.3.3). Each extraction experiment was carried out in triplicate.

III.3.6. Microwave-assisted extraction

Microwave-assisted (MW) extraction was performed on a monomode Microwave apparatus using a closed-vessel system (NEOS-GR, Milestone Sarl, Italy) [4]. 2.5 g of dry plant were placed in a 500 mL flask containing 250 mL of water. The flask was then placed in the MW oven with a 300 W power. Under these conditions, the temperature reached 95°C in 10 minutes. No stirring was applied. Extraction time was 10 min. After filtration, the extracts were concentrated and finally freeze-dried (see section III.3.3). Each extraction experiment was carried out in triplicate.

III.3.7. Percolation extraction

Percolation extraction was performed using a coffee percolator KRUPS equipment (Model, city, Germany) [4]. 2.5 g of dry plant were placed in a filter and 250 mL of water were used. The temperature reached 100°C after a few seconds. No stirring was applied. Extraction time was 10 min. For that, the extracted solution obtained after 5 min percolation was passed again in the percolator for again 5 min. After filtration, the extract was concentrated and finally freeze-dried (see section III.3.3). Each extraction experiment was carried out in triplicate.

III.3.8. Optimized easy-to-use infusion extraction

2.5 g grinded material (see section III.3.2) were infused in 250 mL boiling water using 'French press' (Bistro model, Triengen, Switzerland) [4]. After addition of the boiling water, a good initial mixing of the plant in water was ensured by manually rotating the recipient (with grinded plant, magnetic stirring –which is generally not available at home- was however not required to get optimal extraction). After 10 min, the herbal tea solution was filtrated first with the Bodum® cover to remove the largest particles, then with Whatman filter paper to remove residual solid plant. Finally, the herbal tea solution was concentrated and freeze-dried to get dry extract.

III.3.9. Kinetic monitoring

The kinetic of extraction was monitored by UV absorbance at 198 nm (Perkin-Elmer Model Lambda 20, Wellesley, MA, USA) using UV quartz cells of 1 mL (Hellma GmbH, Müllheim, Germany). 100 μ L of solution were taken and added to 4 mL (or 8 mL if the absorbance values were above 1.7 AU) water. The resulting solution was shortly vortexed before UV measurement. The same volume of fresh water (100 μ L) was added in the reactor (three-necked flask) to keep constant the total volume. Zero absorbance value was set using 100 μ L water instead of herbal tea solution.

All the kinetic curves (absorbance $A(t)$ vs extraction time t) were fitted according to the following equation (see plain lines in Figure III.1A) using Excel solver:

$$A(t) = A_{\infty} - A_1 \exp\left(-\frac{t}{\tau_1}\right) - (A_{\infty} - A_1) \exp\left(-\frac{t}{\tau_2}\right) \quad (1)$$

where A_{∞} is the maximum absorbance at infinite extraction time, A_1 is a fitting parameter corresponding to an intermediate extraction plateau and τ_1 and τ_2 are two characteristic extraction times.

III.3.10. Total polyphenols content (TPC)

The total polyphenols content (TPC) in plant extracts was estimated using the Folin-Ciocalteu's reagent as described by Singleton & Rossi [67]. 100 μ L of a solution prepared by mixing dry plant extract (100 μ L of 20 mg mL⁻¹ in water) with 1 mL water, were added to 200 μ L Folin-Ciocalteu reagent and 2 mL water. After 3 min, 1 mL of 20 % sodium carbonate (20 g/100 mL water) was added. After vortex-mixing for 2 min, followed by incubation at room temperature and in darkness for 90 min, the resulting solution was centrifuged at 8000 rpm for 3 min (Sigma Model 302K, Osterode am Harz, Germany) and the absorbance at 760 nm was measured using the same equipment as in section III.3.9. Gallic acid (0-250 mg L⁻¹) was used for the standard calibration curve. The results were expressed as mg GA equivalent per gram of dry plant and calculated as mean value \pm one SD ($n=3$). Zero absorbance value was set using 100 μ L water instead of herbal tea solution.

III.3.11. Total flavonoids content (TFC)

The total flavonoids content (TFC) in hawthorn extracts was estimated by the aluminium chloride method according to Lamaison & Carnet [68]. 200 μL of plant extract solution (20 mg mL^{-1} in water) were firstly added to 200 μL water and 600 μL methanol. 200 μL of the resulting solution were then added to 800 μL methanol and 1 mL of 2 % $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ methanolic solution (2 g/100 mL in methanol). After vortex-mixing for 2 min, followed by incubation at room temperature and in darkness for 15 min, the absorbance at 430 nm was measured using the same equipment as in section III.3.9. Quercetin (0-35 mg L^{-1}) was used for the standard calibration curve. The results were expressed as mg of equivalent Quercetin per gram of dry plant, and calculated as mean value \pm one SD ($n=3$). Zero absorbance value was set using 200 μL water instead of herbal tea solution.

III.3.12. Total proanthocyanidin oligomers content (OPC)

The total proanthocyanidin oligomers content (OPC) in hawthorn extracts was estimated using the HCl/n-butanol assay of Porter *et al.* [69]. 200 μL of plant extract solution (20 mg mL^{-1} in water) were firstly added to 200 μL water and 600 μL methanol. 250 μL of the resulting solution were then added to 3 mL of a 95 % solution of n-butanol/HCl (95:5 v/v) and 100 μL of a 2 % solution of $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (2 g/100 mL in HCl 2M). After vortex-mixing for 2 min, followed by incubation at 95°C in an oil bath for 40 min and cooling at room temperature, the absorbance at 550 nm was measured using the same equipment as in section III.3.9. Cyanidin chloride (0-30 mg L^{-1}) was used for the standard calibration curve. The results were expressed as mg CY equivalent per gram of dry plant, and calculated as mean value \pm SD ($n=3$). Zero absorbance value was set using 200 μL water instead of herbal tea solution.

III.3.13. UHPLC and UHPLC-ESI-MS analysis

20 mg hawthorn dry extract was dissolved in 1 mL MilliQ water, and finally strongly vortexed for 2 min. The resulting solution was diluted 5 times with MilliQ water, vortexed again for 2 min, and analyzed by UHPLC-DAD and UHPLC-ESI-MS.

The UHPLC-DAD system consisted of a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS equipped with a WPS-3000TBRS auto sampler, and a TCC-3000RS column compartment set at 35°C (ThermoFisher Scientific, Waltham MA, USA). The system was operated using Chromeleon 7 software. A Kinetex C18 100A 100×2.1 mm, 2.6 µm column combined with a security guard ultra-cartridge was used (Phenomenex Inc., Torrance CA, USA). A binary solvent system was used, consisting of water/formic acid (1 ‰, v/v) as solvent A and acetonitrile/formic acid (1 ‰, v/v) as solvent B. The gradient program started with 5 % B, then B was increased to 100 % in 30 min with a convex increase (curve 5 in Chromeleon 7). The flow rate of the mobile phase was 0.4 mL.min⁻¹, and the injection volume was 20 µL. The peaks were monitored at 280, 320 nm and 360 nm. The UV-Vis spectra of the different compounds were recorded between 200 and 500 nm using the DAD.

UHPLC-ESI-MS analysis was performed using a Synapt G2-S (Waters Corp., Milford MA, USA) equipped with ESI operating in resolution mode. The UHPLC column, injection volume, flow rate and gradient program were the same as for UHPLC-DAD. Positive and negative ionization modes and fast DDA MS methods with automatic MSMS intensity-based switching parameters were used. The capillary voltage was set to 2.4 kV, the cone voltage was set to 30 V and the extractor voltage was set to 3 V. The source temperature was 140°C and the desolvation temperature was 450°C. MS spectra were obtained by scanning ions between $m/z = 50$ and $m/z = 1200$. The system was operated using MassLynx 4.1 software.

III.3.14. (-)ESI FT-ICR-MS analysis

All the samples were analyzed at least in duplicate. The dried extract was dissolved into 2 mL ultrapure water in glass vial and put for 2 min in an ultrasonic bath at room temperature. The aqueous solutions obtained for the different samples were recovered and transferred in 2.5 mL vials for 2 min centrifugation at 14000 rpm. The different solutions were then diluted 200 times in methanol for direct injection in the mass spectrometer.

Sample analysis was performed with a 12 T FT-ICR mass spectrometer Solarix (Bruker Daltonics) and the parameters were optimized via software FTMS-Control V2.2.0 (Bruker Daltonics). Prior acquisition, the mass spectrometer was externally calibrated with arginine clusters (10 mg L^{-1} in methanol). The methanolic solutions were infused with a flow rate of $2 \text{ } \mu\text{L min}^{-1}$ in the ESI source (Apollo II, Bruker Daltonics) used in negative-ion mode with a capillary voltage set at 3.6 kV. The temperature and the flow rate of the drying gas were kept at $180 \text{ }^\circ\text{C}$ and 4 L min^{-1} , respectively, and the pressure of the nebulizer gas was 2.2 bar. Mass spectra result from the accumulation of 300 scans over a m/z 122-100 range and with a 4 megaword time-domain.

The achieved mass spectra were processed in Data Analysis 5.0 (Bruker Daltonics). An internal calibration, with a list of well-known $\text{C}_x\text{H}_y\text{O}_z$ (fatty acids and sugars) anions, was performed with mass accuracy values lower than 500 ppb. Peak lists were generated at signal-to-noise ratio ≥ 4 and exported. Algorithm developed by Kanawati et al. was applied to remove signals related to satellite and magnetron peaks [70]. As the samples were analyzed in replicates, only m/z features observed at least 60 % were conserved and aligned in a matrix with a 0.5 ppm tolerance. The achieved matrix was processed for assignment by computing the average m/z values as previously reported with an annotation tolerance of 0.5 ppm [71]. Eventually, CHO, CHOS, CHON, CHONS, and CHOCl molecular series were achieved.

Perseus software was used to perform Principal Component Analysis (PCA). The chemical composition description yielded for the different samples was detailed by means of heteroatom class distribution and van Krevelen diagrams representing the hydrogen-to-carbon and oxygen-to-carbon atomic ratios. According to the location on the van Krevelen diagram, it is possible to distinguish various biochemical families such as lipids, amino acids, carbohydrates, and polyphenols [4,40]. Peaks were putatively assigned via MassTRIX with metabolite compounds observed in the *Arabidopsis thaliana* [72]. Deprotonated and

chlorinated adducts were allowed for putative compound assignment within a 0.5 ppm window.

III.3.15. Enzymatic activities assays

III.3.15.1. Hyaluronidase capillary electrophoresis inhibition assay

The effects of the different plant extracts were assessed towards hyaluronidase activity according to the protocol optimized in our laboratory using a CE/PDA based assay [73]. Briefly, 25 μL of IB was pre-incubated with 10 μL of Hyaluronic acid (HA, 4 mg mL^{-1}) and 10 μL of plant extract (5 mg mL^{-1}) for 10 min at 37°C. Then, 5 μL of enzyme were added to the reaction mixture so that the final concentration of the enzyme in the mixture was 0.2 mg mL^{-1} . Reactions were incubated at 37 °C for 180 min, and then stopped by increasing the temperature to 90 °C using a water bath for 10 min [73,74]. Results were compared to those obtained with EGCG, a referenced inhibitor of hyaluronidase at the same concentration of 1 mg mL^{-1} in the final reaction mixture [74,75].

Blank assays where HA hydrolysis occurred normally in absence of plant extracts have been carried out and stopped using the same protocol described above. More precisely, 35 μL of incubation buffer solution were pre-incubated with an appropriate volume of HA (4 mg mL^{-1}) for 10 minutes at 37 °C and then 5 μL of enzyme solution was added to the reaction mixture. All reactions were carried out in triplicates.

The reaction mixtures and aqueous raw extracts were then analyzed on a PA800+ CE apparatus equipped with a photodiode array detector (Brea, CA, USA). The control of CE was performed using the Beckman 32 karat software (Beckman Coulter, USA). Uncoated fused-silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) were used with a total length of 57 cm (47 cm effective length) and 50 μm inner diameter. The detection wavelength was set at 200 nm (bandwidth 10 nm). Between runs, the capillary was flushed with NaOH (5 min), water (0.5 min) and background electrolyte (BGE) (3 min) to ensure a good cleaning of the inner capillary surface. All rinse cycles were carried out at

50 psi. Hydrodynamic injections were performed from the anodic side of the capillary at 1.5 psi for 5 sec. The separation was performed in normal polarity mode at +15 kV separation voltage. The corrected peak area (CPA), which is the ratio of area to the migration time, was used as a reliable mean for the quantification of the peak of tetrasaccharide, the final product of HA hydrolysis. It was followed to assess the enzyme's activity in the presence of plant extracts and compared to reactions carried out in absence of these extracts. For all tested extracts, the percentage of inhibition was calculated according to Eq. 2.

$$\% I = \left(1 - \frac{A_x}{A_0}\right) \times 100 \quad (2)$$

where A_x is the CPA of the tetrasaccharide product formed in the presence of plant extract and A_0 is the CPA of the tetrasaccharide product formed in the absence of extract.

Buffers and stock solutions for hyaluronidase CE assay: The incubation buffer (IB) was prepared by dissolving the appropriate amount of sodium acetate in deionized H₂O to end up with a 2 mM solution whose pH was adjusted to 4.3 with 1 M glacial acetic acid. The BGE was prepared by dissolving the appropriate amount of ammonium acetate in deionized H₂O to end up with a 50 mM solution whose pH was adjusted to 8.9 with 1 M ammonia. All buffers were filtered using hydrophilic polyvinylidenedifluoride (PVDF) syringe filters before use. Stock solutions of BTH, HA, EGCG and tetrasaccharide were prepared in the IB at a concentration of 10 mg mL⁻¹ and diluted to the appropriate concentrations in the same buffer. The hyaluronidase stock solution was prepared at 10 mg mL⁻¹ in IB and then aliquots of 2 mg mL⁻¹ were stored at -20°C. Extract stock solutions were prepared at 5 mg mL⁻¹ in deionized H₂O, filtered using hydrophilic PVDF syringe filters and stored at + 4°C. All buffer solutions were prepared daily in deionized H₂O and stored at + 4°C.

III.3.15.2. Angiotensin-converting enzyme (ACE) inhibition assay

The effects of the plant extracts on ACE activity were investigated using an ACE-kit WST. Plant extracts were prepared in deionised H₂O at 13 mg mL⁻¹ and then centrifuged at 2000

x g for 10 min at 25 °C. The supernatant was then recovered and stored at -20 °C before use. The concentration of the extracts was 1 mg mL⁻¹ in the reaction media.

The different reagents needed to run the ACE inhibition assay were prepared as instructed in the kit's protocol. Briefly, the enzyme working solution was prepared by dissolving the contents of the vial labelled enzyme B with 2 ml deionised H₂O and then adding 1.5 ml of enzyme B solution to the vial labelled enzyme A. The indicator solution was prepared by dissolving the contents of the vials labelled enzyme C and coenzyme in 3 ml deionised H₂O each and then adding 2.8 ml of each of the solutions to the vial labelled indicator solution. All solutions were stored at -20 °C. The ACE inhibition assay was carried out in 96-well microtiter plates. To screen the inhibitory effect of each extract, 20 µL of the sample solution, 20 µL of the 3-Hydroxybutylyl-Gly-Gly-Gly (3HH-GGG) substrate buffer solution and 20 µL of the enzyme solution were added to the designated wells. The positive control consisted of substituting the 20 µL of the extracted sample solution with deionized H₂O. The reagent blank consisted of substituting the 20 µL of the sample and the enzyme solutions with 40 µL of deionized H₂O. Since the sample solutions were colored, sample blanks were carried out containing only 20 µL of the sample solution and 240 µL of deionized H₂O. The plate was then incubated at 37 °C for 1 hour in a thermostated oven (Heareus, Hanau, Germany) before adding 200 µL of the indicator solution to each well except those wells with sample blanks making the final volume of each well 260 µL. The plate was then incubated for 10 min at room temperature before reading the mixtures' absorbance at 450 nm using a Thermo scientific Multiskan GO UV/Vis microplate spectrophotometer (Thermofisher scientific, MA, USA). ACE inhibition assays were performed in triplicates (*n*=3) and plate readings in duplicates (*n*=2). Percentage of inhibition of ACE by the plant extracts was calculated according to Eq. 3.

$$\% \text{ ACE inhibition} = \frac{A_{\text{blank1}} - (A_{\text{sample}} - A_{\text{blank3}})}{(A_{\text{blank1}} - A_{\text{blank2}})} \times 100 \quad (3)$$

where A_{blank1} is the absorption of the positive control without any extracts, A_{blank2} is the absorption of the reagent blank without any extracts nor enzyme, A_{blank3} is the absorption of the sample blank and A_{sample} is the absorption of the reaction modulated by the plant extracts.

III.3.16. ABTS Antioxidant Assay

The antioxidant capacity of the extracts was investigated using the method described in our laboratory by Messaili. S. et al [75]. Briefly, 7 mM of ABTS and 2.45 potassium persulfate were mixed and agitated in the dark for 16 hr at room temperature to form the blue-green ABTS radical solution. The ABTS radical solution was then diluted with ethanol/water (25/75) at a 1:12.5 volume ratio. To carry out the antioxidant assays, 190 μL of the ABTS diluted solution was mixed with 10 μL of the extract or positive control solution in a 96 well-microtiter plate. Plant extracts were prepared in deionized H_2O and their antioxidant capacities determined at 1, 0.1 and 0.01 mg mL^{-1} . Similarly, Trolox was prepared at 1, 0.1 and 0.01 mg mL^{-1} in pure ethanol and used as a positive control. The absorbance of the plate was recorded at 734 nm after 30 min of incubation in the dark at room temperature. The antioxidant assays were performed in triplicate ($n=3$) and plate readings in duplicates ($n=2$). The antioxidant activities of the different extracts and the positive control were assessed based on their ability to induce decolorization of the ABTS radical solution by electron transfer. This was manifested by a reduction of the absorbance compared to the absorbance of the ABTS radical solution. The percentage reduction in absorbance is calculated according to Eq. 4.

$$\% \text{ reduction of absorbance} = \left(1 - \frac{A_{sample}}{A_{ABTS}}\right) \times 100 \quad (4)$$

where A_{sample} is the absorption of the mixture of the ABTS radical and extract samples or trolox and A_{ABTS} is the absorption of the ABTS radical solution only.

III.4. Conclusions

In this work, we demonstrated that for the three plants considered (i.e. CA, BC and HAW), the extraction by infusion was an efficient and the easiest way to extract the water-soluble components, in less than 3 min provided that the plant was grinded. This easy-to-

use-at-home protocol is based on the grinding of 2.5 g of raw dry plant, the infusion for 3 min with 250 mL of boiling water in a French-press coffee maker. Using this protocol, the global extraction yields are optimized and repeatable (25.9 % for CA; 28.6 % for BC and 21-22 % for HAW, expressed as mass proportion of the extracted compounds relative to the dry plant). 10 min cooling is required before consumption to reach drinkable temperature (60°C); but the process can be easily speed-up down to 4 min by adding one small ice cube to the preparation.

About the chemical composition, it is worth noting that BC extracts contain much more phenolic compounds than the two other plants, while HAW extracts contain much more proanthocyanidin oligomers than the two other plants. CA and BC extracts contain similar amounts of flavonoids. UHPLC revealed that the major compounds detected in UV are flavonol in BC; hydrocinnamic acid derivatives, flavone flavanone and aurone in CA; flavanol, flavonol and flavone in HAW. The main compounds detected in UHPLC were identified by UHPLC-ESI-MS. UHPLC-ESI-MS profiles revealed the presence of Flavanomarein and Martitimein derivatives in CA extracts, while FT-ICR MS revealed the specific presence of Oleanolic or Ursolic acid. In BC, quercetin and kaempferol derivatives were mainly identified. Vitexin-2-O-rhamnoside, hyperoside and isoquercetin were the main components in HAW. In FT-ICR MS, about 2500 hints were obtained for each plant, among which about 1100 hints (25 % of all the features) are common to all 3 plants; about 350 hints are common to any group of two plants; and about 700 hints (about 15 % of all the features) are specific to each plant.

Regarding their enzymatic and antioxidant activities, an interesting hyaluronidase inhibition, superior to 90 %, was reported for HAW extracts (at 1 g L⁻¹) similar to the inhibition observed with the referenced hyaluronidase inhibitor. The inhibition by BC and CA extracts was lower; 64 % and 60 %, respectively. As for the anti-hypertensive activity, CA extracts demonstrated the highest ACE inhibition (91-97 % at 1 g L⁻¹, similar to the compound of reference), followed by HAW extracts (81-92 %); while BC extracts gave the lowest percentage of ACE inhibition (72-76 %). About the antioxidant activity, BC extracts had the highest antioxidant capacity in correlation with the highest contents in TPC and TFC,

while CA extracts presented the lowest contents in TPC and TFC and the lowest antioxidant capacity.

This work highlights the complexity in the composition of these plant extracts, either in number of chemical components and in the chemical structure. It also confirms the interest of these medicinal plants toward a broad range of biological activities. In the future, it seems promising to investigate biological / pharmacological activities that are different from the primary therapeutic intention. Their use as daily nutrient in food toward the prevention of chronicle disease would also be of interest.

Supplementary Materials: The following are available online at www.mdpi.com/. Figure SI-III.1: Pictures of raw and grinded CA and BC materials of various granulometries. Figure SI-III.2: Mass spectra achieved by ESI(-) FT-ICR MS analysis of BC, CA and HAW with their corresponding heteroatom class distribution and van Krevelen diagram. Figure SI-III.3: Chemical structures of all compounds identified by UHPLC-ESI-MS. Figure SI-III.4: UHPLC profiles of two lots (55870 and NH558024) of BC infusion extracts obtained using Bodum® recipient. Figure SI-III.5: UHPLC profiles of three lots (559980, CB44120 and NH558088) of CA infusion extracts obtained using Bodum® recipient. Figure SI-III.6: Example of electropherograms showing the inhibitory effect of two extracts on hyaluronidase. Figure SI-III.7: Decreasing temperature profiles vs time for different volumes of water in the Bodum® container with or without added ice in the container. Table SI-III.1: Some species putatively assigned to m/z peaks observed specifically in BC, CA and HAW samples. Table SI-III.2: Inhibition assays of hyaluronidase and ACE as well as the ABTS antioxidant capacity assay of the different plant extracts.

Author Contributions: conceptualization, L.L. and H.C.; methodology, all authors; software, P.C.N., L.L., J.C.R., J.H., R.N., G.H.D.B., P.S.K., H.C.; validation, all authors; formal analysis, all authors; investigation, L.L., J.C.R., R.N., P.S.K., H.C.; resources, all authors; data curation, P.C.N., L.L., J.C.R., J.H., R.N., G.H.D.B., P.S.K., H.C.; writing—original draft preparation, P.C.N., L.L., R.N., H.C.; writing—review and editing, all authors; visualization, all authors; supervision, L.L. and H.C.; project administration, L.L. and H.C.; funding acquisition, P.C.N., L.L. and H.C.

Funding: P.C.N. PhD fellowship was funded by the Ministry of Education and Training of Vietnam and Campus France.

Acknowledgement: The authors thanks J. Rodriguez (ICGM) for the granulometry experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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SUPPORTING INFORMATION OF CHAPTER III

Foods, 2020, 9, 1978

Article 2: Water-based extraction of Bioactive Principles from Blackcurrant leaves and Chrysanthellum Americanum: a Comparative study with Hawthorn

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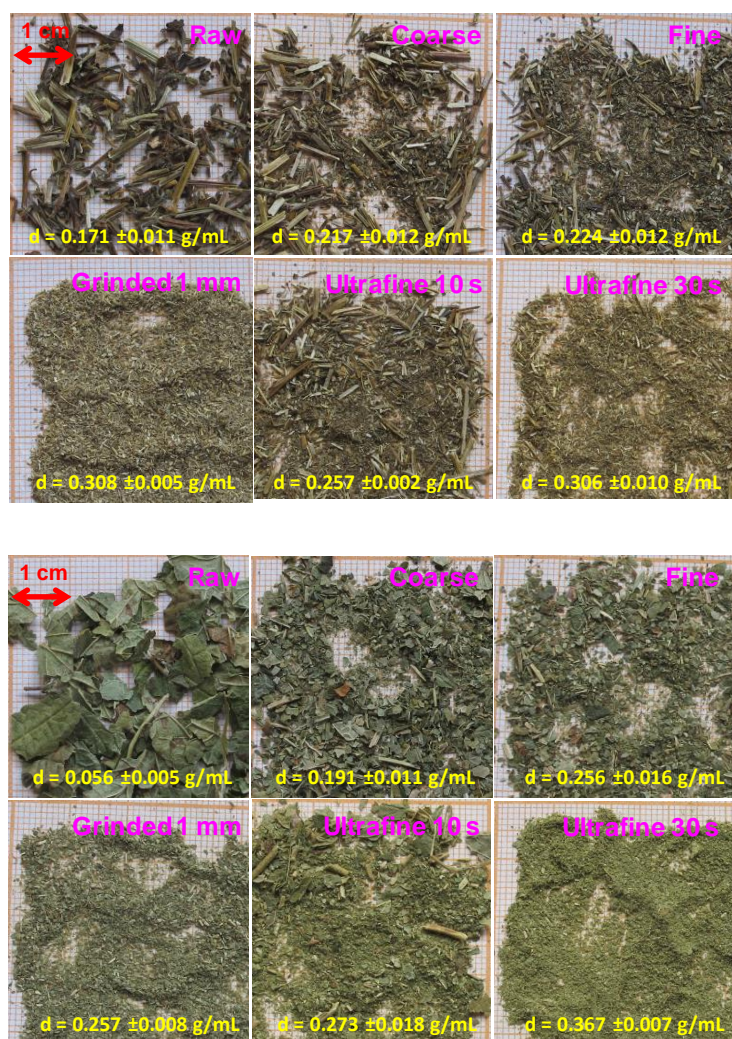
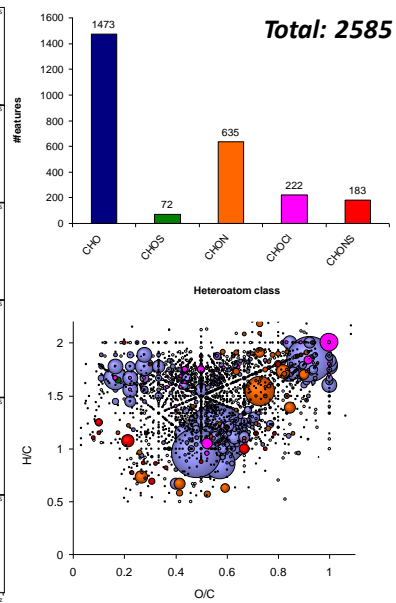
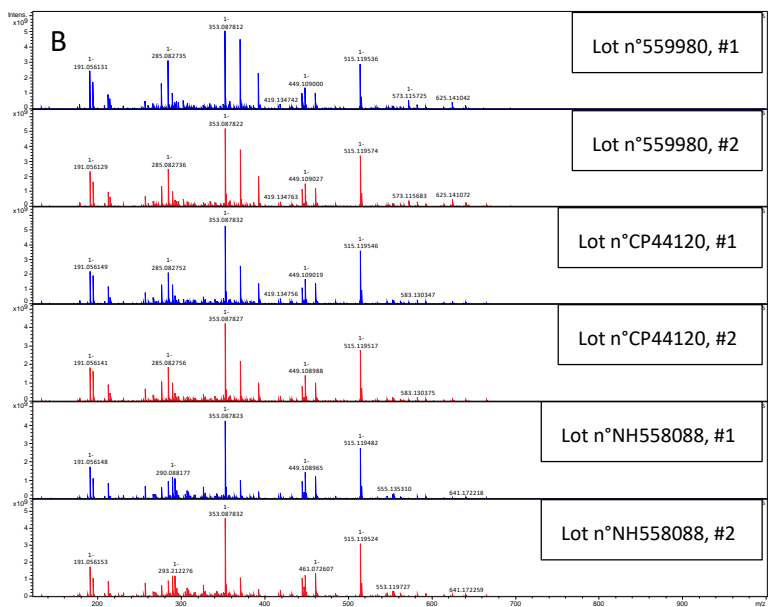
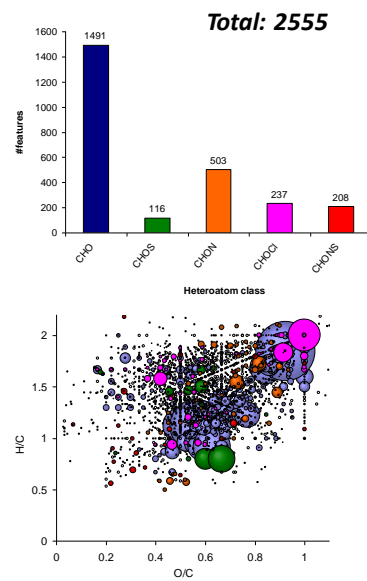
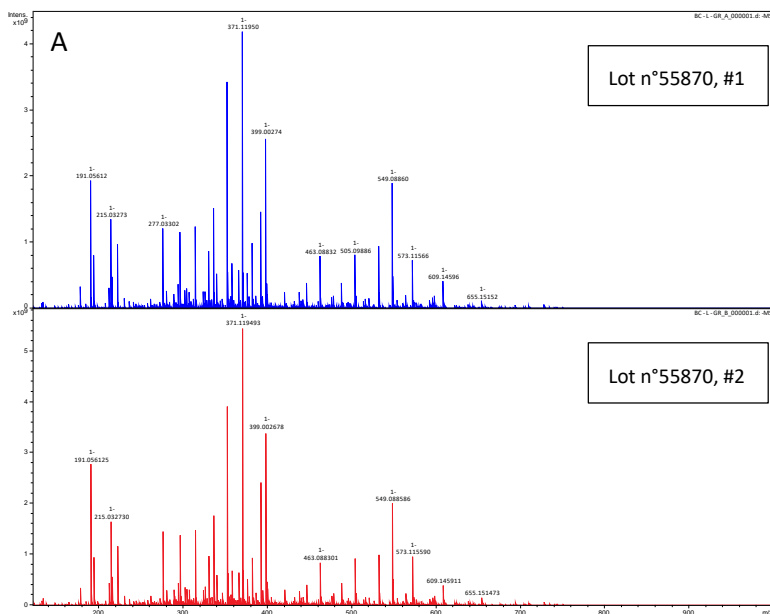


Figure SI-III.1. Pictures of raw and grinded CA (top) and BC (bottom) materials of various granulometries. One small square on the pictures is 1 mm large (see also the scale on the picture).



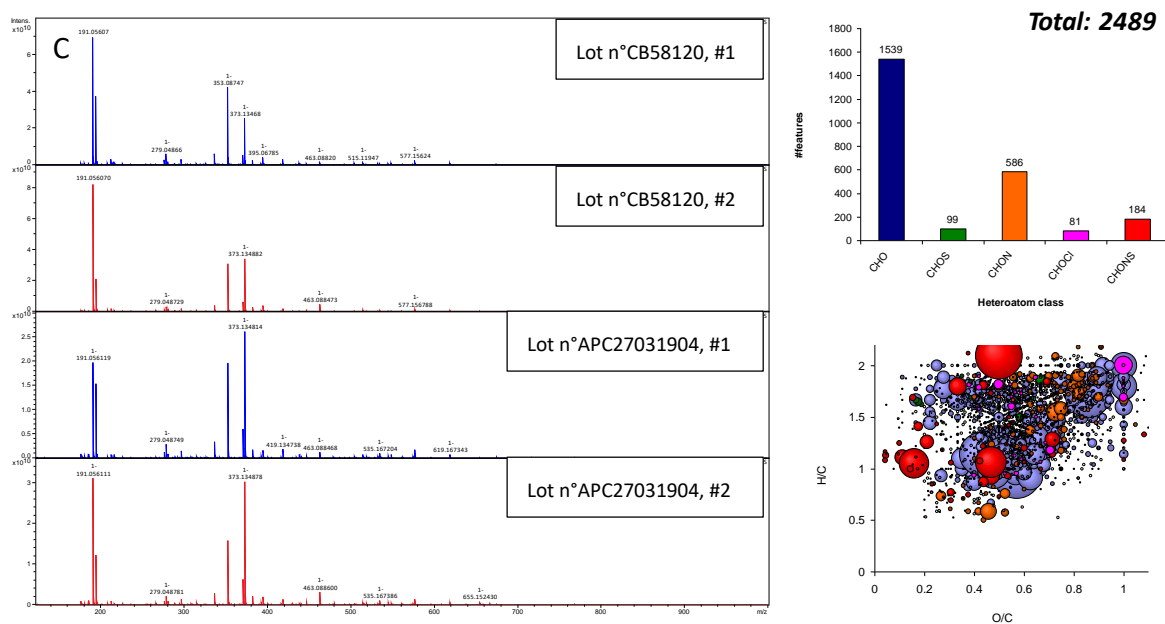
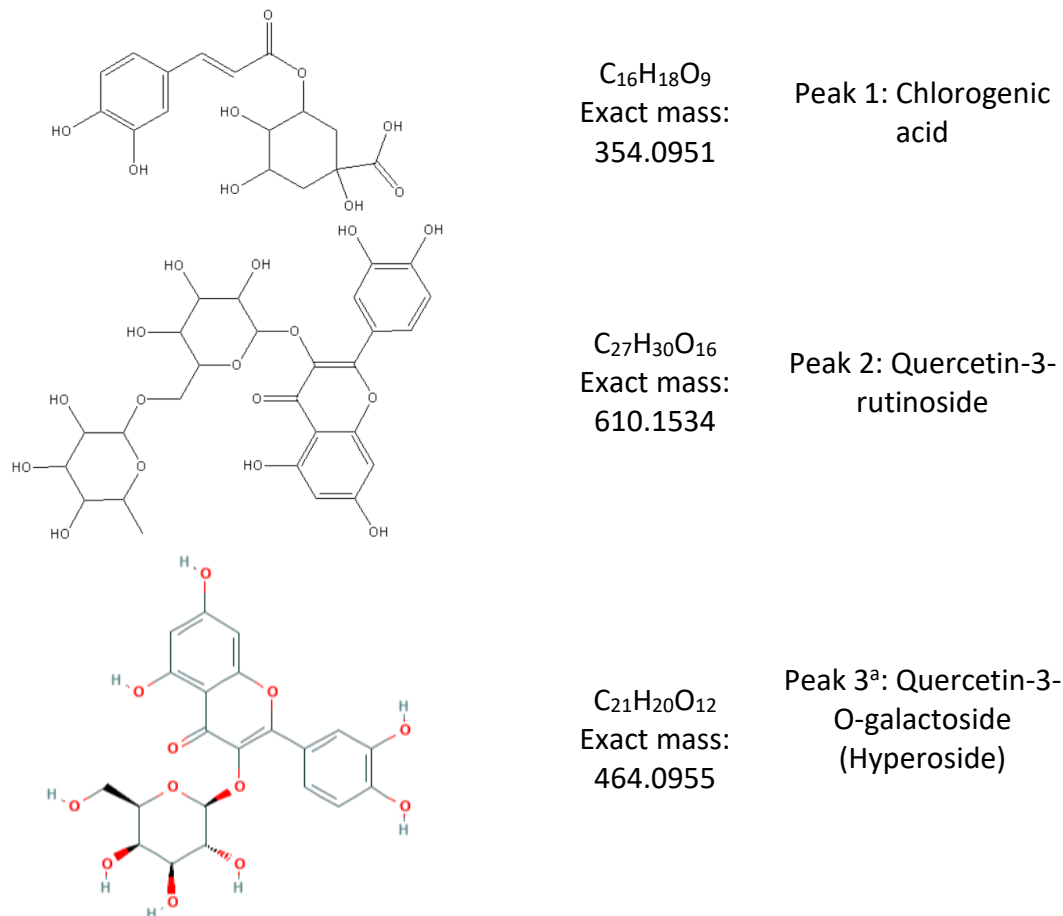
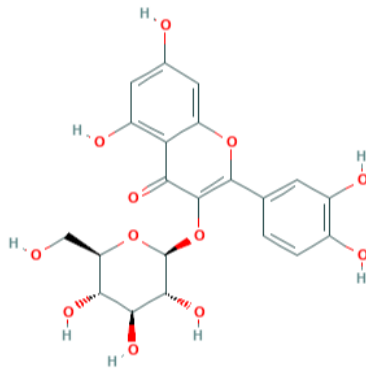


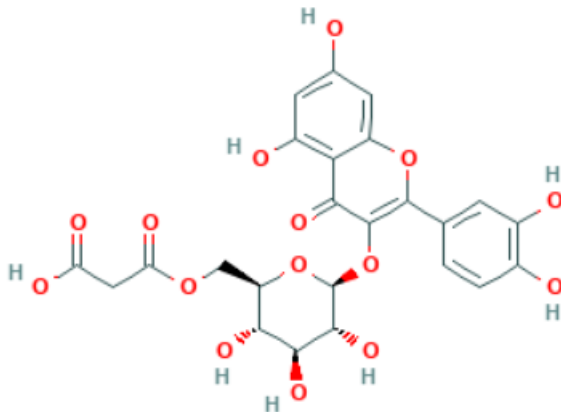
Figure SI-III.2. Mass spectra achieved by ESI(-) FT-ICR MS analysis of BC (A), CA (B), and HAW (C) with their corresponding heteroatom class distribution and van Krevelen diagram. The size of the plots is relative to the peak intensity. See Table III.1 for the list of all lot numbers investigated in this work.





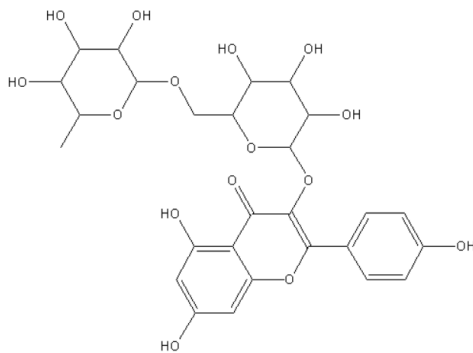
$C_{21}H_{20}O_{12}$
Exact mass:
464.0955

Peak 3^b: Quercetin-3-
O-glucoside
(Isoquercetin)



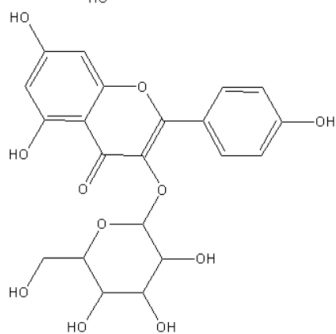
$C_{24}H_{22}O_{15}$
Exact mass:
550.0959

Peak 4^a: Quercetin-3-
6-O-malonyl-glucoside



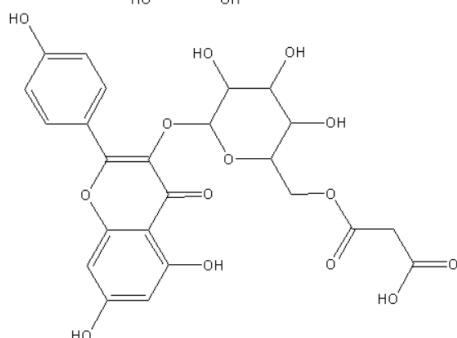
$C_{27}H_{30}O_{15}$
Exact mass:
594.1585

Peak 4^b: Kaempferol-3-
O-rutinoside



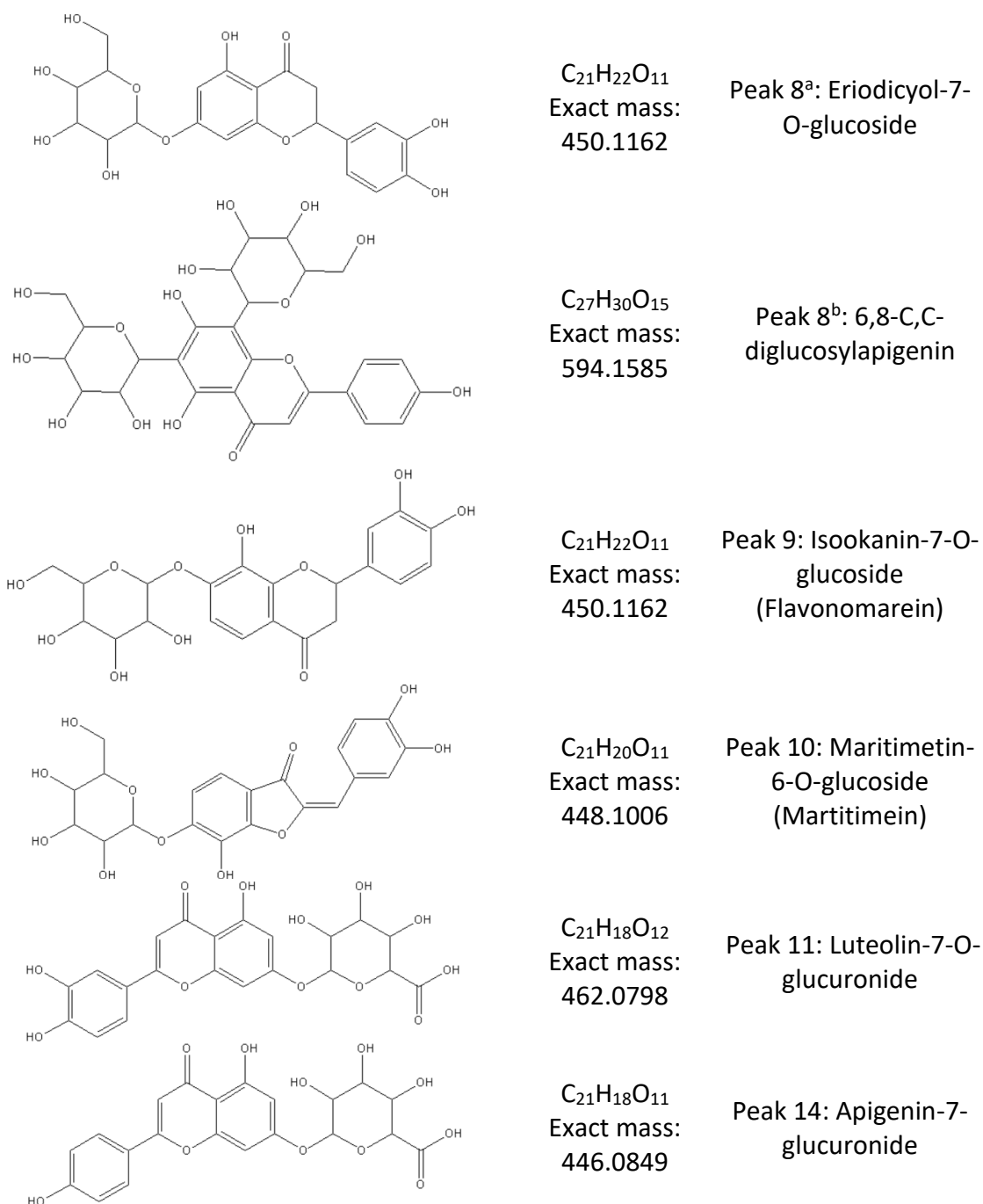
$C_{21}H_{20}O_{11}$
Exact mass:
448.1006

Peak 5: Kaempferol-3-
O-hexoside

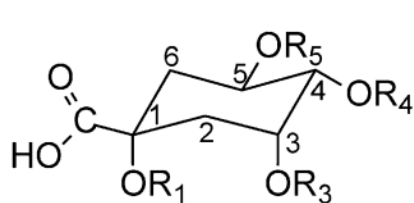


$C_{24}H_{22}O_{14}$
Exact mass:
534.101

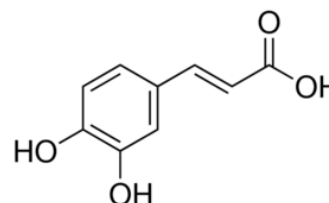
Peak 6 and 7:
Kaempferol-malonyl-
glucoside and
Kaempferol-malonyl-
glucoside isomers



Peak 12, 13 and 15: three of six following isomers of di-caffeoylquinic acids



Quinic acid



C = Caffeic acid

Name

R₁

R₃

R₄

R₅

1,3-di- <i>O</i> - caffeoylquinic acid	C	C	H	H
1,4-di- <i>O</i> - caffeoylquinic acid	C	H	C	H
1,5-di- <i>O</i> - caffeoylquinic acid	C	H	H	C
3,4-di- <i>O</i> - caffeoylquinic acid	H	C	C	H
3,5-di- <i>O</i> - caffeoylquinic acid	H	C	H	C
4,5-di- <i>O</i> - caffeoylquinic acid	H	H	C	C

Figure SI-III.3. Chemical structures of all compounds identified by UHPLC-ESI-MS.

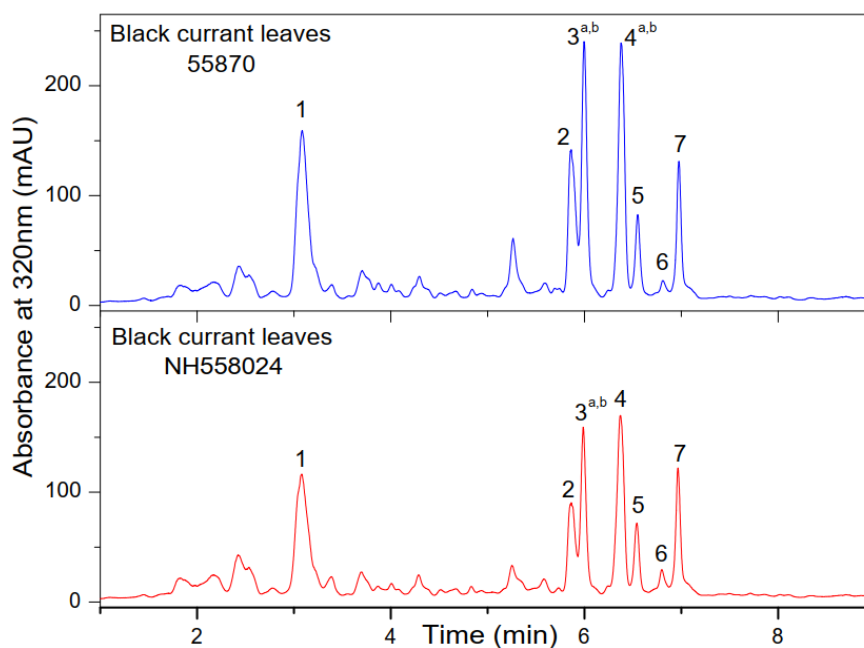


Figure SI-III.4. UHPLC profiles of two lots (55870 and NH558024) of BC infusion extracts obtained using Bodum® recipient.

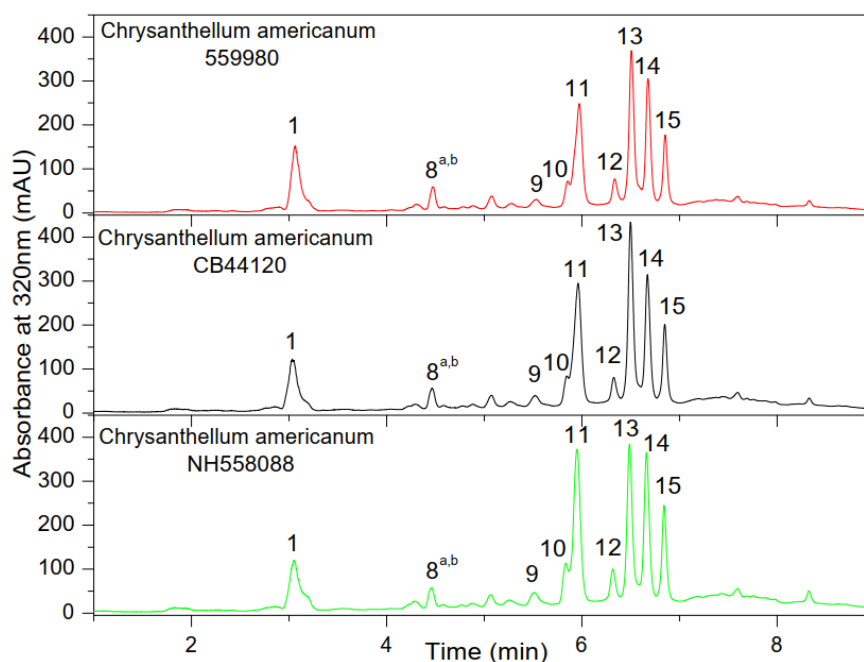


Figure SI-III.5. UHPLC profiles of three lots (559980, CB44120 and NH558088) of CA infusion extracts obtained using Bodum® recipient.

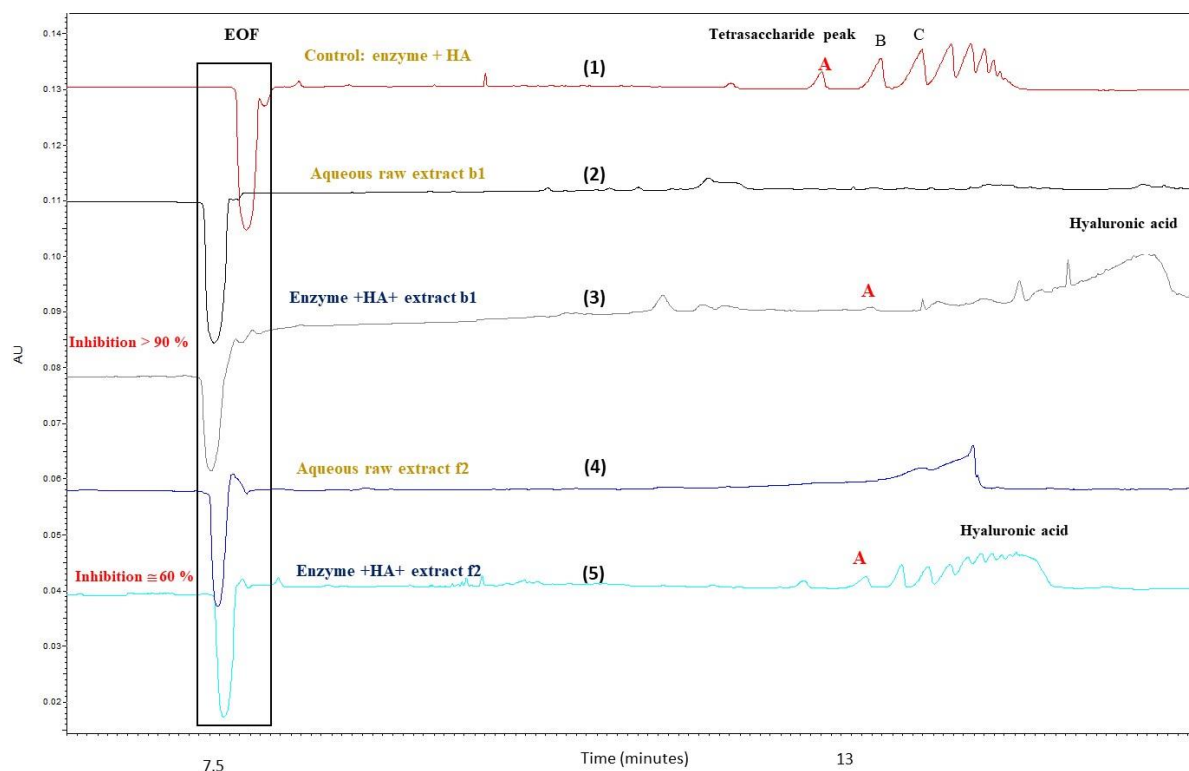


Figure SI-III.6. Example of electropherograms showing the inhibitory effect of two extracts on hyaluronidase. EPG (1) enzymatic assay occurred normally in absence of plant extracts. Electropherograms number 2 and 4 correspond to raw aqueous extracts (b1 and f2, respectively) confirming the absence of interferents with tetrasaccharide (peak A), the final product of HA hydrolysis. Electropherograms number 3 and 5 are respectively the enzymatic reactions carried out

in the presence of plant extracts b1 and f2. Reaction mixture in IB of control: 0.2 mg mL^{-1} hyaluronidase and 0.8 mg mL^{-1} HA. Modulation of hyaluronidase activity experimental conditions: 0.2 mg mL^{-1} hyaluronidase, 0.8 mg mL^{-1} HA and 1 mg mL^{-1} of filtered raw extract. Incubation at 37°C for 180 minutes. IB: 2 mM sodium acetate (pH 4.3). Electrophoretic separation conditions: BGE: 50 mM ammonium acetate (pH 8.9); anodic injection: 1.5 psi for 5 s ; separation: $+15 \text{ kV}$ at 25°C ; detection: $\lambda = 200 \text{ nm}$; rinse between analyses at 30 psi : 5 min NaOH (1 M) , 0.5 min water and 3 min BGE ; bare-silica capillary: 57 cm total length, 47 cm detection length, $50 \mu\text{m}$ i.d. Peaks identification: electroosmotic flow (EOF, $t_m = 7.3 \text{ min}$), peak A: tetrasaccharide ($t_m = 13.5 \text{ min}$), peak B: hexasaccharide, peak C: octasaccharide, Hyaluronic acid (HA, $t_m=17 \text{ min}$). All peaks were identified by CE-HRMS [1].

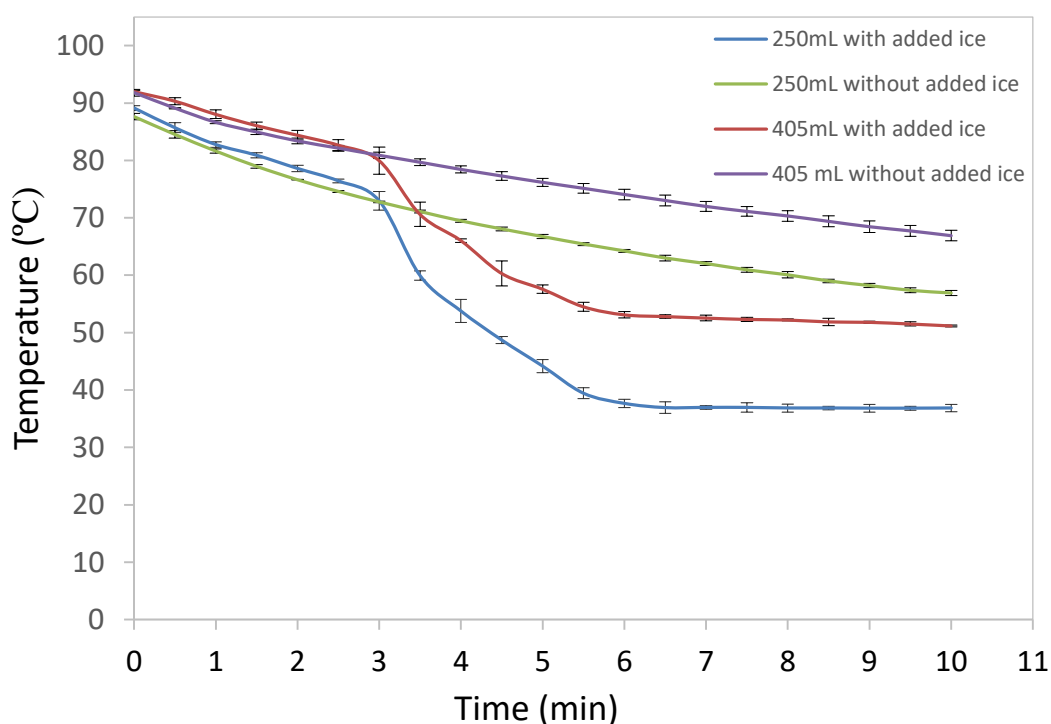


Figure SI-III.7. Decreasing temperature profiles vs time for different volumes of water (corresponding to a mug 250 mL or a bowl 405 mL) in the French-press Bodum® container with or without added ice in the container. At each $3 \text{ min} \pm 10 \text{ s}$, $4 \text{ min} \pm 10 \text{ s}$, $5 \text{ min} \pm 10 \text{ s}$, one piece of ice (ice mass = $23.4 \pm 1.5 \text{ g}$) was added into the Bodum® container. Error bars are \pm one SD on $n=3$ repetitions of independent extractions. The French-press Bodum® container was left open on the top and under stirring. Lines are only guides for the eyes.

Table SI-III.1. Some species putatively assigned to *m/z* peaks observed specifically in BC, CA and HAW samples but also commonly observed in all the samples analyzed by ESI(-) FT-ICR MS. Peaks of highest intensity are selected and putative assignment is based on identical molecular formulae achieved by ESI(-) FT-ICR MS and of identified compounds in *Crataegus*. Names in bold are species identified by UHPLC-DAD.

	<i>m/z</i>	Assignment	Candidate compound	Candidate compound	Category	Ref.
BC specific	329.175898	C20H26O4	Carnosol	[M-H] ⁻	Diterpene	
	331.191497	C20H28O4	Carnosic acid	[M-H] ⁻	Diterpene	
	335.090313	C14H20O7	Salidroside	[M+Cl] ⁻		[2]
	341.043368	C15H14O7	(+)-Galocatechin	[M+Cl] ⁻	Flavonoid	[3]
	343.082382	C18H16O7	Cirsilineol	[M-H] ⁻	Flavonoid	
	345.170802	C20H26O5	Rosmanol	[M-H] ⁻	Diterpene	
	347.075052	C11H20O10	Vicianose	[M+Cl] ⁻	Disaccharide	
	347.186439	C20H28O5	Gibberellin A53/A14/A15	[M-H] ⁻		[4]
	351.018013	C15H12O8S	5,7,4'-Trihydroxyflavanone 7-sulfate	[M-H] ⁻	Flavonoid	
	365.064481	C13H19O10P	Salicin 6-phosphate	[M-H] ⁻		
	383.163030	C20H28O5	Gibberellin A53/A14/A15	[M+Cl] ⁻		[4]
	399.157945	C20H28O6	Phorbol	[M+Cl] ⁻	Diterpene	
	589.192423	C29H34O13	Matteuorientate B	[M-H] ⁻	Flavonoid	
	601.119838	C28H26O15	(2S)-5,7,3',4'-Tetrahydroxyflavanone 7-(6-galloylglucoside)	[M-H] ⁻	Flavonoid	
	609.124844	C30H26O14	Galocatechin-(4α->8)-epigallocatechin	[M-H] ⁻	Flavonoid	[5]
	629.127747	C27H30O15	Kaempferol 3-rhamnosyl-(1->2)-galactoside	[M+Cl] ⁻	Flavonoid	
	635.125281	C28H28O17	Acacetin 7-glucuronosyl-(1->2)-glucuronide	[M-H] ⁻	Flavonoid	
	645.101553	C30H26O14	Quercetin 3-(2''-p-coumarylglucoside)	[M+Cl] ⁻	Flavonoid	
	663.192874	C31H36O16	Pectolarigenin 7-(4'''-acetylrutinoside)	[M-H] ⁻	Flavonoid	
695.146424	C30H32O19	Quercetin 3-(6''-malonylneohesperidoside)	[M-H] ⁻	Flavonoid		
CA specific	231.06627	C13H12O4	Goniothalenol	[M-H] ⁻	Lactone	
	247.1339894	C15H20O3	Parthenolide	[M-H] ⁻	Sesquiterpene lactone	[6]
	251.1652585	C15H24O3	Indicumenone	[M-H] ⁻	Ketone	[7]
	274.129653	C12H21NO6	Glutaryl carnitine	[M-H] ⁻		
	289.104169	C11H18N2O7	N-Succinyl-L-2,6-diaminoheptanedioate	[M-H] ⁻		
	317.066697	C16H14O7	Lecanoric acid	[M-H] ⁻	Polyphenol	

	327.051088	C17H12O7	(-)-Acanthocarpan	[M-H] ⁻	Flavonoid derivative	
	330.0322425	C12H13N3O4S	Acetylsulfamethoxazole	[M+Cl] ⁻		
	347.2439396	C18H36O6	Sativic acid	[M-H] ⁻	Fatty acid	
	371.1347112	C17H24O9	Syringin	[M-H] ⁻	Monosaccharide derivative	[8]
	371.2439485	C20H36O6	19(R)-hydroxy-Prostaglandin F1 α	[M-H] ⁻	Fatty acid	
	433.0958854	C26H42O3S	1 α ,25-dihydroxy-3-deoxy-3-thiavitamin D3 3-oxide / 1 α ,25-dihydroxy-3-deoxy-3-thiacholecalciferol 3-oxide	[M-H] ⁻		
	455.353069	C30H48O3	Oleanolic/ursolic acid	[M-H] ⁻	Triterpene	[9-11]
	457.1140505	C21H20O10	Vitexin	[M+Cl] ⁻	Flavonoid	
	529.135034	C26H26O12	Luteolin 3'-methyl ether 7-(6''-crotonylglucoside)	[M-H] ⁻	Flavonoid	
	561.2397738	C35H34N2O5	Trilobine ([M-H] ⁻)	[M-H] ⁻	Alkaloid	
	563.1767775	C27H32O13	Pinocembrin 7-rhamnosylglucoside	[M-H] ⁻	Flavonoid	
	609.2838795	C32H46O9	Cucurbitacin A	[M+Cl] ⁻	Cholesterol derivative	
	633.2245624	C24H42O19	Lactodifucotetraose	[M-H] ⁻	Polysaccharide	
	725.208519	C36H38O16	Licorice glycoside C1/C2	[M-H] ⁻	Flavonoid	
HAW specific	131.046229	C4H8O3N2	Asparagine	[M-H] ⁻	Amino acid	[12]
	193.035397	C6H10O7	Galacturonic acid	[M-H] ⁻	Saccharide derivative	[13]
	195.066306	C10H12O4	Homoveratric acid	[M-H] ⁻	Phenol	
	201.113254	C10H18O4	Decanedioic acid	[M-H] ⁻	Fatty acid	
	203.0826113	C11H12O2N2	Tryptophan	[M-H] ⁻	Amino acid	[12]
	207.066307	C11H12O4	Sinapaldehyde	[M-H] ⁻	Phenol	
	210.077208	C10H13NO4	3-Methoxytyrosine	[M-H] ⁻	Amino acid derivative	
	217.083013	C8H14N2O5	Glutamylalanine	[M-H] ⁻	Dipeptide	
	355.123586	C19H25ClO2	11 beta-Chloromethylestradiol	[M+Cl] ⁻	Cholesterol derivative	
	369.067285	C12H18O13	Digalacturonic acid	[M-H] ⁻	Disaccharide	[13]
	387.040652	C17H17ClO6	Griseofulvin	[M+Cl] ⁻	Dibenzofuran	
	409.023334	C17H14O10S	Quercetin 3,7-dimethyl ether 4'-sulfate	[M-H] ⁻	Flavonoid	
	473.072564	C22H18O12	Chicoric acid	[M-H] ⁻	Phenol	
	515.226281	C21H41O12P	1-dodecanoyl-glycero-3-phospho-(1'-myo-inositol)	[M-H] ⁻	Monoacylglycero phosphoinositol	

537.192084	C33H30O7	8-trans-[2-(6-Benzoyloxy-4-hydroxy-2-methoxy-3-methylphenyl)ethenyl]-5-methoxyflavan-7-ol	[M-H] ⁻	Flavonoid
613.132937	C27H30O14	Kaempferol 3-rhamnoside-(1->2)-rhamnoside	[M+Cl] ⁻	Flavonoid
663.156574	C30H32O17	Apigenin 7-(6''-malonylneohesperidoside)	[M-H] ⁻	Flavonoid
725.193469	C32H38O19	Schaftoside 6''-O-glucoside	[M-H] ⁻	Flavonoid
737.193737	C33H38O19	Kaempferol 7-methyl ether 3-[3-hydroxy-3-methylglutaryl-(1->6)]-[apiosyl-(1->2)-galactoside]	[M-H] ⁻	Flavonoid
769.198376	C37H38O18	Isovitexin 2''-O-(6'''-feruloyl)glucoside	[M-H] ⁻	Flavonoid

	<i>m/z</i>	Assignment	Candidate compound	Adduct	Category	Ref.
Common features	132.030259	C4H7O4N	Aspartatic acid	[M-H] ⁻	Amino acid	[9-11]
	133.014272	C4H6O5	Malic acid	[M-H] ⁻	Organic acid	[9-11]
	137.024450	C7H6O3	Protocatechuic aldehyde/hydroxybenzoic acid	[M-H] ⁻	Phenol	[9-11]
	146.045903	C5H9O4N	Glutamatic acid	[M-H] ⁻	Amino acid	[9-11]
	153.019355	C7H6O4	Protocatechuic acid	[M-H] ⁻	Phenol	[9-11]
	163.040092	C9H8O3	Coumaric acid	[M-H] ⁻	Coumaric acid	[9-11]
	179.035004	C9H8O4	Caffeic acid	[M-H] ⁻	Caffeic acid	[9-11]
	179.056137	C6H12O6	Glucose/fructose/Inositol	[M-H] ⁻	Monosaccharide	[9-11]
	181.071782	C6H14O6	Sorbitol	[M-H] ⁻	Monosaccharide	[9-11]
	188.035337	C10H7O3N	alpha-Cyano-4-hydroxycinnamic acid (HCCA)	[M-H] ⁻	Phenol	[9-11]
	191.019742	C6H8O7	Citric acid	[M-H] ⁻	Organic acid	[9-11]
	191.056085	C7H12O6	Quinic acid	[M-H] ⁻	Phenol	[9-11]
	193.050666	C10H10O4	Ferulic acid	[M-H] ⁻	Phenol	[9-11]

223.061189	C11H12O5	Sinapinic acid	[M-H] ⁻	Phenol	[9-11]
285.040487	C15H10O6	Kaempferol/Cyanidin (-2H-)	[M-H] ⁻	Flavonoid	[9-11]
289.071827	C15H14O6	Catechin/epicatechin	[M-H] ⁻	Flavonoid	[9-11]
301.035432	C15H10O7	Quercetin	[M-H] ⁻	Flavonoid	[9-11]
315.051075	C16H12O7	Sexangularetin	[M-H] ⁻	Flavonoid	[9-11]
331.067100	C13H16O10	Galloylglucose	[M-H] ⁻	Tannin	[9-11]
341.109006	C12H22O11	Sucrose	[M-H] ⁻	Saccharide	[9-11]
353.087829	C16H18O9	Chlorogenic acid / 5-O-Caffeoylquinic acid	[M-H] ⁻	Phenol derivative	[9-11]
417.082718	C20H18O10	Kaempferol-O-arabinoside (crataegide)	[M-H] ⁻	Flavonoid	[9-11]
431.098376	C21H20O10	Vitexin/Isovitexin/Apigenin-C-hexoside/Kaempferol O-pentoside	[M-H] ⁻	Flavonoid	[9-11]
447.093308	C21H20O11	Orientin/Luteolin-C-hexoside/ Maritimetin-6-O-glucoside/Kaempferol-3-O-hexoside	[M-H] ⁻	Flavonoid	[9-11]
449.108966	C21H22O11	Eriodictyol 7-O-glucoside/Isookanin-7-O-glucoside	[M-H] ⁻	Flavonoid	[9-11]
461.072579	C21H18O12	Luteolin-7-O-glucuronide	[M-H] ⁻	Flavonoid	[9-11]
461.108994	C22H22O11	Methyluteolin-C-hexoside/Methoxykaempferol-pentoside	[M-H] ⁻	Flavonoid	[9-11]
463.088240	C21H20O12	Hyperoside/Isoquercetin/Spiraeoside	[M-H] ⁻	Flavonoid	[9-11]
477.103886	C22H22O12	Sexangularetin 3-O-glucoside	[M-H] ⁻	Flavonoid	[9-11]
489.103836	C23H22O12	O-Acetylorientin	[M-H] ⁻	Flavonoid	[9-11]
505.098780	C23H22O13	Quercetin-O-acetyl hexoside	[M-H] ⁻	Flavonoid	[9-11]
515.119522	C25H24O12	Dicaffeoylquinic acid	[M-H] ⁻	Phenol	[9-11]

533.093590	C24H22O14	Kaempferol-malonylglucoside	[M-H] ⁻	Flavonoid	[9-11]
563.104079	C25H24O15	Sexangularetin 3-O-(malonyl)glucoside	[M-H] ⁻	Flavonoid	[9-11]
577.156128	C27H30O14	Iso/vitexin rhamnoside	[M-H] ⁻	Flavonoid	[9-11]
593.151052	C27H30O15	Kaempferol-3-O-neohesperidoside /Iso/Orientin O-rhamnoside	[M-H] ⁻	Flavonoid	[9-11]
609.145975	C27H30O16	Rutin/Quercetin-3-O-rhamnosylgalactoside/Quercetin 3-rutinoside	[M-H] ⁻	Flavonoid	[9-11]
623.161612	C28H32O16	Sexangularetin 3-neohesperidoside/Metoxykaempferol methylpentosylhexoside	[M-H] ⁻	Flavonoid	[9-11]
625.140986	C27H30O17	6,8-Diglucosylapigenin	[M-H] ⁻	Flavonoid	[9-11]

Table SI-III.2. Inhibition assays of hyaluronidase and ACE as well as the ABTS antioxidant capacity assay of the different plant extracts. For the enzymes inhibition assays, the plant extracts were screened at 1 mg mL⁻¹ and the inhibition percentages of hyaluronidase and ACE were calculated according to Eq.2 and Eq.3, respectively. The antioxidant capacities of the plant extracts were determined at 0.01 mg mL⁻¹ and calculated according to Eq.4. The absorbance of the multi-well plates was read twice for the ACE inhibition and ABTS antioxidant capacity assays. All assays were carried out in triplicates (n=3). Plant extracts were obtained from HAW (#1/#2, grinded 1 mm and #1/#2, grinded 'fine'), BC (#1/#2, grinded 'fine') and CA (#1/#2, grinded 'fine'). *EGCG, hyaluronidase referenced inhibitor, and Trolox, an antioxidant reference, were used to validate the methods. n.r.= Not relevant

	Identification	Average hyaluronidase inhibition \pm SD (%) ; n=3	Average ACE inhibition \pm SD (%) ; n=3		Average reduction of ABTS absorbance \pm SD (%) ; n=3 (Antioxidant Assay)	
			(Read #1)	(Read #2)	(Read #1)	(Read #2)
Hyaluronidase Referenced inhibitor	EGCG*	100 %	n.r.	n.r.	n.r.	n.r.
Reference antioxidant compound	Trolox*	n.r.	n.r.	n.r.	64 \pm 2	64 \pm 2

<i>Hawthorn flowering tops (HAW)</i>	n°20335, #1	96 ± 1	89 ± 2	89 ± 2	45 ± 1	45 ± 1
	n°20335, #2	97 ± 2	81 ± 4	82 ± 4	45 ± 0	45 ± 0
	n°CB58120, #1	97 ± 1	91 ± 2	92 ± 2	34 ± 3	34 ± 3
	n°CB58120, #2	93 ± 1	88 ± 1	88 ± 1	28 ± 0	29 ± 0
<i>Blackcurrant leaves (BC)</i>	n°55870, #1	64 ± 2	76 ± 0	76 ± 0	58 ± 1	58 ± 1
	n°55870, #2	64 ± 3	73 ± 5	72 ± 5	64 ± 2	64 ± 2
<i>Chrysanthellum americanum (CA)</i>	n°559980, #1	61 ± 2	96 ± 2	97 ± 2	19 ± 2	19 ± 2
	n°559980, #2	59 ± 1	91 ± 2	91 ± 2	22 ± 0	22 ± 1

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CHAPTER IV: TEA CREAMING IN HERBAL TEA

CHAPTER IV: TEA CREAMING IN HERBAL TEA

IV.1. Introduction

Tea creaming consists of nanoparticles (with a size generally from 10 to 400 nm) that are forming in infusion with time when the preparation is cooling down. These nanoparticles can reduce the content of active components that are extracted and bioavailable by precipitation. In the meantime, they could be of potential interest to stimulate the immune defense [1], for their use as nutraceuticals [2], and for the production of nanoparticles [3]. Tea creaming formation depends on several factors, including chemical composition of plant material, infusion solid concentration, and temperature. To our knowledge, only tea creaming from green or black tea was already studied in the literature [4-8]. Therefore, we found it interesting to investigate the formation of nanoparticles in herbal tea infusions, from a kinetic and sizing distribution point of view.

IV.2. Materials and methods

IV.2.1. Grinded plants

Dry *Chrysanthellum americanum* (lot n° 559980, origin Ivory Coast), dry blackcurrant leaves (*Ribes nigrum*, lot n° 55870, origin Poland) and dry hawthorn flowering tops (*Crataegus oxyacantha*, lot n° CB58120, origin France) raw materials were purchased from France Herboristerie (Noidans-Lès-Vesoul, France). Dry green tea ([*Camellia sinensis*](#), Phu Ho Tra brand), was purchased from Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phu Tho, Vietnam. No information related to the chemical composition was found on the green tea package. Dry plants (hawthorn, black currant leaves, *Chrysanthellum americanum* and green tea) were grinded using Delonghi (Model KG79, Treviso, Italy) grinder at the position named 'fine', then stored at room temperature.

IV.2.2. Infusion extraction protocol and tea creaming formation

Method 1 (M1): 30 g grinded plant materials were infused in 600 mL boiling distilled or tap water under magnetic stirring at 500 rpm using 'French press' Bodum® commercial equipment (Bistro model, Triengen, Switzerland). After 5 min, the herbal tea solution was filtrated first with the Bodum® cover to remove the largest solid particles, then, centrifuged at 7000 rpm at room temperature for 10 min, and finally filtrated using a Büchner funnel and a vacuum pump (KNF Model N820FT.18, Freiburg, Germany) to remove the residual solid

plants. The resulting infused solution was next divided into 3 tubes of same volume (named tube 1, 2 and 3, see Figure IV.1). Tube 1 content was freeze-dried (Cryotec Model CRIOS-80, Saint-Gély-du-Fesc, France) and the extraction yield was determined. Caffeine at 500 mg/L was added into tube 3. Tube 2 and tube 3 were then stored in the fridge at 4°C for one night (\approx 16 h). The herbal tea solution in tube 2 and tube 3 was centrifuged at 7000 rpm at room temperature for 10 min and the supernatant was removed (Figure IV.1). Finally, the tea creaming particles contained in tube 2 and tube 3 was freeze-dried and weighed. The experiment was carried out in duplicate.

Method 2 (M2): 25 g grinded plant materials were infused in 250 mL boiling distilled water under magnetic stirring at 500 rpm using the same 'French press' Bodum® equipment. After 5 min, the herbal tea solution was filtrated first with the Bodum® cover to remove the largest solid particles, then, centrifuged at 7000 rpm at 40°C for 10 min, and finally filtrated using 0.2 μ m Millipore® filter paper to remove the residual solid plants. The resulting infused solution was next divided into 2 tubes of same volume (named tube 1 and 2, see Figure IV.1) and stored in the fridge at 4°C for one night (\approx 16 h). The herbal tea solution in both tubes was centrifuged at 5000 rpm at room temperature for 10 min and the supernatant was removed (Figure IV.1). The tea creaming particles were washed with 40 mL pure water and centrifuged. The supernatant was removed. This process was then repeated 4 times. Finally, the tea creaming contained in tube 1 was freeze-dried and weighed. The tea creaming contained in tube 2 was dispersed in pure water for the determination of the hydrodynamic size by dynamic light scattering (see section IV.2.3).

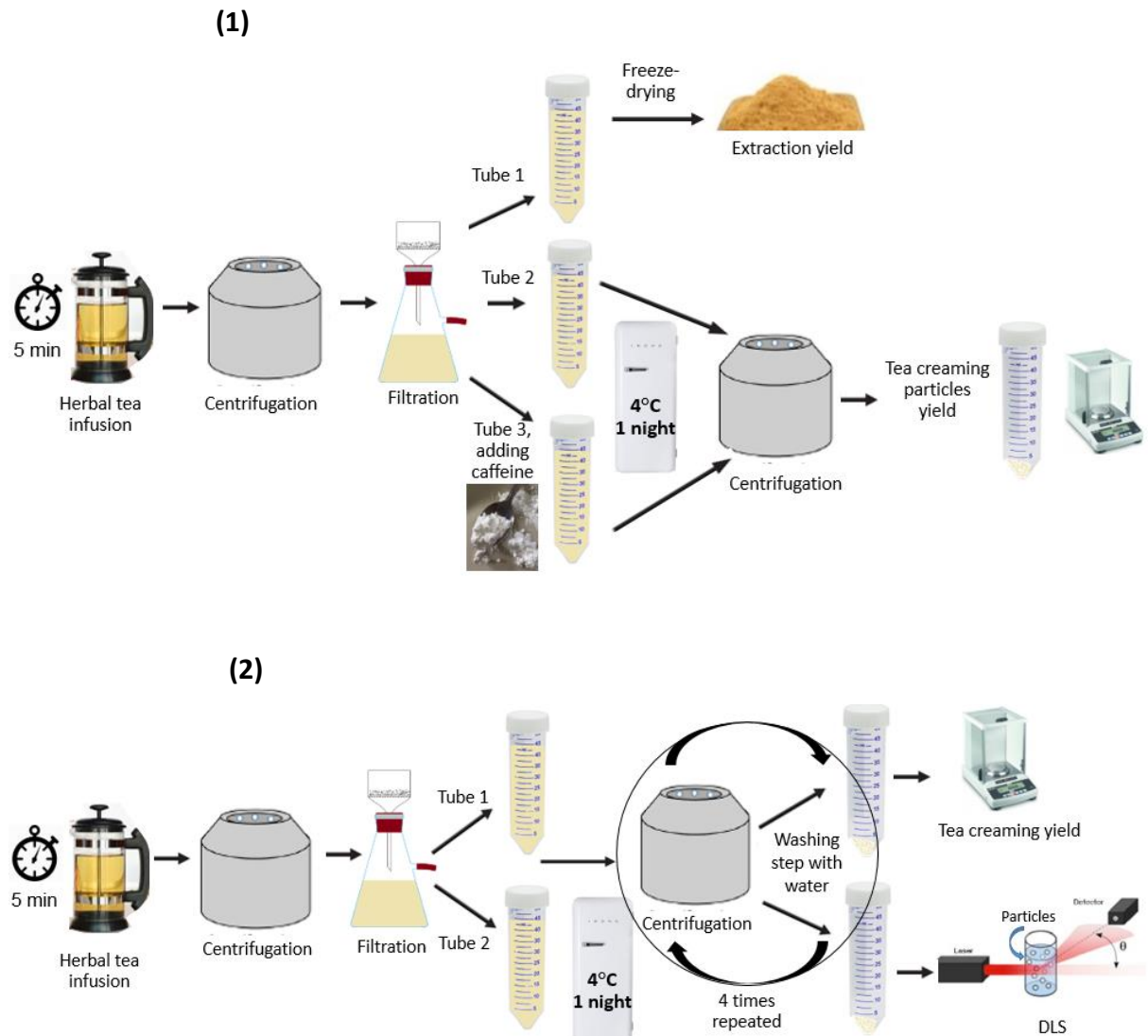


Figure IV. 1: Protocols for the formation and characterization of tea creaming in herbal tea: method 1 (1) and method 2 (2).

IV.2.3. Hydrodynamic size determination

The hydrodynamic diameter of the tea creaming particles was determined at various temperatures using a Litesizer™ 100 & Litesizer™ 500 equipment (Anton Paar GmbH, Graz, Austria). Light scattering angle was set at 150° and the analysis model “Contin” was selected.

IV.3. Results and discussion

Tea creaming was studied from the infusion of various dry ground materials (green tea, hawthorn, blackcurrant leaves, *Chrysanthellum americanum*) using the protocol described in section IV.2.2. As shown in Table IV.1, the tea creaming amount that was

obtained from method 1 (0- 30 mg), was much higher than that from method 2 (0-2 mg) in the case of herbal tea and also was much lower than in the case of green tea (M1: 205.85 mg and M2: 6.5 mg). Tea creaming in green tea (M1: 20.6 mg/g dry plant or M2: 0.52mg/g dry plant) was found higher or lower than that obtained by Kim et al. for green tea leaves harvested in Hwage, Korea (10.74 mg/g dry plant) [4] depending on the method used. The 'real' content in tea creaming in all the infusions is between the two values given by methods 1 and 2, and probably closer to value given by method 1. Method 1 may slightly overestimate the tea creaming mass due to the remaining water in the sample (the solid was not freeze-dried). Method 2 highly underestimate the tea creaming quantity due to loss of particle in the washing process. The origin, the species and variety, and the chemical composition of the plant can have a great impact on the tea creaming formation and yield. For example, it is known that caffeine and phenolic compounds strongly increase tea creaming amount [4]. It is also worth noting that no tea creaming was obtained with *Chrysanthellum americanum*, even after 16 h storage at 4°C.

Using method 1, the addition of caffeine (at 500 mg/L) into herbal tea infusion (BC or HAW) did not increase the tea creaming amount. In contrast, adding caffeine (1g/L) into green tea infusion increased the tea creaming formation by 15 %. When using tap water instead of distilled water, the tea creaming amount increased by 18.3 %, probably due to the presence of metal ions (calcium and or magnesium) that could help precipitating tea creaming particles, as already shown in ref [4].

Table IV.1. Extraction yield, tea creaming amount, average size of tea creaming at room temperature (RT) and corresponding ratio in dry plant. Data obtained from the infusion of 10 g (M1) or 12.5 (M2) dry plant according to the protocol described in section IV.2.2.

^a: data obtained from distilled water, \pm one standard deviation calculated on $n=2$ repetitions, ^b: data obtained from tap water, \pm one standard deviation calculated on $n=2$ repetitions, ^c: data obtained from adding 500 mg caffeine/L, \pm one standard deviation calculated on $n=2$ repetitions, ^d: \pm one standard deviation calculated on $n=3$ repetitions.

Plant	Extraction yield (% M1)	Tea creaming amount (mg)		Average size of tea creaming at RT (M2, nm) ^d	Ratio tea creaming/dry plant (‰) (M1/M2)
		Protocol 1 (M1)	Protocol 2 (M2)		
Green tea	25.13 \pm 0.69 ^a	205.85 \pm 11.8 ^a	6.50	4953 \pm 347	20.59/0.52
Hawthorn (Lot N°: CB58120)	16.43 \pm 0.87 ^a	21.01 \pm 1.24 ^a	0.97	419.5 \pm 8.8	2.10/0.08
		20.02 \pm 1.02 ^c			
Blackcurrant leaves (Lot N°: 55870)	20.74 \pm 0.36 ^a	35.87 \pm 0.71 ^b	2.08	372.3 \pm 13.6	3.03/0.17
		30.33 \pm 2.77 ^a			
		29.10 \pm 0.72 ^c			
<i>Chrysanthellum americanum</i> (Lot N°: 559980)	19.61 \pm 1.41 ^a	0	0	0	0

The average hydrodynamic diameter of tea creaming was followed upon time at different temperatures (4°C, 10°C, 30°C, 40°C) using the method 2 described in section IV.2. Figure IV.2 displays the results obtained for blackcurrant leaves at 4°C. Figure SI-IV.1 shows the results for the other plants (see supporting information of chapter IV). As shown in Figure IV.2, the average hydrodynamic diameter of tea creaming increased significantly from 100 nm ($t=0$) to approximately 400 nm ($t=2h$), then reached a plateau stable till $t=9h$, and finally decreased to around 220 nm after $t=11h$.

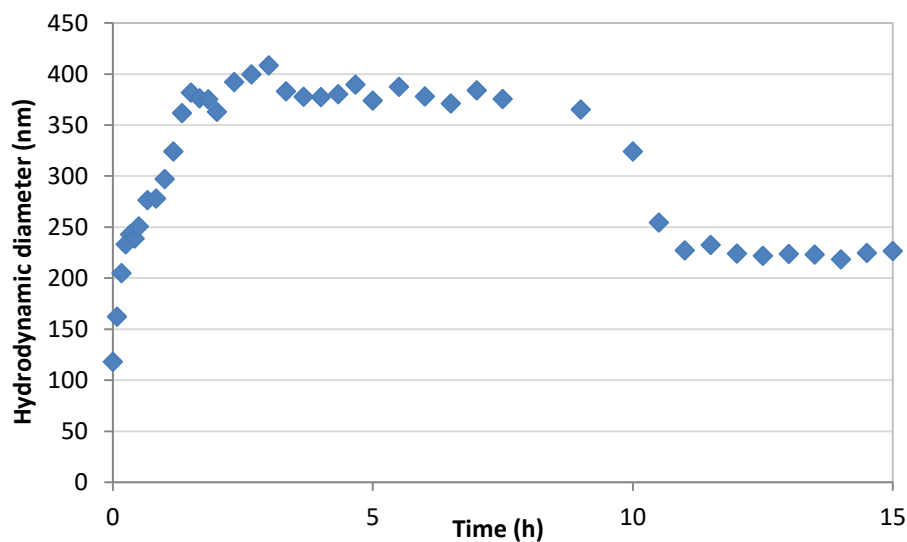


Figure IV.2: Average hydrodynamic diameter of tea creaming issued from 5 min infusion of 12.5 g of blackcurrant leaves in 125 mL water, filtrated using 0.2 μm Millipore® filter paper, and measured at 4°C upon time. See the protocol in section IV.2.2 and Figure SI-IV.2 for the corresponding DLS autocorrelation curves.

Compared to the data obtained at other temperatures (10°C, 30°C, 40°C), it can be observed that the temperature has no significant effect on the size of tea creaming at least below 40°C (Figure IV.3), which is in good agreement with other data reported in the literature [4]. The authors showed that the temperature has only an impact on the relative amounts of the different individual tea creaming components (catechins, caffeine, rutin and proteins) [4].

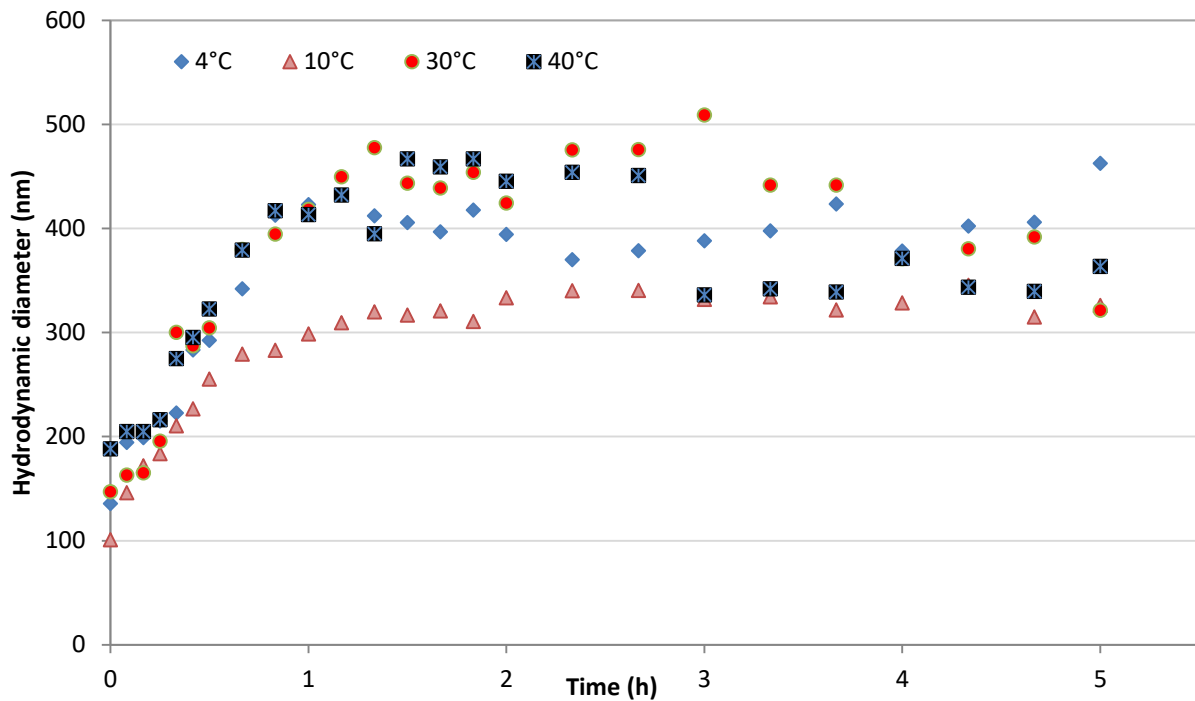


Figure IV.3. Average hydrodynamic diameter of tea creaming issued from 5 min infusion of 12.5 g of blackcurrant leaves in 125 mL water, filtrated using 0.2 μm Millipore® filter paper, and measured at various temperatures upon time. See the protocol in section IV.2.2 and Figure SI-IV.2 for the corresponding DLS autocorrelation curves.

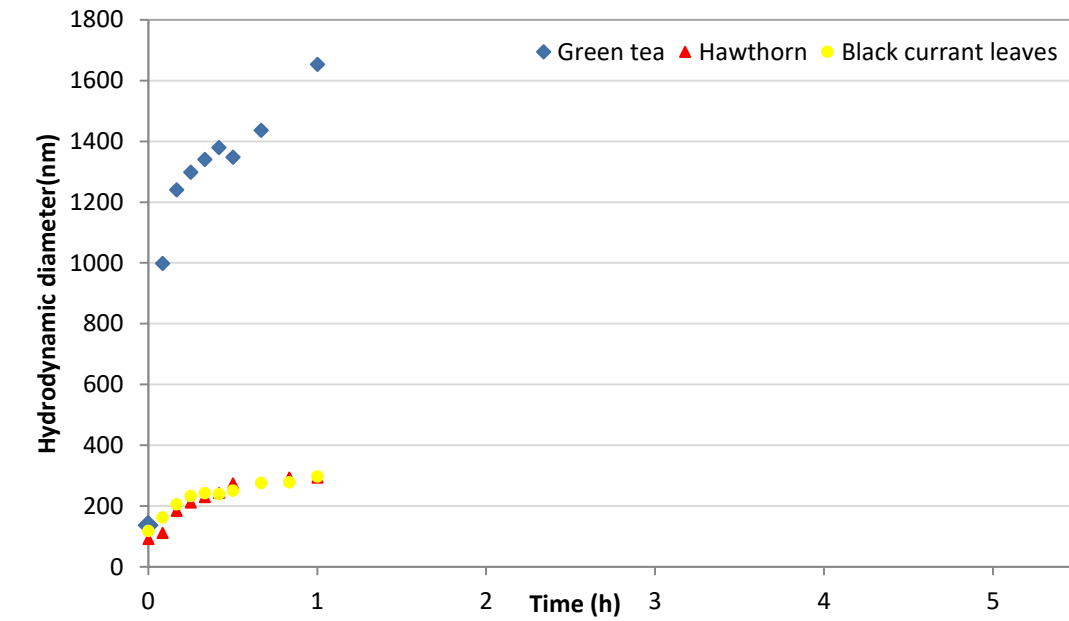
IV.4. Conclusion

To conclude, tea creaming formation has been observed in herbal tea such as hawthorn and blackcurrant leaves, but not in *Chrysanthellum americanum*. However, the tea creaming amount was found much lower than in green tea and was not dependent on the temperature up to 40°C. The addition of caffeine at 500 mg/L into herbal tea infusion (blackcurrant leaves or hawthorn flowering tops) did not increase the tea creaming amount. When using tap water instead of distilled water, the tea creaming amount increased by about 20 %, probably due to the presence of metal ions like calcium or magnesium in tap water. Other investigations like addition of catechine or the use of tap water combined with the addition of caffeine will be carried out in the next future.

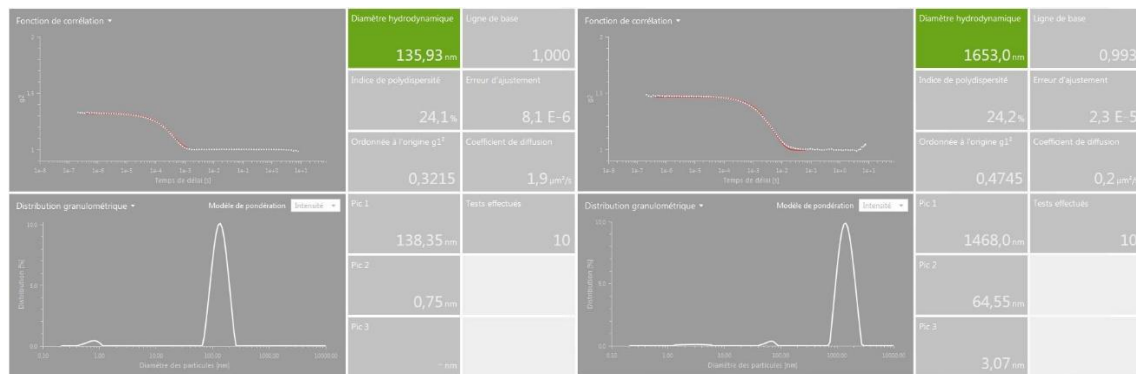
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SUPPORTING INFORMATION OF CHAPTER IV



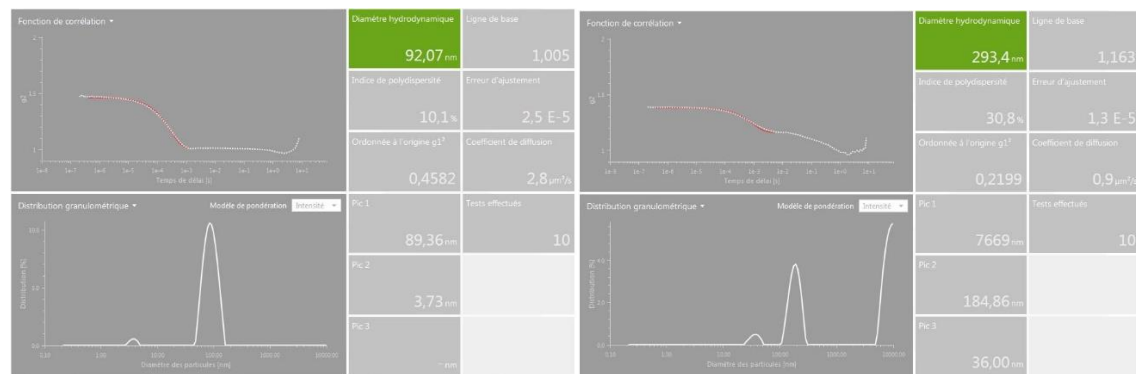
A



t=0

t=1h

B

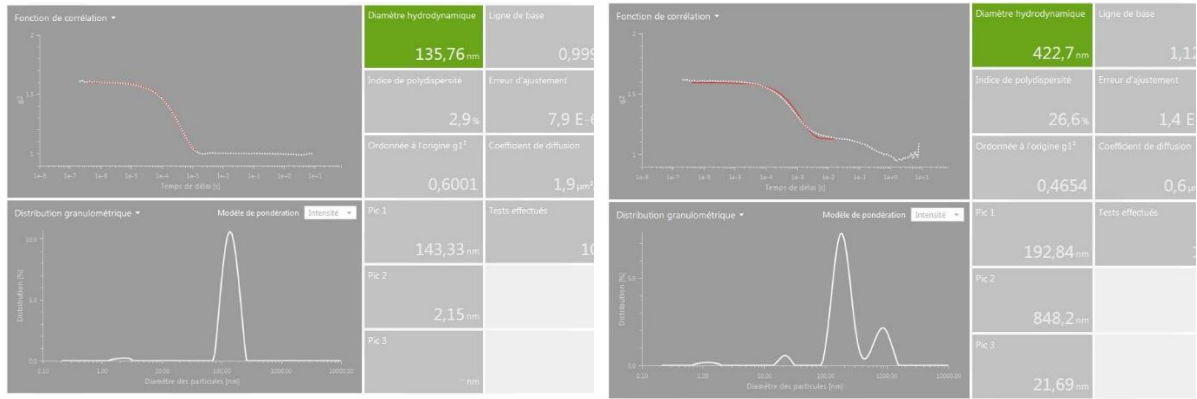


t=0

t=1h

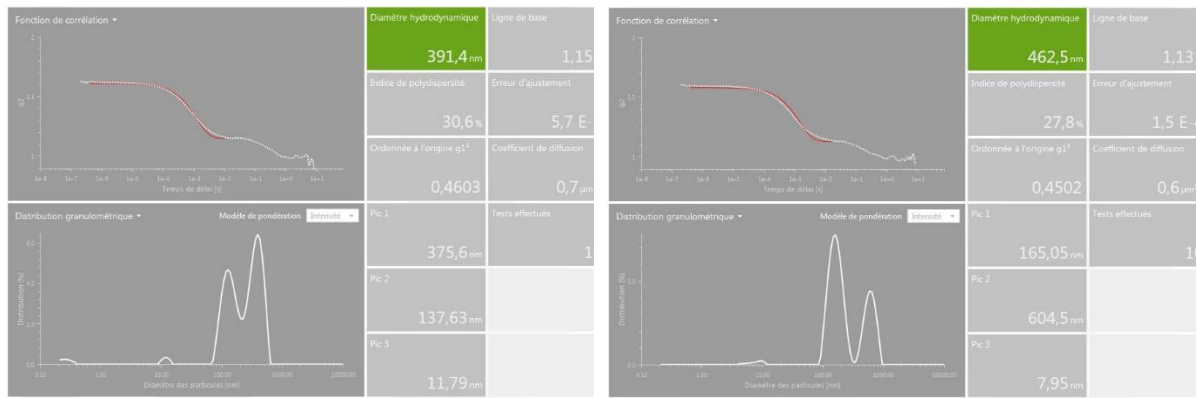
C

Figure SI-IV.1. Average hydrodynamic diameter of tea creaming particles issued from the infusion of green tea, hawthorn and blackcurrant leaves at 4°C upon time (A). Corresponding DLS autocorrelation curves for the case of green tea (B) and hawthorn (C).



t=0

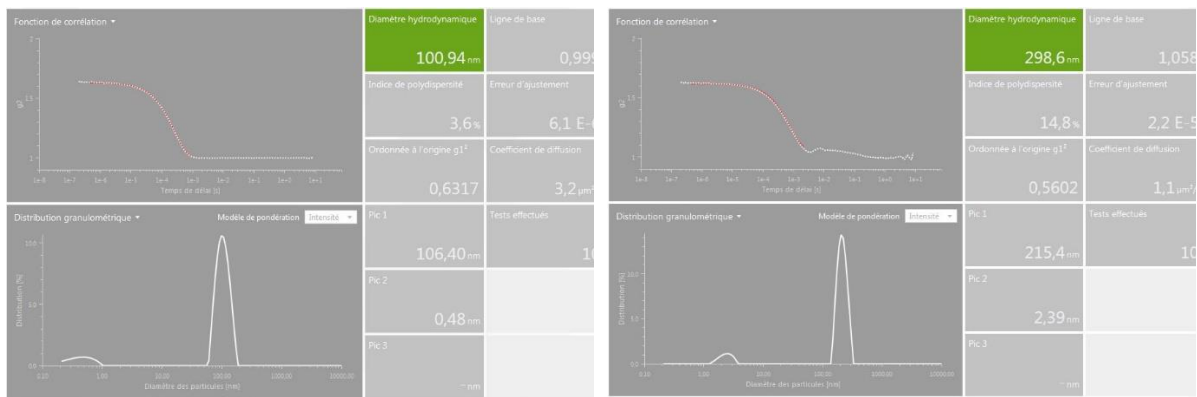
t= 1 h



t= 2 h

t= 5 h

(A) at 4°C

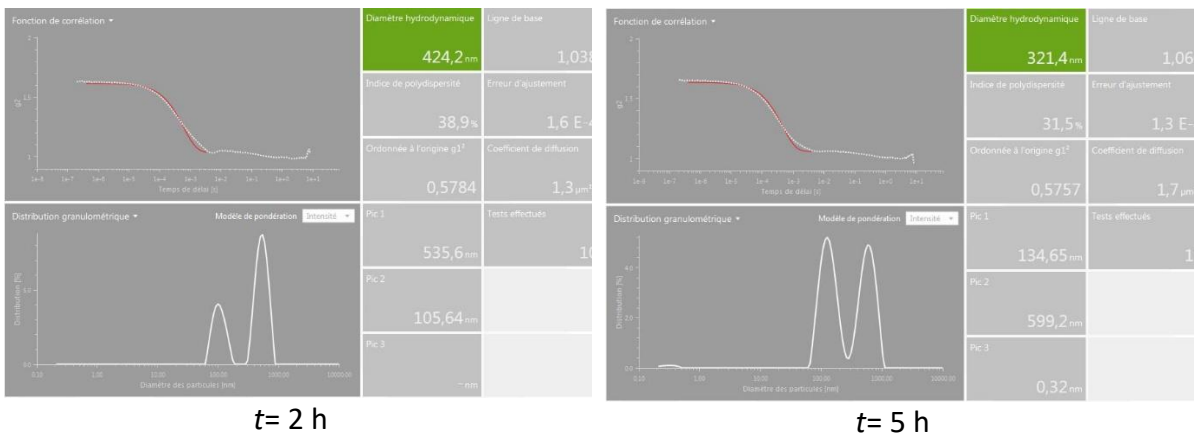
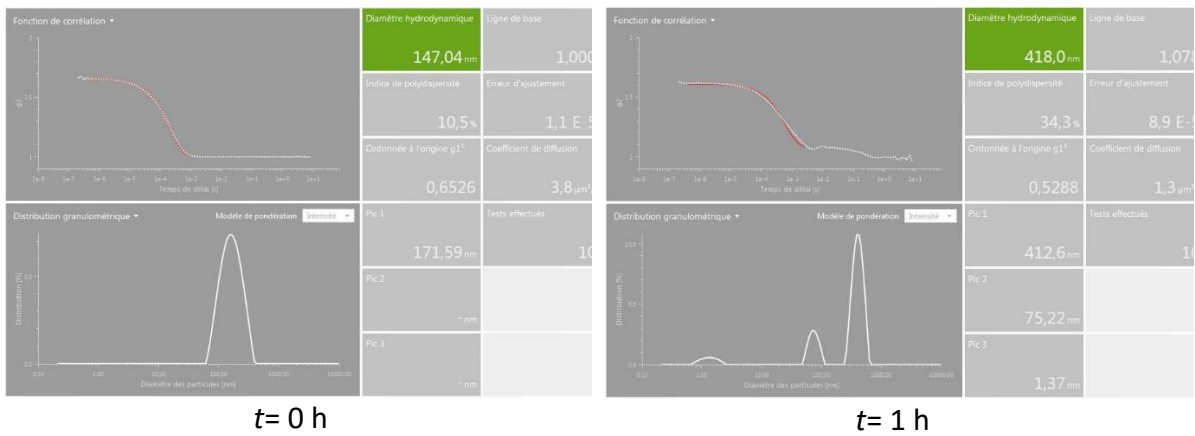


t= 0 h

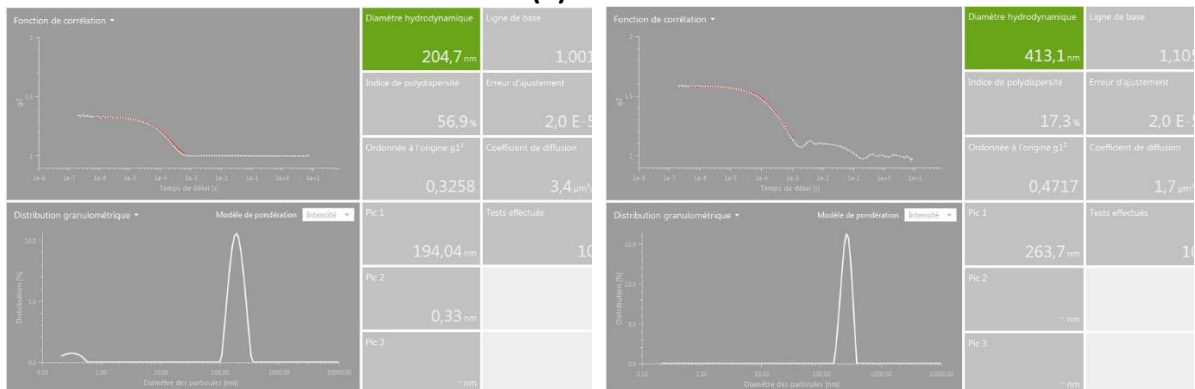
t= 1 h

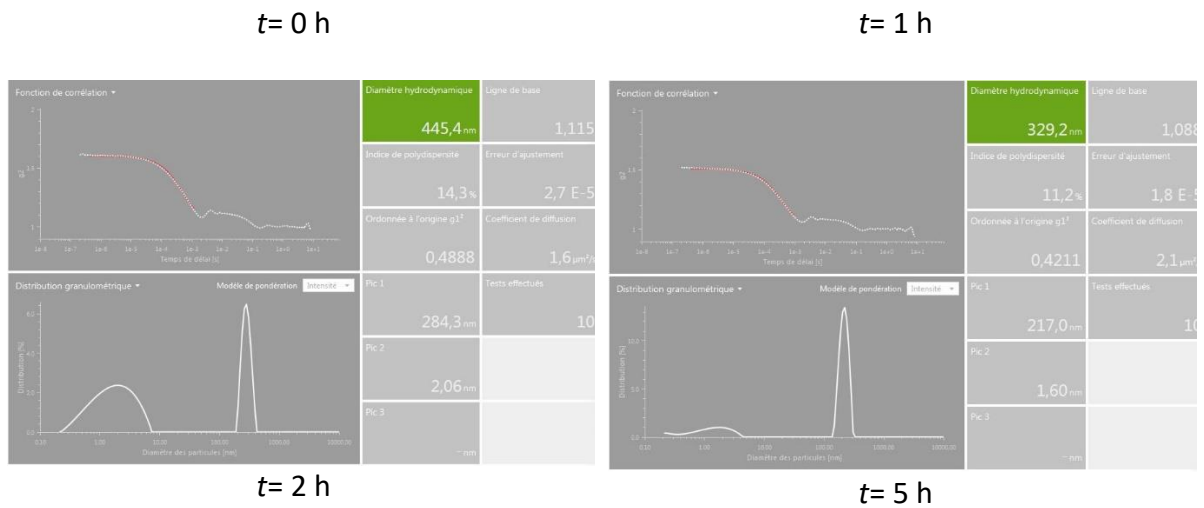


(B) at 10°C



(C) at 30°C





(D) at 40°C

Figure SI-IV.2. DLS autocorrelation curves for the case of blackcurrant leaves at 4°C (A), 10°C (B), 30°C (C) and 40°C (D) upon time.

GENERAL CONCLUSION

In this thesis, the impact of the experimental conditions of extraction on the daily intake dose and its reproducibility was investigated in details, with a special attention to home-prepared extractions at drinkable temperature (≤ 60 °C). Three medicinal plants of complementary pharmacological activities were selected. Hawthorn (HAW), one of the 'best seller', is a world-spread flowering shrub known for its ability to regulate the sympathetic system and for its cardio-tonicity. Blackcurrant leaves (BC) are mostly known for their anti-inflammatory activity. *Chrysanthellum americanum* (CA) is an American and African plant used for its capacity of stimulating the liver, for promoting blood microcirculation and for its cholesterol-lowering activity.

Various extraction modes in water (infusion, maceration, percolation, ultrasounds, microwaves) were compared in terms of extraction yield, of amount of phenolic compounds (TPC), flavonoids (TFC), and proanthocyanidin oligomers (OPC), and of UHPLC profiles of the extracted compounds. On raw material, microwave extraction mode was the best extraction mode, but the overall extraction yield was limited to 17 % for HAW, 20 % for BC and 27 % for CA for 10 min extraction time. Grinding the plant was found the best way to increase the kinetics and the overall yield of extraction (24 % for HAW, 33 % for BC and 31 % for CA for 10 min extraction time). If the plant is ground, infusion is the simplest way to extract bioactive components from medicinal plants, and the other extraction modes did not significantly improve the extraction yield. As far as the plant is ground, the automatic stirring of the infusion was not required and simple manual stirring at the beginning and the end of the extraction was enough to obtain optimal extraction. A 10 min infusion was a good option to reach drinkable temperature, shorter time was also possible provided that a small ice cube was added to cool down the preparation. The use of a tea bag was not recommended since it slowed down the extraction/diffusion process and decreased the extraction yields.

In the case of HAW, the UHPLC profiles were also very similar from one extraction mode to the other, but strongly depended on the part (flowers or flowering tops) and the state (fresh or dry) of the plant. Dry flowers (without leaves) had higher content in hyperoside but lower contents in apigenin-C-hexoside and vitexin-2-O-rhamnoside compared to dry flowering tops. Much higher content of vitexin-2-O-rhamnoside and lower contents in

apigenin-C-hexoside, procyanidins and chlorogenic acid were obtained in freshly harvested flowering tops compared to dry ones. These differences in chemical composition were confirmed by FT-ICR-MS. Fresh flowering tops contained higher contents in aminoacids, aminosugars, lipids, carbohydrates and some flavonoids. Dry flowers contained more amino acids or amino sugars and flavonoids linked to sugars as compared to dry flowering tops.

A simple, fast and optimized protocol that can be used by anyone at home was developed, using a French-press coffee maker and any commercially grinder to get a granulometry < 1 mm. In the case of HAW, this protocol (3 min infusion of 2.5 g ground flowering tops in 250 mL boiling water) can afford a daily intake of polyphenols, flavonoids, and proanthocyanidin oligomers similar to the recommended dose from standardized hawthorn extracts. Extraction yields about 22 % (in wt of the initial HAW dry plant) can be reached in a repeatable and controlled way. It is worth noting that the global extraction yield remained unchanged for all five HAW lots tested, even if the repartition of active compounds (TPC, TFC and OPC) may vary from one lot to the other. The overall cost for one month of daily HAW intake (one infusion per day) is about 2.2 euros, i.e. about 10 times lower than the cost of the standardized plant extract. Using the same protocol, the global extraction yields were about 26 % for CA and about 28.5 % for BC.

About the differences in chemical composition between the three studied plants, BC contained much more phenolic compounds than the two other plants, while HAW contained much more proanthocyanidin oligomers than the two other plants. CA and BC contained similar amounts of flavonoids. UHPLC revealed that the major compounds detected in UV are flavonol in BC; hydrocinnamic acid derivatives, flavone, flavanone and aurone in CA; flavanol, flavonol and flavone in HAW. In CA extracts, UHPLC-ESI-MS profiles revealed the presence of Flavanomarein and Martitimein derivatives, while FT-ICR MS revealed the specific presence of Oleanolic or Ursolic acid. In BC extracts, Quercetin and Kaempferol derivatives were mainly identified. Vitexin-2-O-rhamnoside, Hyperoside and Isoquercetin were the main components in HAW extracts. In FT-ICR MS, about 2500 hints were obtained for each plant, among which about 1100 hints are common to all 3 plants; about 350 hints are common to any group of two plants; and about 700 hints are specific to each plant.

Regarding their enzymatic and antioxidant activities, an interesting hyaluronidase inhibition ($\geq 90\%$) was reported for HAW extracts (at 1 g L^{-1}), similar to the inhibition observed

with the referenced hyaluronidase inhibitor, and much higher than the two other plant extracts. As for the anti-hypertensive activity, CA extracts demonstrated the highest ACE inhibition (91-97 % at 1 g L⁻¹) closely followed by the HAW extracts (81-92 % at 1 g L⁻¹). About the antioxidant activity, BC extracts had the highest antioxidant capacity in correlation with the highest contents in phenolic compounds and flavonoids, while CA extracts presented the lowest contents in those active compounds and the lowest antioxidant capacity.

In collaboration with the University of Campinas (Group of Prof. Mario Roberto Marostica Junior, DEPAN - FEA – UNICAMP, Brazil), a study aiming at determining the preventive effects of HAW extracts in a murine model of inflammatory bowel diseases, was carried out (see Appendix B). It was found that the HAW extracts produced an anti-inflammatory response in rats with TNBS-induced colitis, by reducing inflammatory mediators and regulating oxidative stress pathways, posteriorly resulting in less colonic necrosis. HAW has thus a promising therapeutic indication against chronic intestinal inflammatory disease, in addition to its well-known cardiovascular protective and anti-hypertensive role. Other *in vivo* biological activities are planned to be studied at UNICAMP, for instance about metabolic disorder / obesity, based on our plant extracts.

In collaboration with the University of Orléans (Group of Prof. Reine Nehmé), a study aiming at screening the effect of aqueous extracts of different parts of *Crataegus oxyacantha* (leaves, flowers and flowering tops), *Ribes nigrum* (blackcurrant) and *Chrysanthellum americanum* on pancreatic lipase (PL) inhibitor, was performed (see Appendix C). It was observed that the highest PL inhibition was obtained by fresh hawthorn leaves extracts (37 ± 3 %). Extracts of *Chrysanthellum americanum* only showed slight PL inhibition (14 ± 0.1 %). On the contrary, activation of PL was noticed in the presence of hawthorn flowers extracts as well as of blackcurrant leaves extracts (26 ± 1 % and 37 ± 1.5 %, respectively).

Tea creaming (i.e., nanoparticles with a size generally from 10 to 400 nm) formation was finally investigated in these herbal tea infusions, from a kinetic and size distributions point of view. It is worth noting that tea creaming could be formed in the case of HAW and BC, but not in the case of CA. However, the tea creaming amount was found much lower than in green tea, probably due to different chemical components. The tea creaming amount was also not dependent on the temperature up to 40°C. The addition of caffeine at 500 mg/L into herbal

tea infusion (blackcurrant leaves or hawthorn flowering tops) did not increase the tea creaming amount. When using tap water instead of distilled water, the tea creaming amount increased by about 20 %, probably due to the presence of metal ions like calcium or magnesium in tap water

Finally, this work highlights the complexity in the composition of these three plant extracts, both in number of chemical components and in chemical structures. It also confirms the potential interest of these medicinal plants toward a broad range of biological activities, including those which are different from the primary therapeutic intention. Last but not least, we believe that the home-made optimized protocol described in this work is of very general use for those who are interested in medicinal plants. This work addresses some crucial keys for the promotion and integration of herbal medicine in modern Western medicine, and for the satisfaction of an increasing societal demand in that field.

APPENDIX

APPENDIX A

Chemical composition of dry flowering tops plant (Lot number: APC27031904)

A.I. Materials and Methods

A.I.1. Chemicals

Dry hawthorn flowering tops (lot number: APC27031904) raw materials (*Crataegus oxyacantha*, origin France) were purchased from France Herboristerie (Noidans-Lès-Vesoul, France). Chlorogenic acid were purchased from HWI Group (Rülzheim, Germany). Other chemicals were described in the chapter I (section II.3.1).

A.I.2. Granulometry

“1 mm” hawthorn material obtained as described in the chapter I (section II.3.2)

A.I.3. Infusion Extraction

A sample of 30 g ground material was infused in 1 L boiling water in 30 min using a ‘French press’ Bodum® (Bistro model, Triengen, Switzerland) under 500 rpm magnetic stirring. After 30 min, the herbal tea solution was filtered first with the Bodum® cover to remove the largest particles, then, the next step was described in the chapter I (section II.3.8) to obtain the dry extract. The same experiment was repeated 17 times to get 100 g hawthorn dry extract. Final lyophilized dry extracts were stored at 4°C.

A.I.4. Total Polyphenols Content (TPC)

The total polyphenols content (TPC) in the hawthorn extract was determined and described in the chapter I (section II.3.10).

A.I.5. Total Flavonoids Content (TFC)

The total flavonoids content (TFC) in the hawthorn extract was determined and described in the chapter I (section II.3.11).

A.I.6. Total Proanthocyanidin Oligomers Content (OPC)

The total proanthocyanidin oligomers content (OPC) in the hawthorn extract was determined and described in the chapter I (section II.3.12).

A.I.7. UHPLC and UHPLC-ESI-MS/MS Analysis

The protocol was described in the chapter II (section III.3.13)

A.II. Results and discussion

A.II.1. TPC, TFC and OPC results

Extraction yields and contents of TPC, TFC and OPC from dry flowering tops are presented in Table A.1. The extraction yield is 20% in mass, meaning that 200mg was extracted from 1 g of dry plant. The values of TPC, TFC and OPC are 28.38 mg eq. GA / g dry plant, 3.84 mg eq. Q / g dry plant and 1.063 mg eq. CY / g dry plant, respectively. Those findings are in good agreement with the literature [1,2].

Table A.1. Extraction yield, TPC, TFC, and OPC values in dry flowering tops of hawthorn extracts. (1mm' grinded) issued from infusion mode at 30 min extraction time using easy-to-use infusion extraction (30 g of plant material in 1L water introduced in Bodum® French press recipient, see section A.I.3), (^a: ±1 standard deviation calculated on *n*=3 repetitions. ^b: in mg eq. GA/g dry plant, ±1 standard deviation calculated on *n*=3 repetitions. ^c: in mg eq. Q/g dry plant, ±1 standard deviation calculated on *n*=3 repetitions. ^d: in mg eq. CY/g dry plant, ± 1 standard deviation calculated on *n*=3 repetitions.

Nature	Lot number	Extraction yield (%)	TPC ^b	TFC ^c	OPC ^d
Dry flowering tops	APC27031904	20.03±0.7 ^a	28.38 ±1.18	3.84 ±0.10	1.063 ±0.087

A.II.2. UHPLC and UHPLC-ESI-MS/MS results

Main compounds found in the hawthorn plant are phenolic acid like chlorogenic acid and flavonoids such as epicatechin, vitexin 2-O-rhamnoside, pinnatifinoside A, hyperoside, isoquercetin and apigenin-C-hexoside (Table A.2 for the retention time and their main fragments ions, Figure A.1 for their UHPLC profiles). Especially, chlorogenic acid, epicatechin,

vitexin 2-O-rhamnoside and isoquercetin presented one of major identified peaks for each subfamily (Figure A.2) are later quantified in this study.

Table A.2. Peak identification of the main compounds detected by UHPLC-DAD in the both dry and fresh hawthorn extract. λ_{\max} are the local maximum of absorbance on the UV spectrum. $[M+H]^+$ column provides the m/z value of the precursor ion. Other ions column gives the m/z value of fragments detected in the MS spectra. Identification method using UV spectrum and ESI(+) spectrum of the pure standards (R) or a secondary standard mixture (R1, *Crataegus* spp. Extract) see ref [4], and * Identification using UHPLC-ESI-MS/MS according to the article Zhang et al [3].

Peak	Retention time (min)	λ_{\max} (nm)	$[M+H]^+$	Other ions in the spectrum	Identified compound	Standard used for identification	Ref
1	4.32	204, 218, 260	288		Cyanidin	R	[1]
2	5.81	218, 236, 324	355	377, 711	5-O-Caffeoylquinic acid	R1	[1]
3	9.79	219, 238, 325	355	377, 711	Chlorogenic acid (3-O-caffeoylquinic acid)	R	[1]
4	12.34	227, 280	579	427, 289	Procyanidin B2	R	[1]
5	12.75	224 279	291	147, 139, 123	Epicatechin	R	[1]
6	15.36	280	867	579	Procyanidin C1	R	[1]
7	17.78	216, 269, 338	579	433, 313	Vitexin 2-O-rhamnoside	R1	[1]
8	18.20	206, 262, 348	415	397, 367, 283	Pinnatifinoside A	*	[2]
9	18.80	220, 256, 353	465	303	Hyperoside	R	[1]
10	19.05	202, 257, 353	303	621	Isoquercetin	R	[1]
11	21.66	268, 337	433	621	Apigenin-C-hexoside	R1	[1]

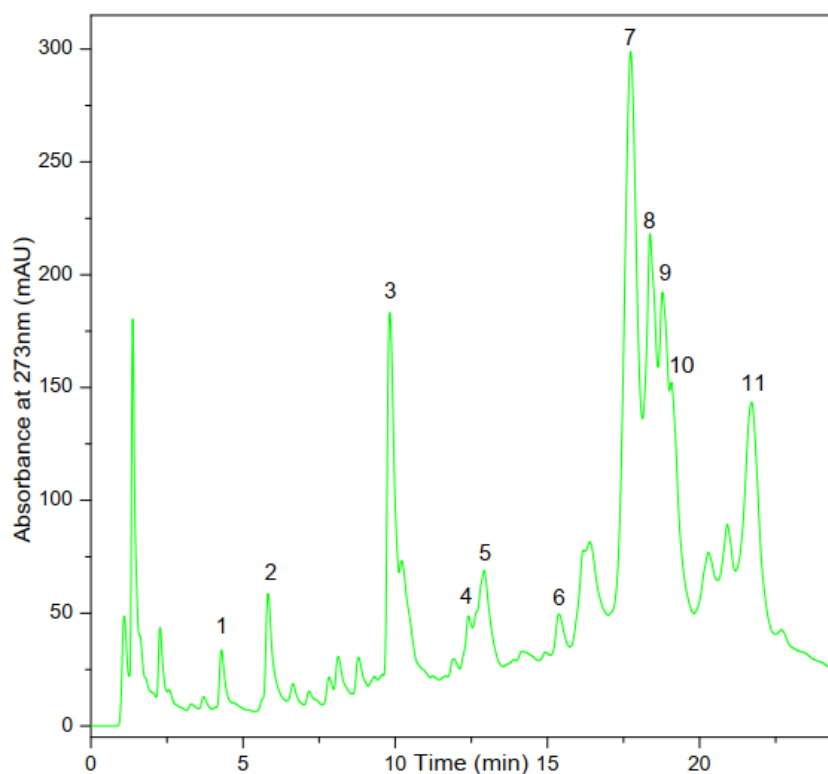


Figure A.1. UHPLC profiles of hawthorn extracts obtained from infusion extraction modes for grinded 1 mm hawthorn flowering tops. Experimental conditions: A Kinetex C18 100A 100×2.1 mm, 2.6 μm column, binary solvent system: water/formic acid (1%, v/v) as solvent A and acetonitrile/formic acid (1%, v/v) as solvent B. Gradient program: 5 % B, then increase of B to 100 % in 35 min with a convex increase, flow rate: 0.3 mL.min⁻¹, injection volume: 20 μL , concentration of injected sample: 4 g/L of dry extract. Column temperature: 20°C, UV monitoring at 273 nm. UV-Vis spectra recorded between 200 and 550 nm. Lot number for grinded 1 mm and 2 mm flowering tops: APC27031904. Peak identification: 1=Cyanidin, 2=5-*O*-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7=vitexin-2-*O*-rhamnoside, 8= Pinnatifinose A, 9=hyperoside, 10=isoquercetin, 11=apigenin C-hexoside.

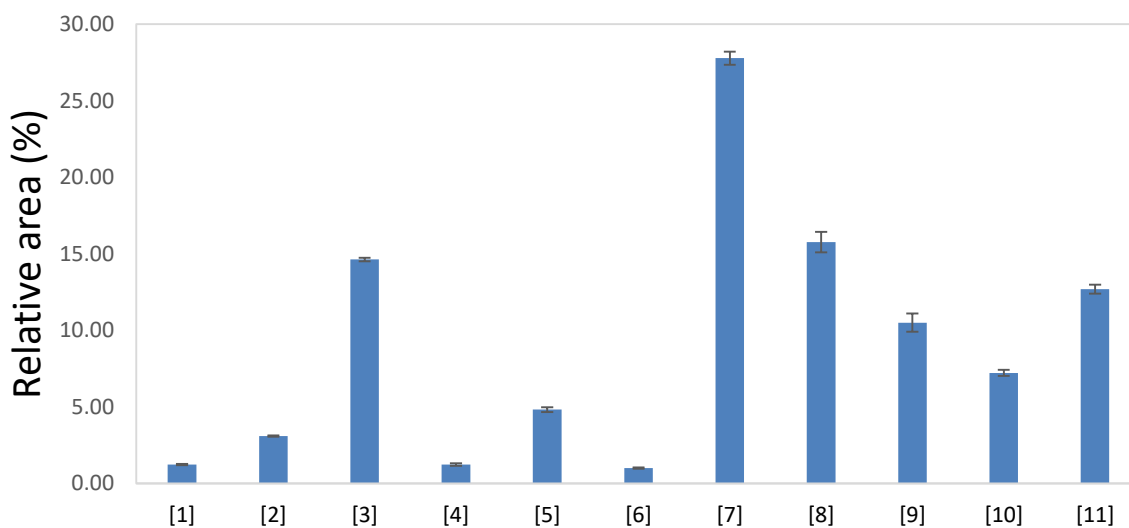


Figure A.2. Relative peak area distributions for the main identified chromatographic peaks according to infusion extraction (lot n° APC27031904)). Same experimental conditions as in Figure A.1. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 11 identified components. Error bars: \pm one standard deviation calculated on $n=3$ repetitions. 1=Cyanidin, 2=5-*O*-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7=vitexin-2-*O*-rhamnoside, 8=Pinnatifinose A, 9=hyperoside, 10=isoquercetin, 11=apigenin C-hexoside.

The quantification of several representative compounds for each subfamily such as chlorogenic acid (peak 3), epicatechin (peak 5), vitexin-2-*O*-rhamnoside (peak 7), isoquercetin (peak 10) was determined by external calibration, using a commercially available standard as presented in Figure A.3. The amount of chlorogenic acid, epicatechin, vitexin-2-*O*-rhamnoside and isoquercetin are 3.10 ± 0.04 , 5.43 ± 0.11 , 4.55 ± 0.14 , 2.55 ± 0.07 mg per g dry plant, respectively, and in good agreement with literature [4-7].

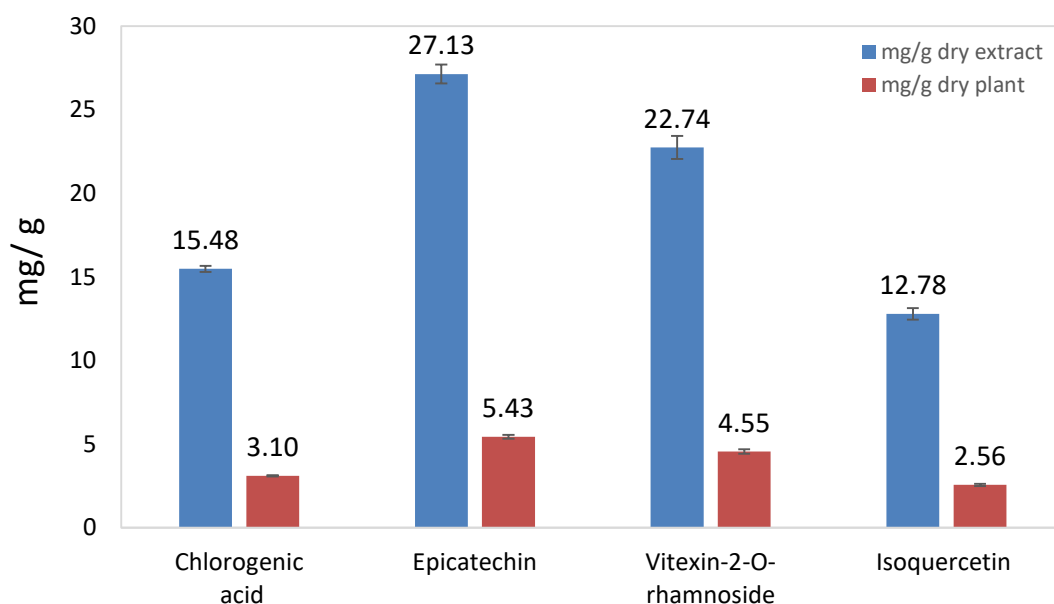


Figure A.3. Quantification of chlorogenic acid (peak 3), epicatechin (peak 5), vitexin-2-*O*-rhamnoside (peak 8), isoquercetin (10) were determined by external calibration, using a commercially available standard. Error bars: \pm one standard deviation calculated on $n=3$ repetitions.

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APPENDIX B**Article 3: Hawthorn extract partially prevents against 2,4,6-trinitrobenzenesulfonic acid-induced colitis***Food research international (Submitted)*

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ABSTRACT

The purpose of the study was to determinate the preventive effects of a phenolic-rich extract from the flowering tops of *Crataegus oxyacantha*, a hawthorn plant, in a murine model of inflammatory bowel diseases (IBDs), with focus in parameters related to inflammation, oxidative stress and microbiome health. To induce colitis, rats were administrated intrarectally with 2,4,6-trinitrobenzene sulfonic acid (TNBS). Hawthorn extract (HE) (100 mg/kg) was given via gavage for 21 days, from which 14 days were preventive, and mesalamine (100 mg/kg), a drug commonly used to treat IBDs, was administrated during the period of disease onset. Dry HE was rich in total phenolic compounds (16.5%), flavonoids (1.8%) and proanthocyanidins (1.5%). Dosage of total phenolic compounds administrated for rats was calculated to be 16.5 mg/kg of body weight. Colitis was successfully induced as seen by high body weight loss, mucosal necrosis and ulceration in colon, infiltration of leukocytes in lamina propria and submucosa, and high levels of inflammatory and oxidative markers ($p < 0.05$). Mesalamine and HE were able to significantly reduce some of these parameters. Specially, the interventions diminished the length of the brownish necrotic lesions formed in distal colon as well as the proportion of the colon affected by the lesions ($p < 0.05$). Also, the drug and the natural extract reduced the levels of the proinflammatory cytokine IL-1 β

($p < 0.05$) and myeloperoxidase ($p < 0.01$), markers typically associated with neutrophil infiltration. Regarding the differences between the effects of the two interventions, only mesalamine was able to reverse the body weight loss induced by TNBS ($p < 0.05$), while only HE promoted a regulation in two enzymes related to oxidative stress, such as glutathione reductase (GR) ($p < 0.001$) and catalase (CAT) ($p < 0.01$). Histological scoring was not changed by the interventions, but it was highly correlated with the area of the necrotic lesion ($r = 0.708$), a parameter found diminished in both mesalamine and HE groups. For the most part, the enzymes of oxidative stress did not show any relevant correlation with others parameters, with exception of the inverse correlation between GR and CAT ($r = -0.732$). Finally, despite being rich in polyphenols, including vitexin-2-O-rhamnoside, HE did not increase the antioxidant capacity of the serum or the concentration of short-chain fatty acids in the feces, what could be related to the dosage administrated to rats. Such results indicate that HE given at 100 mg/kg is partially effective against TNBS-induced colitis mainly due to its anti-necrotic, anti-inflammatory and anti-oxidative effects. More studies need to be performed in order to understand the implications of higher doses or other formulations of the hawthorn plant in animals and humans with intestinal diseases.

Keywords: *Crataegus oxyacantha*, Crohn's disease, chlorogenic acid, flavonoids, inflammation, inflammatory bowel diseases, necrosis, oxidative stress, polyphenols, TNBS, vitexin-2-O-rhamnoside, ulcerative colitis.

ABBREVIATIONS

CAT – Catalase
CD – Crohn's disease
DAD - Diode array detection
DAI - Disease Activity Index
ELISA - Enzyme linked immunosorbent assay
ESI - Electrospray ionization
FID - Flame ionization detector
FRAP - Ferric reducing antioxidant power
GC - Gas chromatograph
GR - Glutathione reductase
GSH – Glutathione
HE - Hawthorn extract
IBD – Inflammatory bowel disease
IL - Interleukin
MDA – Malondialdehyde
MPO – Myeloperoxidase

MS - Mass spectrometry
ORAC - Oxygen-radical absorbing capacity
SCFA – Short-chain fatty acid
SOD - Superoxide dismutase
TFC - Total flavonoids content
TNBS - 2,4,6-trinitrobenzene sulfonic acid
TNF- α - Tumor necrosis factor alpha
TPC – Total polyphenols content
TPOC - Total proanthocyanidin oligomers content
UC - Ulcerative colitis
UHPLC - Ultra-high-performance liquid chromatography

1. INTRODUCTION

In the last 20 years, there was increasingly *in vitro* and *in vivo* evidences of the beneficial health effects of functional foods and extracts from plants. Such alternative or complementary medicine has been suggested as a new tool against modern diseases, which eventually may impact in the future for changes in prevention guidance's and in the treatment of debilitating chronic illness, such as cardiovascular diseases, inflammatory bowel diseases (IBDs) and cancer (Campos, 2019). However, more studies are needed in order to understand and deepen the effective dosages and mechanisms behind the biological action of natural bioactive compounds. Specially, the research on the utilization of the residues of plants, usually commercially discarded, such as stems, leaves and flowers, is probably one of the strands that should be more investigated within the field of medicinal plants and herbs, given their great chemical and nutritional composition (Simpson et al., 2019).

Europe is a continent that possess a wide range of native plants spread thought its territory, with one of the most common ones been hawthorn (*Crataegus spp.*), a tree belonging to the Rosaceae family (Chang et al., 2002). Hawthorn is commonly used as a hedging species in England and Denmark (Billing et al., 1974; Weber, 2010), but also as a favorable herbal remedy trough the continent (Dahmer & Scott, 2010). Specially, the hawthorn extracts (HEs) from the leaves and flowers of *Crataegus oxyacantha* and *Crataegus monogyna*, has been officially approved in Germany to treat patients with mild to moderate chronic congestive heart failure due to its high content in flavonoids and proanthocyanidins leading to cardi tonic activity (Nathan, 1999; Cao-Ngoc et al., 2019). It is known that flavonoids and proanthocyanidins can (1) decrease inflammatory processes, (2) act by augmenting the activity of enzymes that control the oxidation of the cell membrane (Panche et al. 2016; Rauf et al., 2019) and (3) possibly functioning as modulators of the microbiome

environment by being fermented by bacteria (Cardona et al., 2013). Despite that, the effects of HEs rich in polyphenols has been mostly associated with cardiovascular complications and hypertension (Dalli et al., 2011; Walker et al., 2006) and research on their effects in intestinal health and against gut diseases, like IBDs, for example, are lacking.

IBDs, mainly ulcerative colitis (UC) and Crohn's disease (CD), largely affect the gastrointestinal tract, especially the colon, producing a high-grade immune-associated inflammatory and oxidative process (Ungaro et al., 2017). Such diseases possess a high prevalence in Europe and are increasing at a high incidence rate since the 90's in newly industrialized countries, such as Brazil and Taiwan (Ng et al., 2017). Treatment options for IBDs are commonly not well accepted or tolerable for a great part of patients (Horne et al., 2009; Lasa et al., 2020; Pieper et al., 2009), suggesting the need for new studies capable of proving new effective options for such illnesses. From our knowledge, two studies to date has attested the positive effects of HEs in models of IBDs, however these are limited to the fruit. For example, in a study by Fujisawa et al. (2005), the freeze-dried aqueous extract from the fruit of *Crataegi fructus* given in the drinking water was able to prevent against the inflammatory outcome and high mortality of chemically-induced models of colitis (Fujisawa et al., 2005). In another study, by Liu, Zhang, Ji (2020), the freeze-dried hydroalcoholic extract from the fruit of *Crataegus pinnatifida*, rich in flavonoids, inhibited the secretion of proinflammatory cytokines and alleviated the increase of paracellular permeability in Caco-2 cells, therefore, indicating it's protective effects against epithelial barrier dysfunction. Such initial studies indicate the potential of the hawthorn plant to protect against IBDs.

No publication to date investigated the preventive effects of an extract from hawthorn flowering tops (leaves and flower buds) in broader aspects related to IBDs, such as inflammation, oxidative stress and microbiome health. Therefore, in order to understand if the effects of the non-edible residual parts of this plant are extended further than its most-known cardioprotective role, this study aimed to investigate the preventive implications of the administration of a phenolic-rich extract from *Crataegus oxyacantha*' flowering tops in a model IBD-like colitis induced in rats by 2,4,6-trinitrobenzene sulfonic acid (TNBS).

2. MATERIAL AND METHODS

2.1. Chemicals

Aluminum chloride hexahydrate, n-butanol, Folin-Ciocalteu reagent, gallic acid (GA), quercetin (Q), and cyanidin chloride (CY) were purchased from Merck (Saint-Quentin Fallavier,

France). *Crataegus spp.* extract standard (R1) was purchased from HWI Group (Rülzheim, Germany) and standardized at 29 mg of vitexin 2-O-rhamnoside per g of extract. Procyanidin A2, B2 and C1, epicatechin, cinnamtannin A2, and isoquercetin were purchased from Phytolab (Vestenbergsgreuth, Germany). 2,2'-Azobis(2-amidinopropane) dihydrochloride was purchased from Cayman Chemical (Ann Arbor, MI, USA), fluorescein was purchased from Synth (Diadema, SP, Brazil) and metaphosphoric acid was purchased from Vetec Quimica Fina Ltda. (Rio de Janeiro, RJ, Brazil). Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Thiobarbituric acid was purchased from Merck KGaA (Darmstadt, Germany). Enzyme linked immunosorbent assay (ELISA) kits were purchased from Peprotech (Rocky Hill, NJ, United States). 2,4,6-Tripyridyl-S-triazine, 5,5'-dithiobis-(2-nitrobenzoic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or trolox, bovine serum albumin, catalase (CAT), glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO), nitro blue tetrazolium chloride, oxidized glutathione, reduced nicotinamide-adenine dinucleotide phosphate, standards of short-chain fatty acids (SCFAs) (2-ethylbutyric acid, acetic acid, butyric acid and propionic acid), sodium dodecyl sulfate and TNBS were all purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Extraction and chemical characterization of hawthorn

Dry hawthorn flowering tops (*Crataegus oxyacantha*) (mix of stems, leaves and blooms) were purchased from France Herboristerie (Lot number 55849, Noidans-Lès-Vesoul, France). The plants were previously grounded using a Ika grinder (Ika-Werke GmbH, Model MF10 basic, Staufen, Germany) and a "1 mm" grid. Extraction was performed according to Cao-Ngoc et al. (2019). A sample of 50 g was infused in 1 L of boiling water using a 'French press' Bodum® under 500 rpm magnetic stirring. After 30 min, the herbal tea solution was filtered first with the Bodum® cover to remove the largest particles, then with a Whatman filter paper placed in a Büchner funnel using a vacuum pump to remove any residue of solid plant. Finally, the herbal tea solution was concentrated using a rotary evaporator and later freeze-dried (Cryotec Model CRIOS-80, Saint-Gély-du-Fesc, France) to obtain the dry hawthorn extract (HE). The same experiment was repeated 3 times to get a total of 30 g. Lyophilized extracts were stored at 4°C (Cao-Ngoc et al., 2019).

Total polyphenols (TPC) (mg of gallic acid equivalent/g plant), total flavonoids (TFC) (mg of quercetin equivalent/g plant) and total proanthocyanidin oligomers (TPOC) (mg of cyanidin equivalent/g plant) contents were determined according to the Folin-Ciocalteu

method (Singleton & Rossi, 1965), the aluminum chloride method (Lamaison & Carnat, 1990) and the HCl/n-butanol assay (Porter et al., 1985), respectively. Analyses were performed in triplicate.

For the identification of specific phenolic compounds, an ultra-high-performance liquid chromatography associated with diode array detection (UHPLC-DAD) or coupled with electrospray ionization and mass spectrometry (UHPLC-ESI-MS) were utilized. First, 20 mg of the dry extract was dissolved in 1 mL of water and vortexed for 2 min. The resulting solution was diluted five times with water and vortexed for another 2 min prior analysis.

The UHPLC-DAD system consisted of a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS equipped with a WPS-3000TBRS auto sampler and a TCC-3000RS column compartment set at 35°C (ThermoFisher Scientific, Waltham MA, USA). The system was operated using Chromeleon 7 software. A Luna® Omega polar C18 column (1.6 µm, 100 × 2.1 mm) combined with a security guard ultra-cartridge was used (Phenomenex Inc., Torrance CA, USA). A binary solvent system was utilized, consisting of water/formic acid (1%, v/v) as solvent A and acetonitrile/formic acid (1%, v/v) as solvent B. The gradient program started with 5% B, then B was increased to 100% in 30 min with a convex increase (curve 5 in Chromeleon 7). The flow rate of the mobile phase was 0.4 mL/minute and the injection volume was 4 µL. The peaks were monitored at 273 nm. The UV–Vis spectra of the different compounds were recorded between 200 and 550 nm using the diode array detector.

The UHPLC-ESI-MS analysis was performed using a Synapt G2-S (Waters Corp., Milford MA, USA) equipped with a ESI. The column, injection volume, flow rate and gradient program were the same as for UHPLC-DAD. A positive mode was used. The capillary voltage was set to 3 kV, the cone voltage was set to 30 V and the extractor voltage was set to 3 V. The source temperature was 100°C and the desolvation temperature was 450°C. MS spectra were obtained by scanning ions between 100 and 1500 m/z. The system was operated using MassLynx 4.1 software.

The quantification of vitexin-2-O-rhamnoside, a common and abundant flavonoid found in hawthorn species (Alirezalu et al., 2018; Kumar et al., 2012), was determined by external calibration, using a commercially available standard.

2.3. Animal experimentation

2.3.1. Ethics, diet and conditions

The study is in accordance with the ARRIVE guidelines and followed the guide for the care and use of laboratory animals of the National Institutes of Health (NIH Publications No. 8023, revised 1978). The experimental protocol was approved by the Ethics Committee on the Use of Animals of the Universidade Estadual de Campinas (UNICAMP) (number 5042-1/2018).

Male Wistar rats (n=29) were obtained from the Multidisciplinary Center for Biological Research on Laboratory Animal Science at UNICAMP and allocated in an experimentation room with temperature at controlled levels (20-22°C). Rats were submitted to a standard daily 12 h:12 h light-dark cycle. During the acclimatization and experimental periods, rats received a commercial pelleted diet (Nuvilab CR-1, Nuvital) and water *ad libitum*. Animals entered the experimentation room with four weeks and were submitted to the procedures starting at seven weeks of age.

2.3.2. Intervention, colitis induction and clinical evaluation

A total of 29 rats were divided in five experimental groups: *control-C* (n=5), *control colitis-CC* (n=6), *hawthorn-H* (n=5), *hawthorn colitis-HC* (n=6) and *mesalamine colitis-MC* (n=7). Dry HE was diluted in distilled water and given preventively for 14 days at a dosage of 100 mg/kg of body weight via gavage. The dosage chosen was based on the majority of studies found in the literature (Elango et al., 2009; Elango & Devaraj, 2010; Hatipotlu et al., 2015). As other studies tested the effects of HE in larger safe dosages, such as 200 mg/kg, (Aierken et al., 2017; Tadic et al., 2008), we considered ours to be a medium dosage. The intervention with HE continued until the end of the experiment, including the period after colitis induction. Other groups (*C*, *CC*, *MC*) received instead distilled water via gavage.

Acute colitis was induced on day 14. TNBS (10 mg) was dissolved in 250 µL of 50% ethanol (v/v) (Da Silva-Maia et al., 2019) and administrated via rectal in rats previously sedated intraperitoneally with ketamine (75 mg/kg of body weight) and xylazine (10 mg/kg of body weight). Groups without colitis induction (*C*, *H*) received saline solution (0.9%) instead of TNBS.

Mesalamine, a drug commonly used to treat IBDs (Lacucci et al., 2010), was diluted in distilled water and given via gavage at a dosage of 100 mg/kg/day (Da Silva-Maia et al., 2019). Mesalamine was administrated for seven days, from the day of the induction until the end of the experiment.

During the whole experiment, the dietary intake (g) and body weight (g) were measured. Additionally, for the period of colitis, the Disease Activity Index (DAI) was assessed (days 14, 15, 17, 19 and 20), according to described by Gommeaux et al. (2007). The DAI evaluates the body weight loss, anal bleeding and stool consistency (Gommeaux et al., 2007). Photographs of the rat's stool and anus were taken for the analyses of anal bleeding and stool consistency. A researcher, blind to the identification of the groups and with experience in IBDs, did the evaluations. A photographic representation of the scores of bleeding and stool consistency from this study was created similarly to Nascimento et al. (2019) in order to showcase more visually the differences between the scores degrees. A Nikon® SLR D3100 digital camera with 14.2-megapixel resolution and a 18-55 mm lens was utilized.

2.3.3. Euthanasia and tissue measurements

Euthanasia was performed on day 21 by an intraperitoneal administration of ketamine (300 mg/kg of body weight) and xylazine (30 mg/kg of body weight). Exsanguination followed by cervical dislocation was realized to confirm the death.

Colon was dissected, cleaned with saline solution, weighted (g) and measured with a ruler (cm). The colon weight/colon length (g/cm) ratio was analyzed. Brownish necrotic lesions are common features of TNBS-induced colitis (De Almeida et al., 2015; Paiotti et al., 2013), therefore, their width (cm), length (cm) and estimated area (width x length, cm²) were measured for this study. Additionally, the relation between the larger necrotic lesion length (cm) and the colon length (cm) was evaluated as an indicator of the colon proportion (%) affected by the disease. A small portion of the distal colon was separated for histology and the remaining was preserved in -80°C for further analyses (inflammation and oxidative stress).

Other biological tissues were collected and/or evaluated. The blood was centrifuged and the serum collected and stored in -80°C for posterior antioxidant analyses. The feces from cecum and colon were collected and kept on -80°C for the analysis of the concentration of SCFAs. The liver, spleen and kidney were weighted (g). The carcasses of the animals and unused tissues or organs were discarded as biological materials.

2.4. Histopathology

A sample of the distal colon was disposed in a small piece of paper and preserved in formaldehyde 4% (v/v) until histological processing. Briefly, samples were dehydrated with increasing concentrations of ethanol, included in paraffin blocks and subjected to sectioning

follow by insertion in slides. Slides were standardly stained with hematoxylin and eosin and analyzed by a pathologist, blind to the identification of the groups, according to a score modified from Dieleman et al. (1998) (**Table 1**). Samples were analyzed at 100x, 200x and 400x magnifications.

Table 1: Histological grading of colitis.

Score	Leukocyte infiltration	Inflammation extent	Crypt damage
0	None	None	None
1	Discrete	Mucosa	1/3 damaged (basal)
2	Moderate	Mucosa and submucosa	2/3 damaged (basal)
3	Severe	Transmural	Only the surface of the epithelium is intact
4	-	-	Loss of crypt and epithelium

Area involved in percentage (score): 1–25 (1), 26–50 (2), 51–75 (3), 76–100 (4)

For each category of the score (leukocyte infiltration, inflammation extent and crypt damage), points were multiplied by a factor of involvement of the colonic tissue. The sum of the three categories add up to the total score of each section (Dieleman et al., 1998).

2.5. Antioxidant capacity of the serum

The total antioxidant capacity of the serum was evaluated by two methods, the ferric reducing antioxidant power (FRAP) and the oxygen-radical absorbing capacity (ORAC). Trolox was utilized as a standard for both analyses. Before the tests, samples were submitted to a treatment with metaphosphoric acid in order to extract the antioxidant substances (Leite et al., 2011).

For the FRAP analysis, samples were added with a solution composed of 2,4,6-tripyridyl-S-triazine, ferric chloride and acetate buffer at proportions of 1, 1 and 10, respectively. Samples were included in a microplate and incubated for 30 min at 37°C. The absorbance was read at 595 nm using an Epoch™ spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA), which was utilized for all the applicable analyses. The results were expressed in μmol of trolox equivalent/mL of serum (Rufino et al., 2006).

For the ORAC analysis, in a microplate, samples were added with fluorescein and incubated for 10 min at 37°C. After that, 2,2'-azobis(2-amidinopropane) dihydrochloride was added up and the absorbance was read per minute during 80 min with filters set at 520 nm

for emission and 485 nm for excitation. The area under the curve was calculated and the results were expressed in μmol of trolox equivalent/mL of serum (Prior et al., 2003).

2.6. Inflammation and oxidative stress analyses

Colon samples were homogenized in a potassium phosphate buffer (75 mM, pH 7.4) and centrifuged at 10,000 rpm for 30 min. The supernatant was collected and subjected to protein quantification (mg of bovine serum albumin/mL) by the Bradford assay. Supernatants were utilized for the determination of MPO and cytokines concentration, and for the oxidative stress analyses.

The concentration of MPO (U/g protein), tumor necrosis factor alpha (TNF- α) and interleukin (IL) 1 beta (ng/g of protein or mL of serum) were determined as indicators of inflammation. MPO was determined by mixing the diluted homogenates (1:4) or a standard with a cocktail of O-dianisidine dihydrochloride and hydrogen peroxide. MPO was utilized as the standard. Absorbance was read every minute during 10 min at 460 nm and the area under the curve was calculated (Winterbourn et al., 1975). The concentrations of the proinflammatory cytokines TNF- α and IL-1 β in colon homogenates and serum were determined by ELISA, following the protocols of commercial kits.

The activity or concentration of CAT (U/g of protein), glutathione reductase (GR) (consumed NADPH/min/g of protein), GSH (nmol of GSH/mg of protein), MDA (nmol of MDA/g of protein) and superoxide dismutase (SOD) (SOD/g of protein) were determined as indicators more closely related to oxidative stress.

The CAT assay was realized by mixing first the diluted homogenates (1:2) with hydrogen peroxide and incubating for two min in 37°C. After that, ammonium metavanadate was included and the absorbance was read after 10 min at 452 nm. CAT was utilized as a standard. CAT activity was determined by a logarithmic formula, adapted from a recent methodological approach (Hadwan & Ali, 2018).

For the GR analysis, homogenates (diluted at 1:1 or not diluted, depending on the sample) were added with a cocktail containing reduced nicotinamide-adenine dinucleotide phosphate, oxidized glutathione and ethylenediamine tetraacetic acid. The reading was realized at 340 nm per minute for a total of 10 min and the area under the curve was calculated (Carlberg & Mannervik, 1985).

GSH was determined by first mixing a tris plus ethylenediamine tetraacetic acid buffer with diluted homogenates (1:4) or a standard and read at 421 nm. This was followed by the

inclusion of 5,5'-Dithiobis-(2-nitrobenzoic acid) and a 15 min incubation period. The second reading was done also at 421 nm. GSH was utilized as the standard. The calculation was realized by subtracting the first reading data from the second one follow by linear regression (Yoshikawa et al., 1993).

The concentration of MDA was determined by the thiobarbituric acid reactive substances (TBARS) method. Briefly, samples or a standard were mixed with sodium dodecyl sulfate and a solution containing thiobarbituric acid, sodium hydroxide and acetic acid. This mixture was left for 60 min in a boiling water bath and after, cooled for 10 min. Samples were centrifuged at 10.000 rpm for 10 min at low temperature (4°C). The absorbance of the supernatant was read at 532 nm. MDA was utilized as the standard (Ohkawa et al., 1979).

SOD was quantified by mixing the diluted homogenates (1:4) with a cocktail composed of hypoxanthine, xanthine oxidase and nitro blue tetrazolium chloride. Absorbance was read every minute during 10 min at 560 nm and the area under the curve was calculated (Winterbourn et al., 1975).

All analyses were performed in a microplate and the absorbance read in a spectrophotometer.

2.7. Short-chain fatty acids concentration

Feces samples preparation followed the protocol described by Zhao, et al. (2006), with modifications. Approximately 300 mg of stool was diluted in distilled water (1:6) and mixed with HCl for adjustment in pH 2. Afterwards, the samples were centrifuged at 3000 rpm for 30 min. The supernatant was collected and mixed with the internal standard 2-ethyl-butyric acid. Once prepared, the samples were injected into a gas chromatograph coupled to a flame ionization detector. The samples were injected with an auto-injector brand Shimadzu, model Ai20, in a GC-FID Shimadzu, model GC-2010 plus, equipped with a capillary column of fused silica Nukol (30 mx 0.25 mm, inside diameter x 0.25 µm). The chromatographic conditions were as follow: injector at 200°C operating in split mode 1:5 for 1.0 min; helium carrier gas at 1 mL/min; oven temperature ramp starting at 100°C, with an increase of 8°C/min to 190°C, remaining at this temperature for 3.25 min; and detector at 200°C. The analytes were identified by co-injection of authentic standards. All chromatographic analyzes were performed in triplicate. To quantify the area of the analytes of interest and express it in terms of concentration, the internal standard method was used (Zhao et al., 2006).

2.8. Statistical analysis and Pearson's correlation

Results are presented as mean \pm standard deviation (SD) for data from the chemical characterization and as mean \pm standard error of the mean (SEM) for data from the biological analyses.

For data from the biological analyses, first, outliers were discarded after the realization of the Grubbs test (5%). Then, One-way ANOVA followed by Tukey (parametric data) was utilized when making comparisons among all experimental groups. Two-way ANOVA followed by Tukey (parametric data) was utilized for analyses over time (body weight, DAI). Data between groups were considered statistically different when $p < 0.05$.

Data was submitted to Pearson's correlation and included in a correlation matrix. For the correlations, were only considered parameters previously attested to be significantly altered by the induction of colitis or by the intervention with the HE ($p < 0.05$). Groups without colitis induction (*C*, *H*) were not included, as they could biased the results. Pearson's *r* placed between 0.70 and 1 (positive) or -0.70 and -1 (negative) were classified as highly correlated (Hinkle et al., 2003). A heatmap was created to represent the correlation matrix.

Analyses were performed using GraphPad Prism.

3. RESULTS AND DISCUSSION

3.1. Extract characterization and dosage of compounds administrated to rats

Extraction yield was about 20% in mass, meaning that 200 mg of HE was obtained from 1 g of dry ground plant. Contents of TPC, TFC and OPC from both dry plant and dry HE are presented in **Table 2**. The levels found from the three analyses are in accordance with recent studies using similar plant species and portion (Issaadi et al., 2020; Cao-Ngoc et al., 2019). By utilizing only water for the extraction of ground plant by infusion, the results of TPC (33.11 mg/g of dry plant) shown in the present study are comparable or even higher than less green extracts utilized by the literature (Edwards et al., 2012). For example, in a study by Alirezalu et al. (2018), the TPC of different *Crataegus* species (flowers and leaves), extracted with methanol/water (80%, v/v), ranged mostly between 20 to 50 mg/g (Alirezalu et al., 2018). Also, in a study by Keser et al. (2014), the polyphenolic content of the water extract of *Crataegus oxyacantha*' leaves and flowers were found to be higher than the extract with 100% ethanol, while also possessing a high antioxidant activity. Such findings suggest that the

utilization of only water for the extraction of hawthorn can be equally important to other solvents, as far as the dry plant is ground (< 1 mm).

Table 2: Chemical characterization of hawthorn.

Analysis	Dry plant	Dry HE*
TPC (mg of gallic acid equivalent/g)	33.11 ± 0.44	165.55 ± 2.20
TFC (mg of quercetin equivalent/g)	3.76 ± 0.14	18.8 ± 0.70
TPOC (mg of cyanidin equivalent/g)	3.07 ± 0.12	15.35 ± 0.60

HE: hawthorn extract. TPC: total polyphenols content, TFC: total flavonoids content, TPOC: total proanthocyanidin oligomers content. Analyses were performed in triplicate. Results are presented as mean ± SD. *Considering an extraction yield of 20%.

Main compounds found in the hawthorn plant were flavonoids, like chlorogenic acid, vitexin 2-O-rhamnoside, pinnatifinoside A, hyperoside and apigenin-C-hexoside, as detected by the UHPLC retention time (**Table 3**) and peak (**Figure 1A**) profiles. Especially, vitexin 2-O-rhamnoside presented the highest relative peak area (**Figure 1B**), being later quantified. The content of this flavonoid by UHPLC was determined to be 3.53 ± 0.036 mg/g of dry plant or 17.66 ± 0.18 mg/g of HE. Vitexin 2-O-rhamnoside has been found to be the main flavonoid found in hawthorn species, with content reaching up to 6.6 mg/g of dry plant (Martino et al., 2008) or 26 mg/g of the extract (i.e. 5.2 mg/g of dry plant, Cao-Ngoc et al., 2019). Studies has shown the capacity of vitexin 2-O-rhamnoside to protect against induced-oxidative stress damage (Wei et al., 2014) and endothelial injury (Zhu et al., 2006), making it also a probable candidate to inflammatory intestinal conditions. However, such flavonoid has poor oral bioavailability in rats (less than 5%) (Gao et al., 2016), what could indicate the possibility of such compound to be actually fermented by the microbiota in similarity to other prebiotic-like flavonoids (e.g., anthocyanins) (Kawabata et al., 2019), but studies are needed to investigate such assumption.

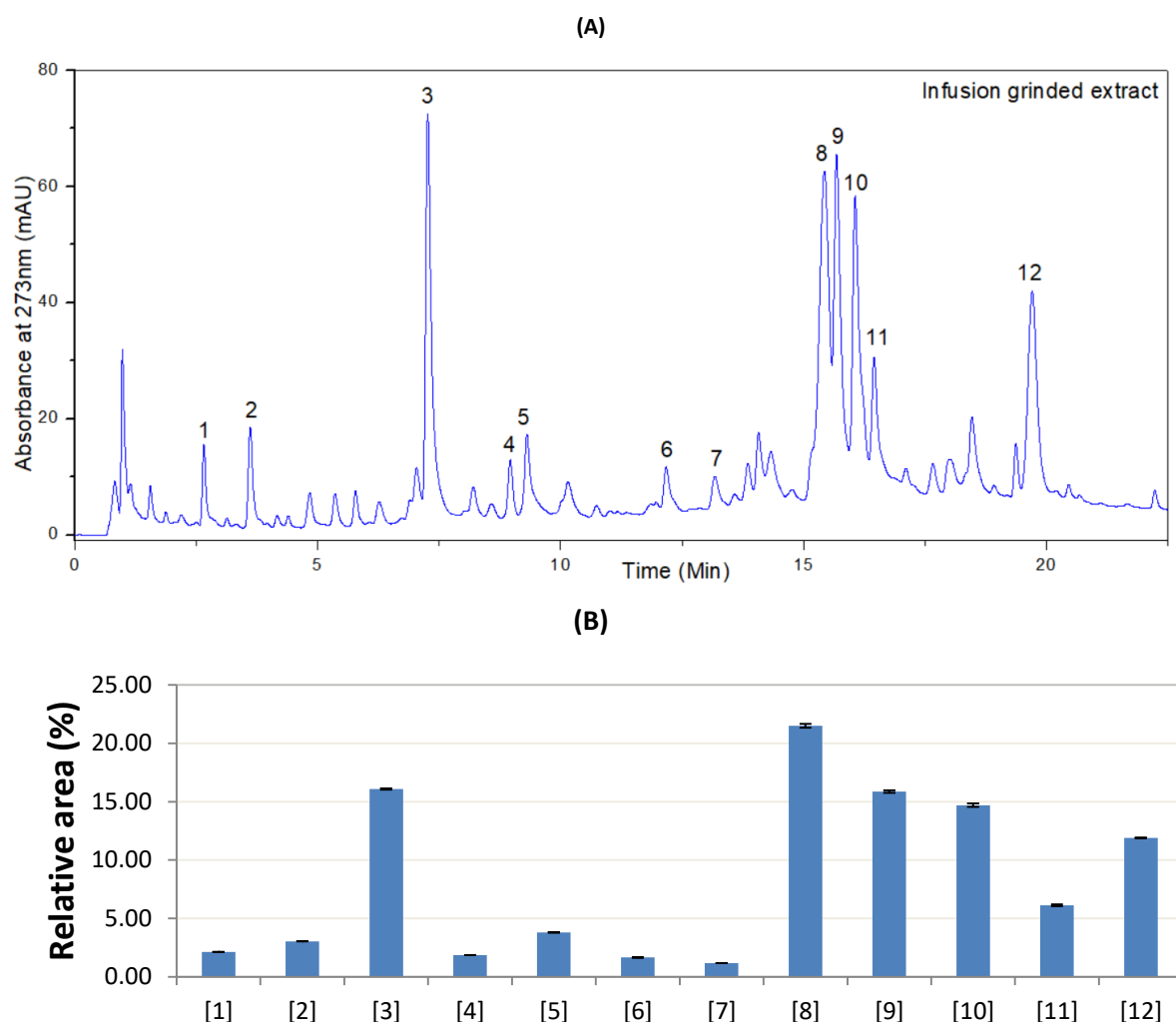


Figure 1: UHPLC profile of the main identified polyphenols in HE.

Peak or relative area (%) identification: 1: Cyanidin, 2: 5-*O*-caffeoylquinic acid, 3: chlorogenic acid, 4: procyanidin B2, 5: epicatechin, 6: procyanidin C1, 7: cinnamtannin A2, 8: vitexin-2-*O*-rhamnoside, 9: pinnatifinose A, 10: hyperoside, 11: isoquercetin, 12: apigenin C-hexoside. The analysis was performed in triplicate. **A.** Peak profile of compounds from hawthorn extract by UV monitoring at 273 nm. **B.** Relative peak area (%) of compounds peaks from hawthorn extract. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 12 identified components. [Color figure.](#)

Table 3: Peak identification of the main compounds detected by UHPLC-DAD.

Peak	Retention time (min)	λ_{\max} (nm)	$[M+H]^+$	Other ions in the spectrum	Identified compound
1	2.71	204, 218, 260	288		Cyanidin
2	3.73	218, 236, 324	355	377, 711	5- <i>O</i> -Caffeoylquinic acid
3	7.49	219, 238, 325	355	377, 711	Chlorogenic acid (3- <i>O</i> -caffeoylquinic acid)
4	9.1	227, 280	579	427, 289	Procyanidin B2

5	9.45	224 279	291	147, 139, 123	Epicatechin
6	12.24	280	867	579	Procyanidin C1
7	13.37	219, 280	1155	287, 413, 575	Cinnamtannin A2
8	15.42	216, 269, 338	579	433, 313	Vitexin 2-O-rhamnoside
9	15.68	206, 262, 348	415	397, 367, 283	Pinnatifinoside A
10	16.13	220, 256, 353	465	303	Hyperoside
11	16.52	202, 257, 353	303	621	Isoquercetin
12	19.85	268, 337	433	621	Apigenin-C-hexoside

λ_{\max} is the local maximum absorbance in the UV spectrum. $[M+H]^+$ provided the m/z value of the precursor ion. Other ions provided the m/z value from fragments detected in the mass spectrometry.

Considering the results from the chemical characterization of the hawthorn plant, for the animal experimentation rats received daily an estimated 16.5 mg of total phenolic compounds/kg of body weight and 1.76 mg of vitexin 2-O-rhamnoside/kg of body weight during 14 days for prevention and the following 7 days while with colitis.

3.2. Macroscopic and microscopic parameters

Colitis was successfully induced in rats, as seen by an increased body weight loss (**Figure 2A**) and high DAI starting three days from the induction ($p < 0,01$) (data not shown). In general, animals with colitis presented softening of the stools and little bleeding, as shown by the photographic representation of their respective scores in **Figures 3** and **4**, respectively. As expected, the treatment with 100 mg/kg of mesalamine recovered the body weight of the animals at the end of the experiment ($p < 0.001$). Mesalamine is a common drug utilized for IBDs, as it can block cyclooxygenases, ILs and TNF- α , therefore controlling the inflammatory process and contributing to preventing against common intestinal symptoms and body weight loss (Lacucci et al., 2010). HE was not able to significantly reverse the body weight loss ($p = 0.0560$), but rats from this group tended to return to its initial body weight, before colitis induction (**Figure 2A**).

Only the rats in the CC group presented high colon weight/length ratio ($p < 0,05$) when compared to C group (**Figure 2B**), potentially indicating the anti-inflammatory effects of the interventions with mesalamine and HE. The occurrence of necrotic lesions was evident in all

rats that received TNBS (**Figure 2C**). Both mesalamine and HE significantly decreased the length ($p < 0,001$) and the area ($p < 0,01$) of the largest brownish necrotic lesion, as well as the proportion of the colon affected by it ($p < 0,05$) when compared to the CC group. No differences were found between the MC and HE groups regarding such parameters (**Table 4**).

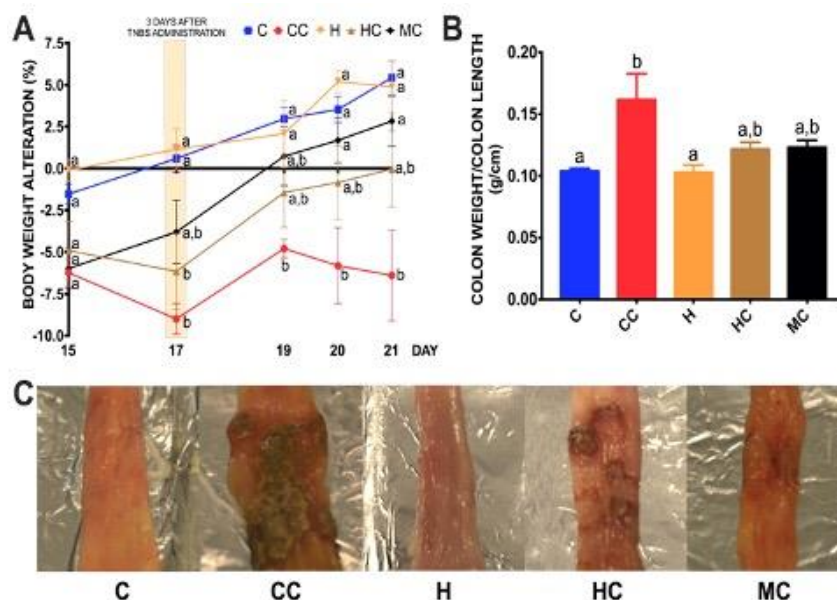


Figure 2: Body weight, colon weight/length ratio and appearance of necrotic lesions.

C: control, CC: control colitis, H: hawthorn, HC: hawthorn colitis, MC: mesalamine colitis. Results are presented as mean \pm SEM. One-way or Two-way ANOVA followed by Tukey. Different letters indicate statistical significance ($p < 0,05$). **A.** Body weight alteration (%) after colitis induction (days 15 to 21). Calculation was determined in relation to day 14. **B.** Colon weight/colon length (g/cm) ratio as an inflammatory index. **C.** Photographic representation of the necrotic lesions on the colonic mucosa of rats with colitis. [Color figure.](#)

Table 4: Macroscopic and microscopic parameters.

Largest necrotic lesion					
Parameter	C	CC	H	HC	MC
Width (cm)	0a	2.04 \pm 0.31b	0a	1.05 \pm 0.28b	1.13b \pm 0.27b
Length (cm)	0a	2.38 \pm 0.17b	0a	0.96 \pm 0.22c	0.92 \pm 0.27c
Area (width x length, cm ²)	0a	5.02 \pm 1.13b	0a	1.31 \pm 0.53a	0.72 \pm 0.27a
Lesion/colon length (%)	0a	11.51 \pm 1.40b	0a	4.67 \pm 1.20a	4.83 \pm 1.75a
Histology*					
Leukocyte infiltration	0a	8.20 \pm 1.80b	0a	7.33 \pm 0.98b	4.85 \pm 0.73b
Inflammation extent	0a	7.40 \pm 1.69b	0a	7.00 \pm 1.12b	5.14 \pm 0.59b

Crypt damage	0a	8.00 ± 2.21b	0a	6.00 ± 2.11b	4.74 ± 0.60b
TOTAL (sum)	0a	23.60 ± 5.41b	0a	16.40 ± 1.03b**	14.71 ± 1.79b

C: control, CC: control colitis, H: hawthorn, HC: hawthorn colitis, MC: mesalamine colitis. Results are presented as mean ± SEM. One-way ANOVA followed by Tukey. Different letters indicate statistical significance ($p < 0,05$). *Score calculation: each parameter (crypt damage, inflammation extent and leukocyte infiltration) was multiplied by the factor of involvement of the mucosa and the total is the sum of the three results. **The sum of this group was not 20.33 ± 4.21 , as the sum of the three parameters suggest (sum of the scores of leukocyte infiltration, inflammation extent and crypt damage); this happened due to an outlier only found in the statistical analysis of the TOTAL.

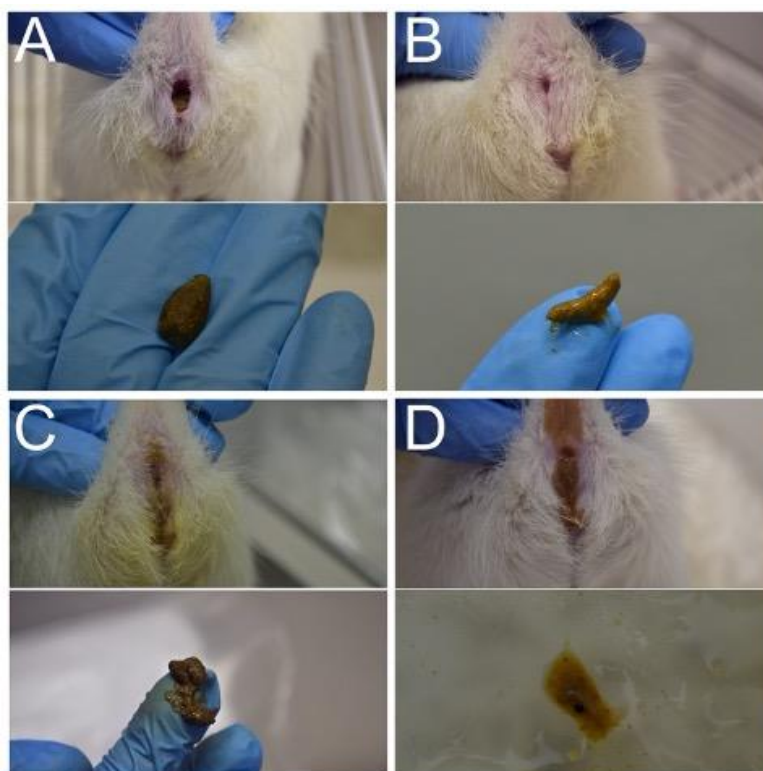


Figure 3: Photographic representation of the score of stool consistency.

A. Score 0 - Normal stools. **B.** Score 1 - Soft pellets not adhering to the anus. **C.** Score 2 - Very soft pellets adhering to the anus. **D.** Score 3 - Liquid stool; wet anus. Score based on Gommeaux et al. (2007) and photographic scheme based on Nascimento et al. (2020). Color figure.



Figure 4: Photographic representation of the score of bleeding.

A. Score 0 – No bleeding. **B.** Score 1 - Small spots of blood in stool; dry anal region. **C.** Score 2 - Large spots of blood in stool; blood appears through anal orifice. **D.** Score 3 - Deep red stool; blood spreads largely around the anus. Score based on Gommeaux et al. (2007) and photographic scheme based on Nascimento et al. (2020). Color figure.

The anti-necrotic potential of extracts from hawthorn is still poorly documented (Fujisawa et al., 2005; Zapatero, 1999), but evidence indicates that flavonoids and phenolic acids (e.g. chlorogenic acid), which were found in abundance in the HE of our study, possess such properties. For example, chlorogenic acid, a polyphenol found in coffee beans, apple and hawthorn species (Alirezalu et al., 2018), and the vitexin-rich plant *Tragopogon graminifolius*, has been shown to be effective in reducing the typical macroscopic and necrotic lesions of TNBS-induced colitis (Farzaei et al., 2015; Zatorski et al., 2015; Zhou et al., 2016). Although necrosis is not a typical documented finding of patients with IBDs, it can work as a parameter to measure the possible anti-inflammatory and prohomeostatic effects of natural products.

Although alterations in the liver, spleen and kidney are occasionally described by studies that used the model of TNBS-induced colitis (Jang et al., 2018; Patel & Trivedi, 2017; Zhi et al., 2017), in the present study, no statistical differences were found between the experimental groups regarding the weight of such organs.

As far as the histological analysis, TNBS provoked an extensive and severe ulceration in epithelium with loss of crypt and goblet cells; a high inflammatory income in mucosa, submucosa and muscular layers; and increased vascularization though the whole tissue (figure not shown). Results from the histological grading significantly shown this for all colitis

groups ($p < 0,05$). Mesalamine ($p = 0.103$) and HE ($p = 0.318$) did not statistically decreased the histological score (**Table 4**), what could probably be related to two possibilities: 1. as expected for the TNBS model (Antoniou et al., 2016), the microscopic severity was extremely high, especially in the area selected for histology (distal colon and rectum), possibly making it difficult for a clear observation of the benefits of the treatments; 2. the HE only had a mild preventive effect that could not be translated in the microscopical analysis. We believe that a later study utilizing other models of colitis should be performed in order to understand if HE could have preventive effects in the histology of the damaged intestinal mucosa. In studies by Cazarin et al. (2014, 2015, 2016), for example, by using the polyphenol-rich residues of *Passiflora edulis* (peel and leaf), the authors could not find statistical significance in the microscopic score of rats with TNBS-induced colitis (Cinthia B.B Cazarin et al., 2014; Cinthia Bau Betim Cazarin et al., 2015), but when they utilized the dextran sodium sulfate model, it was observed a significant reduction in the histological damage in the colon (Cinthia Bau Betim Cazarin et al., 2016). The dextran sodium sulfate model results in less severe inflammation and more superficial ulcers in rats when in comparison with the TNBS acute model (Catana et al., 2018), which typically triggers extensive transmural inflammation (Antoniou et al., 2016).

3.3. Antioxidant capacity of the serum

No statistical differences were found between all groups regarding the results from the FRAP and ORAC methods, meaning that the TNBS model did not worsen the antioxidant capacity of serum and neither the intervention with HE increased it (**Table 5**). In a study by Maurer et al. (2020), similarly, ORAC and FRAP data were not changed by the TNBS-colitis model or by the intervention with a rich source of polyphenols (Luana H. Maurer et al., 2020). Since this study utilized an acute model of colitis, we believe that the serum could not have been deeply affected. Also, a higher dosage of HE may be necessary to achieve significance for such analyses.

Table 5: Antioxidant capacity of the serum, inflammatory profile, and activity or concentration of oxidative stress' enzymes.

Antioxidant analyses (serum)					
Parameter	C	CC	H	HC	MC
FRAP ($\mu\text{mol trolox/mL}$)	0.62 \pm 0.06a	0.55 \pm 0.04a	0.60 \pm 0.05a	0.56 \pm 0.01a	0.59 \pm 0.06a

ORAC ($\mu\text{mol trolox/mL}$)	4.31 \pm 0.71a	3.82 \pm 0.81a	7.26 \pm 2.08a	5.63 \pm 0.47a	4.04 \pm 0.65a
Inflammatory profile (colon and serum)					
MPO (colon) (U/g protein)	9.40 \pm 0.50a	39.8 \pm 11.27b	9.80 \pm 1.49a	11.67 \pm 2.74a	8.66 \pm 0.61a
IL-1 β (colon) (ng/g protein)	111.20 \pm 3.25a	529.90 \pm 127.30b	81.34 \pm 14.20a	271.30 \pm 39.32a	208.00 \pm 39.78a
IL-1 β (serum) (ng/mL)	0.40 \pm 0.04a	0.44 \pm 0.08a	0.44 \pm 0.06a	0.47 \pm 0.09a	0.34 \pm 0.03a
TNF- α (colon) (ng/g protein)	86.27 \pm 2.37a	77.64 \pm 16.17a	85.73 \pm 4.25a	106.50 \pm 15.38a	105.80 \pm 19.70a
TNF- α (serum) (ng/mL)	0.07 \pm 0.01a	0.17 \pm 0.06a	0.06 \pm 0.01a	0.13 \pm 0.06a	0.08 \pm 0.01a
Oxidative stress enzymes (colon)					
CAT (U/g protein)	87.6 \pm 10.49a	211 \pm 33.48b	56 \pm 13.33a	86.5 \pm 20.63a	209.9 \pm 21.85b
GR (consumed NADPH/min/g protein)	106.2 \pm 19.7a	41.6 \pm 5.11b	2.25 \pm 0.94b	324.5 \pm 19.47c	58.71 \pm 9.38ab
SOD (SOD/g protein)	144.4 \pm 45.57a	56 \pm 16.83a	142.8 \pm 43.34a	105 \pm 32.83a	54.67 \pm 4.81a
TBARS (nmol MDA/g protein)	66.78 \pm 12.42a	77.58 \pm 24.24a	37.66 \pm 10.84a	68.30 \pm 10.49a	56.41 \pm 8.02a
GSH (nmol GSH/mg protein)	14.25 \pm 2.85a	9.39 \pm 4.06a	16.7 \pm 2.08a	11.22 \pm 2.21a	13.74 \pm 1.94a

C: control, CC: control colitis, H: hawthorn, HC: hawthorn colitis, MC: mesalamine colitis. FRAP: ferric reducing antioxidant power, ORAC: oxygen-radical absorbing capacity. CAT: catalase, GR: glutathione reductase, MPO: myeloperoxidase, SOD: superoxide dismutase, TBARS: thiobarbituric acid reactive substances, MDA: malondialdehyde, GSH: glutathione. IL-1 β : interleukin 1 beta, TNF- α : tumor necrosis factor alpha. Results are presented as mean \pm SEM. One-way ANOVA followed by Tukey. Different letters indicate statistical significance ($p < 0.05$).

3.4. Concentration or activity of inflammatory proteins and oxidative stress' enzymes

As expected, MPO and IL-1 β levels were significantly increased by the inflammatory process in colon ($p < 0.01$). Both mesalamine and HE decreased the activity of MPO and IL-1 β ($p < 0.05$). Concentrations of IL-1 β in serum and TNF- α in colon and serum were not changed (Table 5).

It is known that besides the common pathway of release of IL-1 β by macrophages (caspase-1 activation by inflammasome), neutrophils proteases can also cleave the precursor of this cytokine into its biological active forms (Guma et al., 2009), following tissue injury and cell necrosis. In addition to helping create an IL-1 β -dependent inflammatory response (Lopez-

Castejon & Brough, 2011), neutrophils can express MPO, a local mediator of tissue damage and inflammation ignition (Aratani, 2018). Patients with IBDs possess high serum and feces levels of IL-1 β and/or MPO (Peterson et al., 2007; Vasilyeva et al., 2016) and the typical inflammation of IBDs is largely attributed to the accumulation (ulcerative colitis) or defective function (Crohn's disease) of neutrophils (Wéra et al., 2016). In the present study, HE was able to reverse the augmented levels of IL-1 β and MPO, indicating its potential to blockage or regulate neutrophil-associated intestinal inflammations.

Regarding the results of oxidative stress' enzymes, CAT activity was increased by the colitis model ($p < 0.01$), while GR was reduced ($p < 0.05$). Only the group treated with the HE shown a normalization in CAT levels ($p < 0.01$) (**Table 5**), demonstrating for the first time in this study, a superior effect of a natural alternative intervention in comparison with a drug. Additionally, only the HE highly increased the levels of GR ($p < 0.001$) in response to the TNBS-induced colitis model (**Table 5**).

Both CAT and GR are known for they role against lipid peroxidation. CAT acts as a first line defense against the formation of free radicals, while GR is responsible for the regeneration of GHS, the body master's antioxidant (Fagan & Palfey, 2010; Ighodaro & Akinloye, 2018; Pérez et al., 2017). According to recent studies, GR can be found increased in rats with improved TNBS-induced colitis and that received rich sources of flavonoids (jaboticaba peel and grape peel residues) (Da Silva-Maia et al., 2019; Luana Haselein Maurer., 2019). This scenario suggests the importance of GR restoration in order to ameliorate intestinal conditions. On the other side, despite the well-known regulating role of CAT, in the present study its activity was actually increased after colitis induction. This probably happened due to adaptative or defective mechanisms, as this enzyme has also been found highly expressed in neutrophils from the intestinal mucosa of patients with IBDs (Kruidenier et al., 2003). Therefore, we believe that, besides acting in the MPO-IL-1 β axis, HE can also positively interfere in the mechanics of CAT and GR, first by avoiding the excessive and/or defective action of CAT, then by highly increasing the levels of GR in order to control the propagation of reactive oxygen species and restore redox balance.

The levels of SOD, MDA and GSH in colon were not changed either by the colitis model or the interventions (**Table 5**).

3.5. Concentration of short-chain fatty acids

It is suggested that the extract of the leaves of *Crataegus oxyacantha* could increase the growth of species of *Bifidobacterium* and *Lactobacillus* and the concentration of acetic acid, as shown by an *in vitro* study using skim milk as a fermentable matrix (Khaleel & Haddadin, 2013). Also, flavonoids, including chlorogenic acid, which was found abundant in the HE utilized in our study, has been shown to lightly increase the counts of *Bifidobacterium spp.* and the concentration of SCFAs *in vitro* (Parkar et al., 2013). However, in the present study, the concentration of SCFAs (total, acetic, butyric, propionic) in feces from both cecum and colon of rats was not altered by HE. Additionally, it was neither affected by the TNBS-colitis model (data not shown). HE at a medium dosage or the amount of vitexin 2-O-rhamnoside or chlorogenic acid administered to rats in this study may not be sufficient to increase the levels of SCFAs. Considering that our polyphenolic extract was probably a poor source of prebiotic-potential carbohydrates, even less would be the chance for modulation of the microbiome of rats.

3.6. Correlation matrix

Parameters that presented the most numerous and diverse correlations were colon weight/length ratio, area of the necrotic lesions and IL-1 β . On the contrary, the DAI at its peak day had overall poor correlations with all other parameters (**Figure 5**).

The colon ratio is considered an inflammatory index (Sánchez-Fidalgo et al., 2010), therefore, it was highly correlated with almost all the lesion parameters and the levels of IL-1 β . On the other side, this cytokine was positively correlated with the levels of MPO ($r=0.846$), probably due to mechanisms previously mentioned, associated with neutrophil infiltration and release of inflammatory markers in the colonic mucosa. MPO also presented a high negative correlation with the body weight alteration ($r=-0.708$). In a trial with patients with ulcerative colitis, MPO and IL-1 β had good correlations with the histological, endoscopic and clinical severity of the disease (Peterson et al., 2007). This scenario suggests the possibility of measuring such markers in order to improve prognostic in IBDs, in addition to targeting compounds capable of specifically blocking them, such as the ones found in the HE.

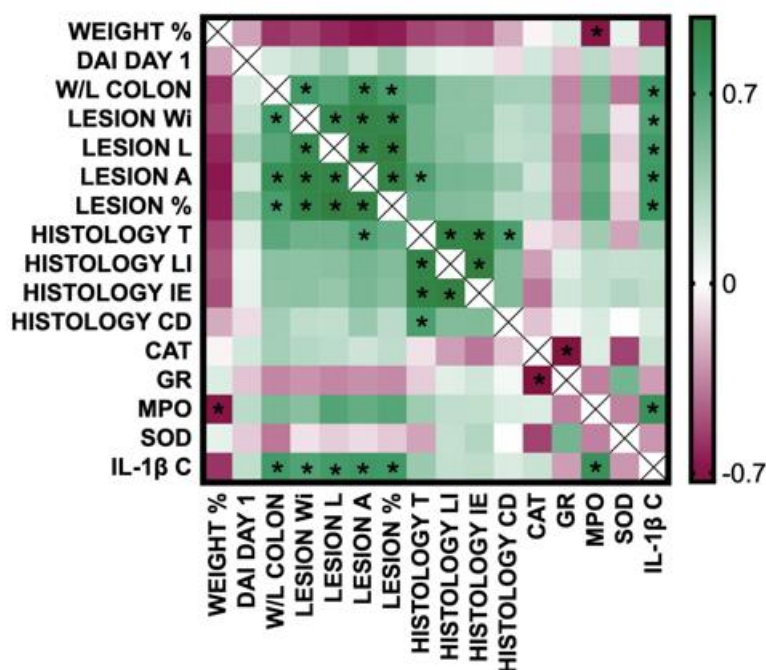


Figure 5: Heatmap correlation matrix.

Pearson's correlation analysis. Correlations considered high (values between 0.7 and 1 or -0.7 and -1) are marked with an asterisk (*). Parameters: WEIGHT %: body weight alteration on euthanasia; DAI DAY 1: Disease Activity Index after one day of the administration of 2,4,6-trinitrobenzene sulfonic (peak day of symptoms); W/L COLON: colon weight/length ratio; LESION Wi: width of the largest necrotic lesion; LESION L: length of the largest necrotic lesion; LESION A: area of the largest necrotic lesion; LESION %: proportion of the colon affected by the largest necrotic lesion; HISTOLOGY T: total score of the histology; HISTOLOGY LI: score of the leukocyte infiltration; HISTOLOGY IE: score of the inflammation extent; HISTOLOGY CD: score of the crypt damage; CAT: catalase levels in colon; GR: glutathione reductase levels in colon; MPO: myeloperoxidase levels in colon; SOD: superoxide dismutase levels in colon; IL-1β: interleukin 1 beta levels in colon. [Color figure.](#)

There was also a negative correlation ($r=-0.732$) between the levels of CAT and GR. This shows, once again, as previously mentioned, that mechanistically, HE is also acting through an oxidative stress pathway, mainly by affecting in opposite ways GR and CAT in order to prevent against lipid peroxidation.

Although the interventions were not able to significantly reduce the histological score, there was a high positive correlation of such parameter with the area of the necrotic lesion ($r=0.708$), which was diminished by the HE.

4. CONCLUSIONS

Mechanistically, it is proposed that the hawthorn plant and its polyphenols may produce an anti-inflammatory response by blocking the nuclear factor kappa B and reducing the expression of genes related to adhesion molecules (ICAMs), cytokines (TNF- α , IL-1 β , IL-6)

and chemokines (e.g., IL-8). However, such observations are mainly collected as part of studies with cardiovascular complications (Wu et al., 2020). Animal studies directed towards IBDs are lacking in analysis (Fujisawa et al., 2005) or do not present an adequate experimental model (e.g., acetic acid-induced colitis) (Malekinejad et al., 2013). Therefore, we believe that the present study is so far the most reliable and complete investigation of the effects of HE in IBDs.

From the present study, we found that, in rats with TNBS-induced colitis, the polyphenol-rich extract from the flowering tops of HE acted by reducing inflammatory mediators (IL-1, MPO) and regulating oxidative stress (CAT, GR) pathways, posteriorly implicating in less colonic necrosis and disease-affected health. Such findings suggest that the hawthorn plant may have utility as an alternative or complementary natural remedy that extends beyond its well-known cardiovascular protective and anti-hypertensive roles.

ACKNOWLEDGMENTS

This study was financed in part by the 1. *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)* - Finance Code 001; 2. *Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)* - processes 140812/2019-9, 403328/2016-0 and 301496/2019-6; 3. *Fundação de Amparo à Pesquisa do Estado de São Paulo* – processes 2018/11069-5, 2017/23657-6, 2015/50333-1 and 2015/13320-9; 4. Ministry of Education and Training of Vietnam; and 5. Campus France. Special thanks to *Red Iberoamericana de Alimentos Autoctonos Subutilizados (ALSUB-CYTED, 118RT0543)*.

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APPENDIX C

Article 4: Screening for pancreatic lipase natural modulators by capillary electrophoresis hyphenated to spectrophotometric and conductometric dual detection

TALANTA (Submitted)

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Keywords

Capillary electrophoresis lipase assay; Contactless conductivity detector; Natural inhibitor screening; TDLFP based on-line enzymatic assay; UPLC/MS molecular characterization of water plant extracts

Abstract

Orlistat has been since its approval the only pancreatic lipase (PL) inhibitor used for the management of obesity. Nonetheless, it manifests several unpleasant side-effects shifting the balance between its efficiency and undesirability towards the latter. The search for novel PL inhibitors has gained increasing attention in recent years. Capillary electrophoresis (CE) present interesting advantages over other techniques such as economy in consumption, superior separation efficiency and the availability of several detection and separation modes. A dual detection CE-based homogeneous enzymatic assay was developed employing both the offline and online reaction modes. Tris/MOPS (10 mM, pH 6.6) was used as the background electrolyte for CE analyses as well as the incubation buffer for enzymatic reactions. The effect of dipalmitoylphosphatidylcholine vesicles on PL reaction kinetics was found to be minimal showing only a 4-5 fold increase in maximum velocity V_{max} and 3 times increase in the Michaelis constant K_m . Once validated, the

method was applied to screen aqueous extracts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysanthellum americanum* as well as 11 novel PL inhibitors and activators purified from natural extracts of oak wood, grapes and wine. The promising potential of using hawthorn leaves as herbal tea infusions for PL inhibition was demonstrated ($37 \pm 3\%$ at 1 mg mL^{-1}) while hawthorn flowers tend to promote PL activity. Two triterpenoids purified from extracts of oak wood used in wine aging were identified for the first time as potent PL inhibitors demonstrating 51 and 57 % inhibition at 1 mg mL^{-1} .

Introduction

Pancreatic lipase (PL) (EC: 3.1.1.3) is a hydrolytic enzyme that catalyzes the digestion of the ester linkages of triglycerides (TGs) hydrolyzing them into monoglycerides (MGs) and free fatty acids in the small intestine thus playing an important role in facilitating the absorption of dietary fats through the intestinal wall. TG digestion by PL contributes to 50-70% of dietary fat digestion [1]. This digestion incorporates contributors such as bile salts and the pancreatic protein co-lipase both of which enhance PL activity in physiological conditions and thus ensure complete TG digestion and absorption [2,3]. Its contribution to TGs digestion and absorption has made PL and its inhibition a trending topic for the last century as a way to combat obesity and its ensuing diseases. The community's interest in the topic also arises from the scarcity of approved PL inhibitors in pharmaceutical markets. Xenical, also known as orlistat, is the only approved PL inhibitor in Europe and the USA used for long term weight management [4]. It is a chemically synthesized derivative of lipstatin, produced by the gram positive bacteria *Streptomyces toxytricini* [3,5]. Orlistat covalently binds to the active serine residue of PL hindering its TGs hydrolyzing faculty [6,7]. Despite orlistat's ability of depleting 30 % of TGs absorption [8,9], several unpleasant secondary effects such as flatulence, oily stool, diarrhea [10,11] in addition to more grave but rare reports of hepatic and renal damage have been reported amongst patients [12-14]. As a result, there has been a large scale search for new natural and synthetic molecules capable of inhibiting PL but with more tolerable side effects. The extracts of many plants and fruits are considered as rich sources of biomolecules that serve as potential orlistat substitutes. For instance, epigallocatechin gallate (EGCG), a flavonoid abundant in green tea demonstrated promising PL inhibition with a half maximal inhibitory concentration (IC_{50}) of $0.8 \pm 0.1 \mu\text{M}$, lower than that determined for orlistat ($IC_{50} = 32 \pm 8.5 \mu\text{M}$) [15]. Saponins, a class of triterpenoids isolated from *Acanthopanax senticosus*, had an IC_{50} ranged between 220-290 μM compared to 40 μM for orlistat [16]. On the other hand, some synthetic

molecules have been shown to induce PL inhibition. Recent examples include rhodanine-3-acetic acid derivatives ($IC_{50} = 5.16 \pm 0.31 \mu\text{M}$) [17] or indole glyoxylamide analogues ($IC_{50} = 4.92 \pm 0.29 \mu\text{M}$) vs $0.99 \pm 0.11 \mu\text{M}$ for orlistat [18].

Several techniques have been described for assaying the activities of enzymes such as PL. These biocatalysis assays can be divided into two categories namely, homogeneous and heterogeneous assays. Homogeneous biocatalysis involves the use of free enzymes in a homogeneous phase with the other reactants. In heterogeneous biocatalysis, the enzymes are often immobilized onto a phase distinct from that of the other reactants [19-23]. It has been reported that in comparison to free enzymes, immobilized enzymes maintain better storage stabilities and are more tolerant to pH and temperature changes [24]. Nonetheless, immobilization may bring about modifications in the enzymes' structure that may reduce its catalytic activity [25]. The fact that several substrates besides TG can be used for assaying PL activity permits the use of a variety of detection techniques [26-28]. A commonly used technique is spectrophotometry using either fluorescence or ultra-violet/visible (UV-Vis) detectors. Microtiter plates are commonly used in conjugation with the latter technique for assaying PL activity or screening for novel modulators [29]. Moreover, spectrophotometric techniques have also been hyphenated to analytical separative techniques such as capillary electrophoresis (CE).

CE relies on ions' charge to size ratios for their separation in an electric field applied across a capillary. The technique excels in separation efficiency, economy of reagents and samples, versatility of operating modes and availability of multiple hyphenation options [30,31]. Assaying enzymatic activity and screening for modulators using CE has been widely reported in the literature [32-37]. Two common operating modes of CE enzymatic assays are the offline (pre-capillary) [38,39] and online (in-capillary) [40] modes. With the offline mode, the enzymatic reaction takes place in a microvial and the reaction mixture is thereafter injected into the capillary [41-43]. The offline reactions are rather easy to control and optimize. The online mode involves the capillary acting as a nanoreactor into which few nanoliters of the different reactants are injected and mixed. Several modes are available for in-capillary mixing of analytes. Electromigration microanalysis (EMMA) [44] and pressure mediated microanalysis (PMMA) [45] are used to mix analytes according to their electrophoretic mobilities and diffusion coefficients, respectively. In this case, the mixing step can be inefficient when more than two reactant plugs are injected [34,46]. Transverse diffusion of laminar flow profiles (TDLFP) [47] is a more suitable approach to mix multiple reactant plugs. In this

mode, the reactants are injected sequentially using high injection pressures and short injection times giving rise to parabolic plugs. The diffusion and mixing of the different plugs is almost exclusively transversal with limited longitudinal diffusion. TDLFP can be easily used to mix more than two plugs. However, dilution of reactants inside the capillary is a significant drawback of this approach. Compared to offline ones, online reactions consume even less reactants (few tens of nanoliters) and it is possible to automate the enzymatic assay minimizing the need for manual intervention [48,49].

As mentioned previously, several types of detectors can be hyphenated to CE. The default detection technique is typically spectrophotometric with UV/PDA detectors usually supplied by the vendor as the built-in detector with the CE instrument. Other examples of CE-hyphenated detection techniques include laser-induced fluorescence (LIF) [50], mass spectrometry (MS) [51,52] and capacitively coupled contactless conductivity detection (C^4D) [53]. C^4D was first described as a CE hyphenated detector in 1998 [54,55]. Since then, the CE/ C^4D couple have seen further development and their fields of application ranged from the analysis of various inorganic [56] and organic [57] molecules to their adaptation onto miniaturized CE microchips for spatial exploration [58,59]. C^4D is excellent in detecting ions with low or no molar extinction coefficient. Furthermore, its use in duality with spectrophotometric detection have been proven to be a powerful way to enhance CE selectivity towards various analytes. Our team has recently developed a CE-UV- C^4D method for monitoring gold nanoparticle functionalization after conducting enzymatic reaction [60]. Spectrophotometric detection was used to monitor the hydrolysis of the thiol glucosinate catalyzed by myrosinase in addition to the evaluation of the stability of nanoparticles in the capillary. On the other hand, C^4D was used as a complementary detector to monitor the increase of inorganic sulfate, another product of the thiol glucosinate hydrolysis, in order to confirm the results obtained by UV detection.

Recently, online and offline CE PL assays have been described. An offline CE- C^4D assay was developed by A. Schuchert-Shi *et al.* [61] to demonstrate the superior selectivity of PL towards the methyl ester substrate of L-threonine compared to L-serine. Y. Tang *et al.* [22] screened PL inhibition potential of ten natural extracts using a heterogeneous, online CE enzymatic assay with PL immobilized onto the capillary walls. Using 4-nitrophenyl acetate (4-NPA) as the substrate, the reaction was monitored by detecting the 4-nitrophenol (4-NP) hydrolysis product using spectrophotometric detection at a wavelength of 400 nm. Six of the screened extracts showed

inhibition of immobilized PL with the ethanolic *Fructus Crataegi* extract at 10 mg mL⁻¹ demonstrating the most relevant inhibition (~ 70 %). In this study, we describe a homogeneous CE-UV/C⁴D dual detection method for assaying PL activity. Both offline and online homogeneous CE-PL assays were developed. 4-nitrophenyl butyrate was chosen as a substrate of the PL which leads to the production of both 4-nitrophenolate (4-NP) and butyrate, detected by spectrometry and by C⁴D respectively (Fig. 1).

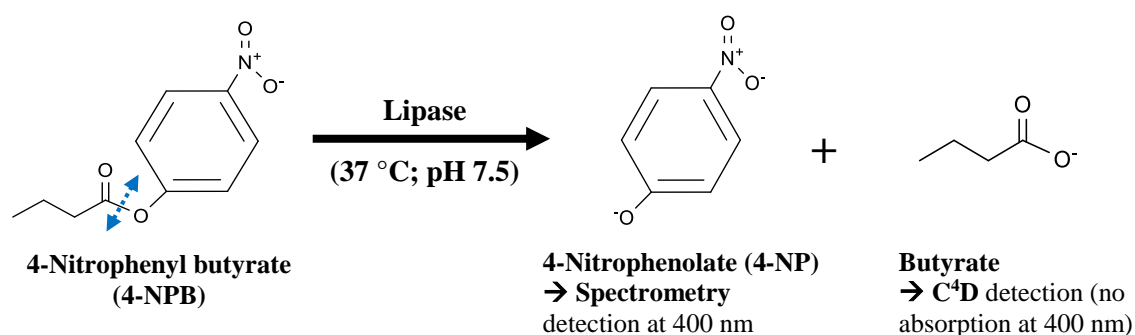


Figure 1: Hydrolysis of 4-NPB into 4-NP and butyrate catalyzed by pancreatic lipase (PL). The dashed double-sided arrow indicates the enzyme cleavage site.

Several parameters were investigated such as the nature of the separation and reaction buffers, preparation of the 4-NPB substrate solution and the effect on PL kinetics of dipalmitoylphosphatidylcholine (DPPC), a zwitterionic surfactant with a critical micellar concentration of 0.46 mM in water [62]. In fact, PL is well known to be an enzyme with an interfacial activation mechanism. It is active at the lipid-water interface such as that present in the intestine where TGs are hydrolyzed. This happens due to the presence of a lid over the enzyme's active site. This lid is opened upon increasing the hydrophobicity of the media or in the interface between aqueous and organic media facilitating access to the enzyme's active site [63]. Orlistat was used as the reference inhibitor to validate the inhibition assays. Furthermore, the developed enzymatic assays were carried out to test the modulatory potential of 11 compounds purified from oak wood, grapes and wine extracts in addition to the aqueous extracts of three different plants, Hawthorn (*Crataegus oxyacantha*), blackcurrant (*Ribes nigrum*) and *Chrysanthellum americanum* extracted by infusion in water to be eventually consumed as herbal tea. We have recently [64] demonstrated promising angiotensin-converting enzyme (ACE) inhibition by *C. americanum* extracts (> 90 %), hyaluronidase inhibition by hawthorn extracts (~ 100 % relative to reference) as well as antioxidant capacities of blackcurrant extracts (~ 100 % relative to reference). The molecular features of the extracts were also characterized and compared to identify the molecules or families of molecules

responsible for the observed bioactivities. The extracts were thus screened for PL modulation given the promising biological and antioxidant activities they have previously demonstrated.

Experimental

Chemicals

Tris(hydroxymethyl)aminomethane (Tris) ($C_4H_{11}NO_3$, $\geq 99.8\%$), N-cyclohexyl-2-aminoethanesulfonic acid (CHES) ($C_8H_{17}NO_3S$, $\geq 99\%$), 3-(N-morpholino)propanesulfonic acid (MOPS) ($C_7H_{15}NO_4S$, $\geq 99.5\%$), sodium butyrate ($C_4H_7NaO_2$, $\geq 98.5\%$), 4-nitrophenol (4-NP) ($C_6H_5NO_3$, spectrophotometric grade), 4-nitrophenyl Butyrate (4-NPB) ($C_{10}H_{11}NO_4$, purity $\geq 98\%$), lipase from porcine pancreas (EC 3.1.1.3, Type II), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) ($C_{40}H_{80}NO_8P$, $\geq 99\%$), orlistat ($C_{29}H_{53}NO_5$, $\geq 98\%$) and lithium hydroxide monohydrate ($LiOH.H_2O$, $\geq 98\%$) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Hydrochloric acid (HCl, 37%) was purchased from Fisher Scientific (Loughborough, UK). HPLC grade methanol (MeOH) was purchased from VWR International (Fontenay-sous-Bois, France). HPLC grade acetonitrile (ACN) and N,N-dimethylformamide (DMF, $\geq 99.8\%$) were purchased from Carlo Erba (Val de Reuil, France). Water used throughout this study was ultra-pure (18 M Ω -cm) produced by an Elga apparatus (Elga, Villeurbanne, France). Different parts of hawthorn (*Crataegus oxyacantha*, origin France), *i.e.* dry flowering tops (2 lots, number 20335 and number CB58120) and dry hawthorn flowers (lot number: 20334), dry *Chrysanthellum americanum* (origin Ivory Coast, lot number: 559980), and dry blackcurrant leaves (*Ribes nigrum*, origin Poland, lot number: 55870) raw materials were purchased from France Herboristerie (Noidans-Lès-Vesoul, France). Fresh hawthorn leaves, flowers and flowering tops were harvested on April 24, 2020 on a wild isolated tree in Crès (France).

Instrumentation

Experiments were carried out using a Beckman-Coulter P/ACE MDQ CE instrument (Fullerton, CA, USA) equipped with a photodiode array (PDA) detection system and an on-capillary TraceDec C⁴D detection cell (Innovative Sensor Technologies GmbH, Strasshof, Austria). The C⁴D parameters were fixed as follows: frequency medium, voltage 0 dB, gain 100%, offset 010, filter: frequency 1/3 and cut-off 0.02. CE control and the analysis of electropherograms was performed using the 32 Karat software (Beckman Coulter). C⁴D signals were acquired by the Tracemon software (Istech, version 0.07a). Uncoated fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillaries had a total length of 61 cm, an internal diameter of 50 μ m and effective length

of 37 cm to the C⁴D detection cell and 51 cm to the optical detection window. The capillary was contained in a system of connecting tubings and reservoirs used for circulating a coolant liquid in a closed circuit within the capillary cartridge. A lab-made 3D printed scaffold was constructed using an Ender 3 printer (Creality 3D, Shenzhen, China) and adapted to fix the various components of the C⁴D detection cell and to avoid coolant liquid leakage (supplementary Fig. S.1). New capillaries were conditioned with 5 mM LiOH, H₂O and BGE for at least 15 min each followed by the application of +30 kV for 15 min. Between analyses, the capillary was rinsed with 5 mM LiOH (5 min), H₂O (1 min), 5 mM HCl (3 min), H₂O (1 min) and BGE (5 min). At the end of each working-day, the capillary was rinsed with 5 mM LiOH and H₂O for at least 15 min each before storing its ends overnight dipped in H₂O vials. All rinse cycles were carried out at 50 psi. The electrophoretic separations were conducted under +30 kV and at 37 °C. The volumes of the injected plugs were estimated using CE expert Lite from Sciex (<https://sciex.com/ce-features-and-benefits/ce-expert-lite>).

UHPLC-DAD system was composed of a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS equipment with WPS-3000TBRS auto sampler, TCC-3000RS column compartment set at 35°C and Chromeleon 7 software, (ThermoFisher Scientific, Waltham MA, USA). UHPLC-ESI-MS system was composed of a Synapt G2-S equipment with ESI operating in resolution mode and MassLynx 4.1 software (Waters Corp., Milford MA, USA). For both methods, a Kinetex C18 100A 100×2.1 mm, 2.6 μm column in association with a security guard ultra-cartridge was used (from Phenomenex Inc., Torrance CA, USA). A binary solvent system was used, consisting of water/formic acid (1 ‰, v/v) mixture as solvent A and acetonitrile/formic acid (1 ‰, v/v) mixture as solvent B. The gradient program started with 95 % A, then A was progressively decreased to 0 % in 30 min with a convex increase (curve 5 in Chromeleon 7). The flow rate of the mobile phase was set to 0.4 mL.min⁻¹ and the injection volume was 20 μL. For the analysis by UHPLC-DAD, the peaks were monitored at 280, 320 and 360 nm and the UV-Vis spectra of the various compounds were recorded between 200 and 500 nm. For the analysis by UHPLC-ESI-MS, positive and negative ionization modes and fast DDA MS methods with automatic MS/MS intensity-based switching parameters, were used. The cone voltage, the extractor voltage and the capillary voltage were set to 30 V, 3 V and 2.4 kV, respectively. The source temperature and the desolvation temperature were 140°C and 450°C, respectively. Ions were scanned between $m/z = 50$ and $m/z = 1200$ to obtain MS spectra.

Solutions

Unless otherwise stated, all solutions were prepared in H₂O and filtered through polyvinylidene fluoride (PVDF) syringe filters of 0.2 µm pore size purchased from Agilent Technologies (Waldbronn, Germany).

Background electrolyte (BGE) and incubation buffer (IB): Tris/MOPS (ionic strength = 10 mM, pH 6.6) was used as the BGE for CE analyses as well as the IB for enzymatic reactions. The pH of solutions was measured using a MeterLab PHM201 Portable pH-Meter (Radiometer Analytical, Villeurbanne, France) and their properties obtained by Phoebus software (Analis, Namur, Belgium) and PeakMaster 5.3. [65]

Reactants: PL stock solutions were prepared daily by suspending the lyophilized enzyme powder in the IB at 10 mg mL⁻¹ followed by 15 min agitation before storage at 0 °C. 4-NPB was chosen as the PL substrate due to its similarity to the natural TG substrate in addition to the possibility of following its hydrolysis products using dual detection. The 4-NPB solution was prepared in 100 % acetonitrile at a concentration of 1 mg mL⁻¹. Unless otherwise stated, orlistat stock solution was prepared in 70 % DMSO at 3 mg mL⁻¹ and was later diluted to 1.5 mg mL⁻¹ in H₂O to prepare the working solution. The PL modulation by **11** compounds (Table 1) purified from oak wood, grapes and wine extracts was investigated. These compounds were prepared at different percentages of organic solvents taking into account their solubilities. The preparation of the different compounds was as follows: compounds **1** to **7** in 70 % methanol, compound **8** in 50 % ethanol and compounds **9** to **11** in 80 % ethanol. All of the purified molecules were prepared at a stock concentration of 40 mg mL⁻¹ and were then diluted to 5 mg mL⁻¹ in H₂O to prepare the working solutions. The modulation of different water extracts from three plants was also investigated. All extracts were prepared before use at 5 mg mL⁻¹ in H₂O and centrifuged at 2000 × g for 10 min before isolating the supernatant. The different modulators (purified molecules + aqueous extracts) were assayed at 1 mg mL⁻¹.

Extraction by infusion of different plant parts: The extracts of different parts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysanthallum americanum* plants were obtained as previously described by P. Cao-Ngoc *et al.* [66]. Unless otherwise stated, plant material was grinded before extraction using a commercial MKM6003 Bosch grinder.

Fresh hawthorn extractions: 20 g of fresh plant material (leaves, or flowers) were infused in 1 L of boiling water using a 'French press' Bodum® (Bistro model, Triengen, Switzerland) under 500 rpm magnetic stirring. After 30 min, the herbal tea solution was filtered first with the Bodum® cover to

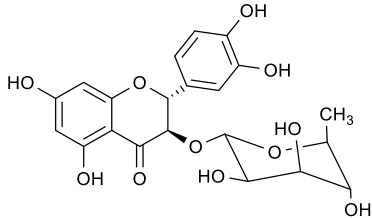
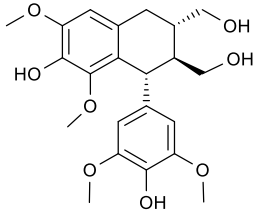
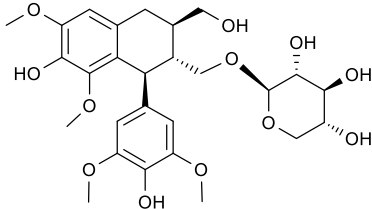
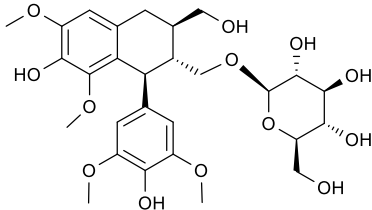
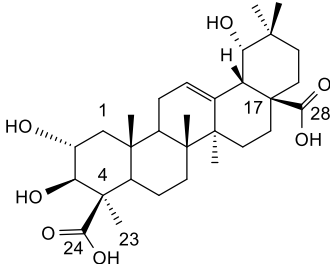
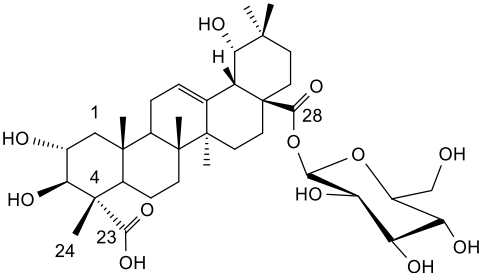
remove the largest particles, then with Whatman filter paper placed on a Büchner funnel using a vacuum pump (KNF Model N820FT.18, Freiburg, Germany) to remove any residual solid plant particles. Finally, the herbal tea solution was concentrated using a rotary evaporator (down to 20 mL volume) and finally freeze-dried (Cryotec Model CRIOS-80, Saint-Gély-du-Fesc, France) to obtain the dry extract. Lyophilized dry extracts were stored at 4 °C.

Dry hawthorn extractions: 2.5 g plant material (flowers only: lot No 20334, used as is without grinding, or grinded flowering tops lot No 20335 (sample A) and lot No CB58120 (sample B)) were infused in 250 mL boiling water for 10 min using the same equipment as above. The herbal tea solution was then filtered, concentrated, freeze-dried and stored in the same way described above. Blackcurrant and *Chrysanthellum americanum*: the extraction protocol was similar to that of dry hawthorn.

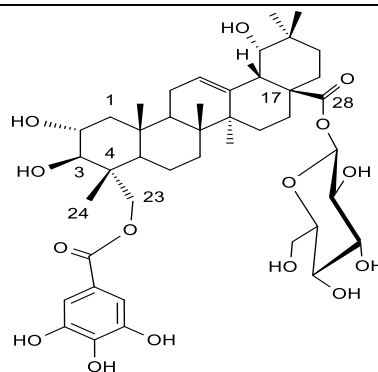
Chemical composition of the plant extracts: The total polyphenols content (TPC), flavonoids content (TFC) and proanthocyanidin oligomers content (OPC) in the various plant extracts were determined by classical colorimetric methods, as previously described [64,66]. UHPLC-DAD and UHPLC-ESI-MS/MS analyses were performed according to the protocol described in our previous paper [64]. Briefly, 20 mg of plant extract was dissolved in 1 mL water, then strongly vortexed for 2 min. The resulting solution was diluted five times with water, vortexed again for 2 min, and finally analyzed.

Extraction and purification of molecules from oak wood, grapes and wine: 11 natural molecules (Table 1) were extracted from oak wood, grapes and wine using centrifugal partition chromatography fractionation and HPLC purification [67-71]: **1.** Astilbin ($C_{21}H_{22}O_{11}$, 450 g mol⁻¹), **2.** (-)-Lyoniresinol ($C_{22}H_{28}O_8$, 420 g mol⁻¹), **3.** (+)-Lyoniside ($C_{27}H_{36}O_{12}$, 552 g mol⁻¹), **4.** (+)-lyoniresinol 9'-O- β -glucopyranoside ($C_{28}H_{38}O_{13}$, 582 g mol⁻¹), **5.** Bartogenic acid ($C_{30}H_{46}O_7$, 518 g mol⁻¹) and its glucosyl derivative **6.** 28-O- β -D-glucopyranosylbartogenic acid (Glu-BA) ($C_{36}H_{56}O_{12}$, 680 g mol⁻¹), **7.** 23-O-galloylarjunglucoside (Quercotriterpenoside-I) ($C_{43}H_{62}O_{15}$, 818 g mol⁻¹) and its isomer **8.** (Quercotriterpenoside-III) ($C_{43}H_{62}O_{15}$, 818 g mol⁻¹), **9.** 3-O-[(6-Ogalloyl)- β -D-glucopyranosyl]bartogenic acid ($C_{43}H_{60}O_{16}$, 832 g mol⁻¹), **10.** 3-O-galloylrobural A ($C_{43}H_{60}O_{16}$, 832 g mol⁻¹) and **11.** 3-O-galloylbarrinic acid ($C_{37}H_{50}O_{11}$, 670 g mol⁻¹).

Table 1: Structures of **11** compounds purified from oakwook and wine extracts and screened for PL modulation potential extracted. The reference PL inhibitor used in this study is orlistat (compound **12**).

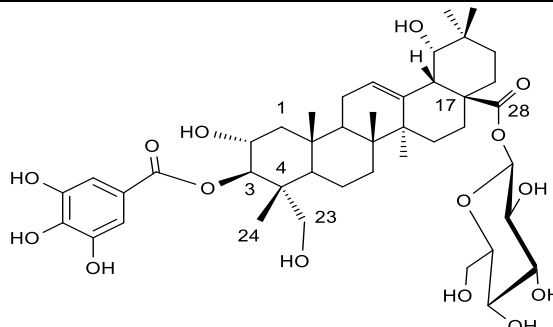
Number	Name	Structure	M _w (g mol ⁻¹)
1	Astilbin		450
2	(-)-Lyoniresinol		420
3	(+)-Lyoniside		552
4	(+)-lyoniresinol 9'-O-β-glucopyranoside		582
5	Bartogenic acid		518
6	28-O-β-D-glucopyranosylbartogenic acid		680

7 Quercotriterpenoside-I



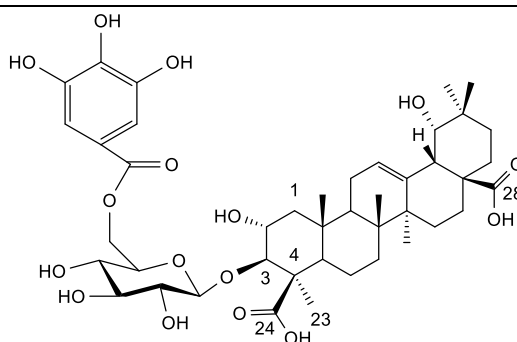
818

8 Quercotriterpenoside-III



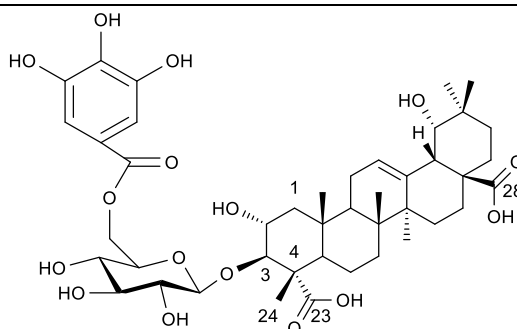
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9 3-O-[(6-Ogalloyl)-β-D-glucopyranosyl]bartogenic acid



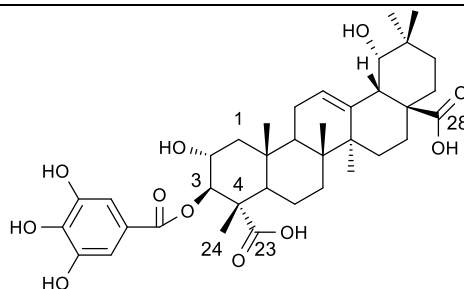
832

10 3-O-galloylrobural A



832

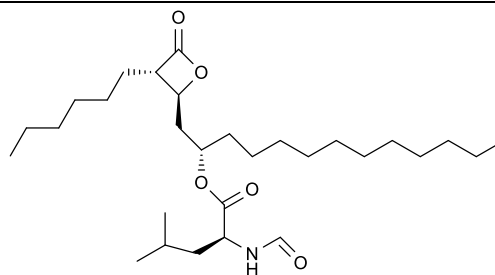
11 3-O-galloylbarrinic acid



670

12
(Reference
inhibitor)

Orlistat



495

CE-enzymatic assays

All reactions were performed in triplicates ($n = 3$) at 37 °C. The corrected peak areas (CPA) of the reaction products represented the peak areas corrected for migration time. Blank reactions were carried out in the absence of the enzyme, which was substituted with the IB, to estimate the non-enzymatic spontaneous hydrolysis of the 4-NPB substrate. Control reactions were conducted to set the baseline activity of PL which corresponds to 0 % PL modulation. The modulatory molecules in control reactions were substituted with their respective solvent. The modulation of PL activity was then calculated relative to the activity of PL observed in control reactions after correction for spontaneous hydrolysis observed in the blank assays. The modulation of PL activity (activation and inhibition) was calculated as follows (Eq.1):

$$\% \text{ Modulation} = \left| \left(1 - \left(\frac{A_M - A_B}{A_C - A_B} \right) \right) \times 100 \right| \quad (1)$$

Where A_M and A_C are the CPA of the hydrolysis products in presence and absence of the modulator molecules, respectively. A_B is the CPA of the hydrolysis products in the blank assays. This calculation is conducted for UV as well as for C^4D detection.

The residual PL activity is calculated after incubation with inhibitors as follows (Eq.2):

$$\text{Residual activity \%} = \left(1 - \frac{I}{100} \right) \times 100 \quad (2)$$

Where I is the percentage of PL inhibition, calculated using the equation (1).

Offline CE-enzymatic assay: 6 μL of PL (1.2 mg mL^{-1}) was pre-incubated with 10 μL of the investigated compounds *i.e.* orlistat (0.3 mg mL^{-1}), natural purified molecules or plant extracts (1 mg mL^{-1}) in 29 μL of Tris/MOPS IB for 5 min. Addition of 5 μL of 4-NPB (0.1 mg mL^{-1}) initiated the enzymatic reaction *i.e.* the incubation step. The reaction mixture was incubated for 5 min to avoid excess substrate hydrolysis ($\leq 10 \%$). At the end of the incubation period, the reaction mixture was moved to a boiling

water bath (95 °C) for 5 min to terminate the reaction. Then, a bench-centrifuge (model number 250711) (Quirumed, Valencia, Spain) was used to centrifuge the reaction mixture tube at $2000 \times g$ for 5 min. Hydrodynamic injections were carried out at 0.7 psi for 5 sec (9 nL).

Influence of DPPC vesicles on PL kinetics: The effect of DPPC vesicles on the kinetics of 4-NPB hydrolysis by PL was investigated using the offline CE-based assay. DPPC vesicles were prepared at 1 mg mL^{-1} in the IB and then added to the enzymatic reaction. The enzymatic reactions were carried out using a range of 4-NPB substrate concentrations (0.25–2 mM). The initial reaction rate V_i (mM min^{-1}) was calculated as the ratio of products formed *per* time interval in the presence or absence of DPPC vesicles. The nonlinear curve fitting program PRISM® 5.04 (GraphPad, San Diego, CA, USA) was used to determine V_{max} and K_m according to following equation (Eq.3):

$$V_i = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \quad (3)$$

where V_i is the reaction rate, K_m is the Michaelis constant, V_{max} is the maximal reaction velocity and $[S]$ is the substrate concentration.

Online CE-enzymatic assay: In the optimized conditions, the investigated modulatory compounds (represented by M for online assays) and the enzyme (PL) were mixed together and injected as a single plug. Simulations of plug overlapping by TDLFP and their position in the capillary were done using a software developed by S. Krylov's group [72]. Injection sequence was carried out from the long capillary end (furthest from the optical detection window) as follows: PL + M (0.5 psi \times 3 sec, 4 nL), 4-NPB substrate (S) (0.5 psi \times 6 sec, 8 nL), PL + M (0.5 psi \times 3 sec, 4 nL) and IB (0.5 psi \times 90 sec, 120 nL) (Fig. 2). Between each injection, the capillary inlet was dipped into the IB to minimize sample carry over [73]. The reactant plugs were mixed inside the capillary and incubated for 5 min. Then, the separation voltage was applied to separate the reaction components and terminate the enzymatic reaction by separating the enzyme from the substrate.

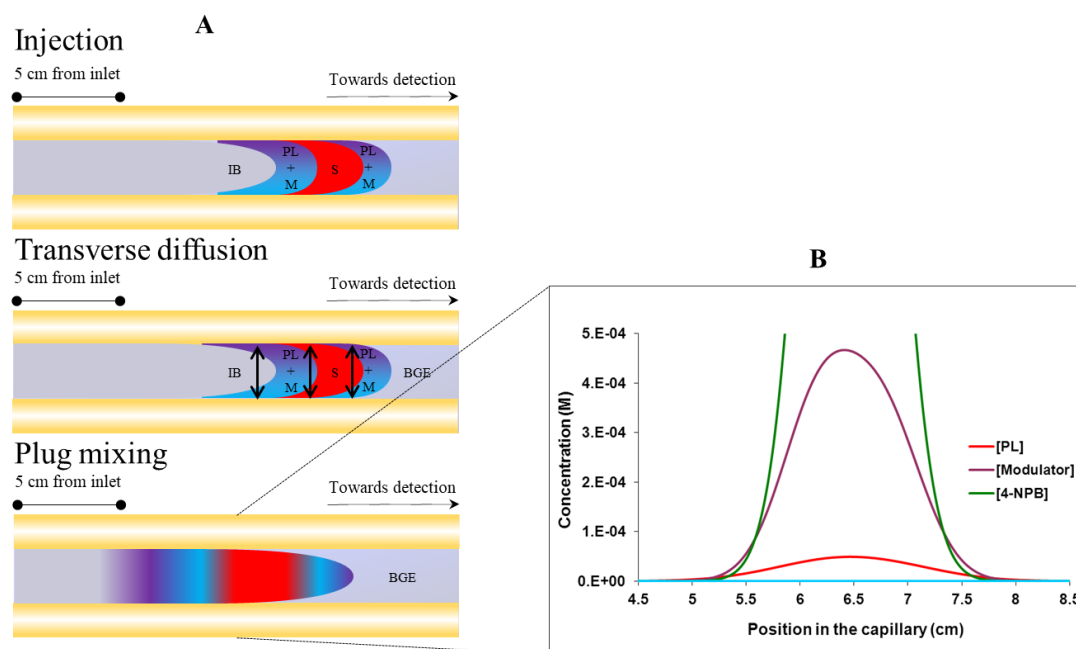


Figure 2. Schematic representation of the different steps of the online enzymatic reaction **A**). Injection of the different reactants sequentially into the capillary filled with the BGE as follows; 8 mg mL^{-1} PL + 1 mg mL^{-1} of the modulator molecule (**M**) ($0.5 \text{ psi} \times 3 \text{ sec}$, 4 nL), 1 mg mL^{-1} 4-NPB (**S**) ($0.5 \text{ psi} \times 6 \text{ sec}$, 8 nL), PL + **M** ($0.5 \times 3 \text{ sec}$, 4 nL) and IB ($0.5 \text{ psi} \times 90 \text{ sec}$, 120 nL). Transverse diffusion of the different reactant plugs into each other. Plug mixing by incubation of the plugs for 5 min at 37°C allowing them to mix to initiate the enzymatic reaction. Simulation of the concentrations of the different reactants relative to their position in the capillary in addition to the overlapping of their plugs **B**).

Results and discussion

Before conducting CE-based enzymatic assays, plant extracts were prepared and characterized. The molecular features of *Chrysanthellum americanum*, blackcurrant and dry hawthorn were thoroughly studied in our previous papers [64,66]. In this study, fresh hawthorn was characterized and its biological activity against lipase was evaluated. Moreover, the characterization of all tested extracts was conducted.

Molecular characterization of plant extracts

Global extraction yields, TPC, TFC and OPC results are presented in Table 2. Concerning hawthorn, the extraction yield was similar for fresh leaves, fresh flowers, and dry and fresh flowering tops (20-24 %). The values of TPC and TFC from fresh leaves and fresh flowering tops were much lower ($107 \text{ mg eq. gallic acid/g dry plant extract}$ for TPC and $10 \text{ mg eq. quercetin/g dry plant extract}$ for TFC) than those from fresh or dry flowers ($121\text{-}171 \text{ mg eq. gallic acid/g dry plant extract}$ for TPC and $16 \text{ mg eq. quercetin/g dry plant extract}$ for TFC) and those from dry flowering tops ($96\text{-}150 \text{ mg eq. gallic}$

acid/g dry plant extract for TPC and 14-15 mg eq. quercetin/g dry plant extract for TFC). However, the value of OPC from dry plant (8-20 mg eq. cyanidin/g dry plant extract) was much higher than that from fresh plant (4.5-5 mg eq. cyanidin/g dry plant extract). Those findings are in good agreement with the literature [74,75]. The global extraction yield of *Chrysanthellum americanum* (26 %) and of blackcurrant leaves (28.5 %) is higher, the former containing much less phenolic compounds (60 mg eq. gallic acid/g dry plant extract for TPC) and proanthocyanidin oligomers (1.6 mg eq. cyanidin/g dry plant extract for OPC) than the two other plants.

Table 2. Extraction yield, TPC, TFC, and OPC values in various plant extracts issued from the infusion of ground plant materials (see experimental part for the details). ^a: calculated on 1 experiment. ^b: data extracted from [66]. ^c: in mg eq. gallic acid/g dry extract, ± 1 standard deviation calculated on $n=3$ repetitions. ^d: in mg eq. quercetin/g dry extract, ± 1 standard deviation calculated on $n=3$ repetitions. ^e: in mg eq. cyanidin/g dry extract, ± 1 standard deviation calculated on $n=3$ repetitions. ^f: data extracted from [64].

Nature	Lot number	Extraction yield (%)	TPC ^c	TFC ^d	OPC ^e
Hawthorn fresh leaves	Harvested 2020	19.8 ^a	107.7 \pm 4.1	9.8 \pm 0.5	5.0 \pm 0.3
Hawthorn fresh flowers		24.0 ^a	121.3 \pm 6.0	15.7 \pm 0.1	4.8 \pm 0.1
Hawthorn dry flowering tops - Sample A -	20335	23.2 \pm 0.6 ^b	149.5 \pm 4.0	15.4 \pm 0.3	20.0 \pm 1.7
Hawthorn dry flowering tops - Sample B -	CB58120	22.5 \pm 0.3 ^b	96.0 \pm 1.8	13.9 \pm 0.4	8.0 \pm 0.4
Hawthorn dry flowers	20334	21.7 \pm 0.6 ^b	171.4 \pm 3.2	15.9 \pm 0.4	9.0 \pm 0.2
<i>Chrysanthellum americanum</i> ^f	559980	25.9 \pm 0.9	59.9 \pm 0.2	12.8 \pm 0.2	1.6 \pm 0.1
Blackcurrant leaves ^f	55870	28.6 \pm 0.5	138.5 \pm 1.7	15.5 \pm 0.6	5.8 \pm 0.3

UHPLC-DAD and UHPLC-ESI-MS/MS analyses showed that the main compounds found in hawthorn are phenolic acids such as chlorogenic acid and flavonoids such as epicatechin, vitexin 2-O-rhamnoside, pinnatifinoside A, hyperoside, isoquercetin and apigenin-C-hexoside (see Fig. 3, Fig S.2 and [66]).

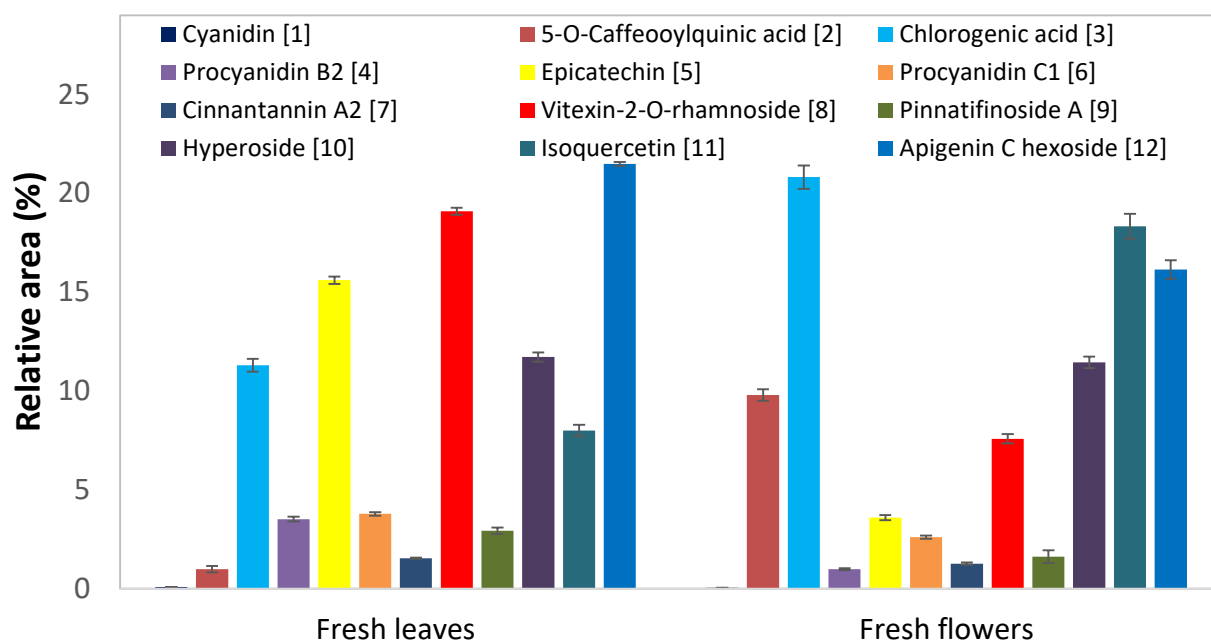


Figure 3. Relative peak area distributions for the main identified chromatographic peaks according to the nature of hawthorn plant part (grinded fresh leaves or flowers). Same experimental conditions as in Figure S.2. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 12 identified components. Error bars: ± 1 standard deviation calculated on $n=3$ repetitions. 1=Cyanidin, 2=5-O-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7= cinnamtannin A2, 8=vitexin-2-O-rhamnoside, 9=pinnaftinoside A, 10=hyperoside, 11=isoquercetin, 12=apigenin C-hexoside.

The quantification of several representative compounds for each subfamily such as chlorogenic acid (peak 3 in Fig. S.3), epicatechin (peak 5), vitexin-2-O-rhamnoside (peak 8), isoquercetin (peak 11) was determined by external calibration, using commercially available standards as presented in Fig. S.3. Interestingly, for fresh hawthorn leaves, the quantities of epicatechin and vitexin-2-O-rhamnoside are 103.2 ± 3.2 mg per g of fresh leaves extract and 17.6 ± 0.5 mg per g fresh leaves extract, respectively, which is higher than that in the flowers and flowering tops extracts. On the contrary, chlorogenic acid and isoquercetin are major components in the fresh flowers, with 32.7 ± 0.4 mg per g fresh flowers extract and 45.87 ± 1.3 mg per g fresh flowers extract, respectively.

Nine major peaks were detected in blackcurrant leaves, two of them (chlorogenic acid and quercetin 3-O-glucoside (isoquercetin)) were unambiguously identified, while the seven others were tentatively assigned to quercetin 3-rutinoside, quercetin 3-O-galactoside, quercetin-3-6-malonylglucoside, kaempferol-3-O-rutinoside, kaempferol-3-O-hexoside, kaempferol-malonylglucoside, and kaempferol-malonylglucoside isomer [64]. Concerning *Chrysanthellum americanum* and blackcurrant leaves, the main compounds have been listed in [64]. Ten major peaks were detected

in *Chrysanthellum americanum*, one of them (chlorogenic acid) was unambiguously identified and the nine others were tentatively assigned to eriodicyol-7-O-glucoside, 6,8-C,C-diglucosylapigenin, isookanin-7-O-glucoside (flavanomarein), maritimetin-6-O-glucoside (maritimein), luteolin-7-O-glucuronide, di-caffeoylquinic acid, apigenin-7-glucuronide, and di-caffeoylquinic acid isomers.

Optimization of the electrophoretic separation buffer (BGE)

Before conducting CE-based enzymatic reactions, it is important to ensure that the reaction can be monitored and quantified using suitable separation and detection conditions of both products, 4-NP and butyrate. The choice of the BGE should be carried out carefully considering its compatibility with the technique of detection as well as the enzymatic reaction. BGE is influential to the sensitivity of analyte detection, peak shape and symmetry and the duration of the analysis [76]. For direct detection using dual conductometric and spectrophotometry and in order to limit noise, the buffer should have low background conductivities and minimal absorption at the wavelength of analyte detection. With conductometric detection, the analytical response is proportional to the difference between the analyte's and the BGE's co-ion mobilities [77]. However, this difference results in the decline of peak symmetry which relies on the equivalence of these mobilities [78].

The properties of two BGE, 100 mM Tris/50 mM CHES (12 mM, alkaline pH 9.0) and 10 mM Tris/40 mM MOPS (10 mM, slightly acidic pH 6.6) were compared using PeakMaster 5.3 (Table S.1). Firstly, both buffers had low conductivities and thus were suitable for direct conductometric detection. Additionally, they did not absorb at the wavelength of interest ($\lambda = 400$ nm).

The limits of detection (LOD) of the products were evaluated by injecting different concentrations of standard solutions. The 4-NP peak detected at 400 nm was symmetrical with Tris/MOPS (peak asymmetry of 0.9) and LOD was found to be 30 μ M. Peak symmetry in this case could be explained by the equivalence of the 4-NP mobility and the BGE co-ion's (MOPS) mobility. 4-NP peak was completely distorted with Tris/CHES. In addition, C⁴D was a lot more sensitive to butyrate with Tris/MOPS as BGE (LOD = 0.5 μ M vs 10 μ M). Comparing the mobilities of the butyrate ion to the co-ions mobilities shows that the difference between both mobilities is almost identical in both buffers (Table S.1). The higher sensitivity with Tris/MOPS is thus probably due to the lower conductivity of this BGE couple compared to Tris/CHES. It is worthy to note that the effective mobility of 4-NP ($\mu_{\text{eff}} = -7.9 \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$) is very close to that of MOPS ($\mu_{\text{eff}} = -5.7 \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$) which explains that the Tris/MOPS is not suitable to the conductometric detection of the 4-NP.

Additionally, the velocities of the 4-NP and butyrate ions, calculated by dividing the distance from the capillary inlet to the respective detector of the ion over the migration time of each ion, were $0.93 \pm 0.004 \text{ cm s}^{-1}$ and $0.57 \pm 0.002 \text{ cm s}^{-1}$ using Tris/MOPS and $0.03 \pm 1.66 \times 10^{-5} \text{ cm s}^{-1}$ and $0.15 \pm 0.003 \text{ cm s}^{-1}$ when using Tris/CHES as BGE, respectively. The greater velocities with Tris/MOPS are reflected by shorter migration times and therefore permit rapid analyses (less than 7 min, Fig. 4). Finally, non-enzymatic hydrolysis of nitrophenyl esters is common especially at alkaline pH due to their high reactivities [79]. The extent of spontaneous hydrolysis of the 4-NPB substrate was acceptable with Tris/MOPS at pH 6.6 (< 10 %) compared to Tris/CHES at pH 9 where it was about 30 %.

For all these reasons, the remainder of the study was carried out using Tris/MOPS (10 mM, pH 6.6).

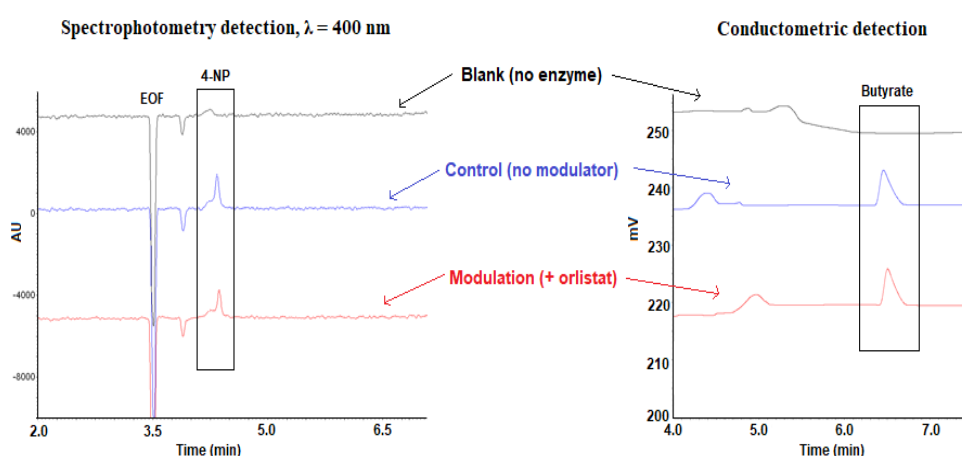


Figure 4. Electropherograms obtained after monitoring 4-NPB hydrolysis by PL using online CE-UV/C⁴D assay. The injection sequence was carried out as follows: PL + Orlistat (0.5 psi × 3 sec, 4 nL), 4-NPB (0.5 psi × 6 sec, 8 nL), PL + Orlistat (0.5 psi × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL). The reactants were incubated for 5 min at 37 °C before the application of + 30 kV. BGE/IB: Tris/MOPS (10 mM, pH 6.6). For more details: see “Experimental section”. EOF: electroosmotic flow, AU: absorbance units and mV: millivolts.

Optimization of the enzymatic reaction conditions

After having chosen the suitable conditions to monitor the enzymatic reaction, the preparation of the 4-NPB substrate was optimized and a suitable IB was chosen. We mainly focused on the stability of the substrate in the stock solution as well as in the final incubation mixture.

Choice of the substrate solvent and of the IB: In Fig. 5.A, the spontaneous non-enzymatic degradation of 4-NPB prepared in either water or ACN and then diluted in Tris/MOPS buffer at pH 7.5 and pH 6.6 (which corresponds to the BGE tested in the previous section) was observed. The

incubation of the different 4-NPB solutions correspond to the offline enzymatic reaction steps (see section “CE-enzymatic assays”) for comparison between spontaneous and enzymatic hydrolysis of 4-NPB. When working at pH 7.5, the degree of spontaneous hydrolysis of the 4-NPB, dissolved in water or ACN, was significant as concluded by comparing the intensities of the yellow colored solutions. Conventionally, Tris adjusted to pH 7.5 is used as the IB to assay PL activity since this conforms to the optimal pH of PL activity [80,81]. At lower pH 6.6 no spontaneous degradation was visually observed with the stock solution prepared in ACN. Owing to its chemical nature, it is a common practice to prepare 4-NPB in organic solvents such as ACN. It is worthy to note that significant hydrolysis of 4-NPB was observed when prepared in DMF, even at 1 % (v/v) (results not shown). To further investigate the spontaneous degradation, the 4-NPB solution in ACN diluted in Tris/MOPS pH 6.6 was analyzed by CE at 400 nm.

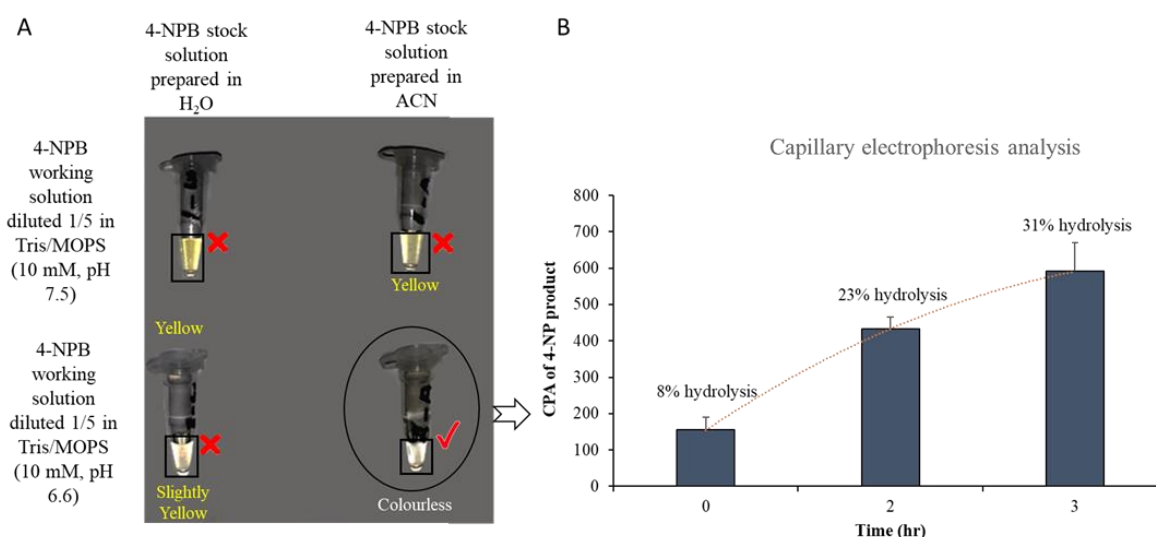


Figure 5. Visual spontaneous hydrolysis of 4-NPB in the absence of PL. **A)** The yellow color represents the release of 4-NP as a result of spontaneous 4-NPB hydrolysis. The substrate was prepared at a stock concentration of 5 mM in either water (left) or ACN (right). 10 μ L of 5 mM 4-NPB was then mixed with 40 μ L of 10 mM Tris/40 mM MOPS (ionic strength= 10 mM, pH 7.5 (top row) or pH 6.6 (bottom row)) to have a final substrate concentration of 1 mM and a final volume of 50 μ L. The mixtures were incubated at 37 $^{\circ}$ C for 5 min and then at 95 $^{\circ}$ C for another 5 min before the color of each tube was observed. **B)** CE injection at different time points of 4-NPB solution prepared in ACN and diluted in Tris/MOPS (10 mM, pH 6.6) to estimate time dependent spontaneous hydrolysis.

The results (Fig. 5.B) show a time-dependent increase in 4-NP corrected peak areas (CPA) corresponding to an increase of 4-NPB spontaneous degradation from only 8 % (immediately after

incubation) to 31 % over a period of three hours (n=3). This emphasizes the importance of short CE analysis times in order to analyze rapidly the reaction mixtures after their preparation. Accordingly, the 4-NPB substrate was prepared in pure ACN and diluted in the Tris/MOPS (10 mM, pH 6.6) for the remainder of the study. The ACN quantity in the final reaction was estimated to be 10 %. Being enzymes that function superiorly in the aqueous-organic interfaces, lipases show improved stability in relatively highly organic media rather than purely aqueous ones due to the interfacial activation phenomenon [63,82].

The Tris/MOPS (10 mM, pH 6.6) was also used as the IB. Using 5 min of incubation time confirmed that the activity of the PL in this buffer was satisfactory. Moreover, using the same solution as both the BGE and IB is simpler and of great interest particularly when adapting the online enzymatic assay.

K_m and V_{max} evaluation and influence of DPPC phospholipidic vesicles on PL activity: As before mentioned, PL is active at a lipid-water interface. For this, the offline CE-based assay was conducted using the optimized conditions above presented in order to investigate the effect of DPPC vesicles on 4-NPB hydrolysis by PL in terms of molecular affinity (K_m) and of reaction rate (V_{max}). Both UV results *i.e.* quantification of 4-NP and C⁴D results *i.e.* quantification of butyrate were used.

The results obtained in the absence of DPPC confirmed the substrate-enzyme relationship between 4-NPB and PL as confirmed by K_m values obtained using UV (K_m = 0.57 mM) and C⁴D (K_m = 0.59 mM) (Table 3). K. Shirai *et al.* [83] found similar K_m value of 0.52 mM for the hydrolysis of 4-NPB by lipoprotein lipase (LPL) when using fluorescence polarization spectroscopy. V_{max} was slightly higher for the hydrolysis of 4-NPB with PL in this study as compared to the hydrolysis with LPL (Table 3).

Table 3: Kinetic constants of 4-NPB hydrolysis by PL in the presence or absence of 1 mg mL⁻¹ DPPC. The constants were calculated by following the formation of the hydrolysis products, 4-NP and butyrate, using spectrophotometric and conductometric detection, respectively. The constants were compared to those obtained for 4-NPB hydrolysis by LPL as described by K. Shirai *et al.* [83]

(n=3)		4-NP	Butyrate	4-NP
		UV	C ⁴ D	Lit. [83]
K _m (mM)	- DPPC	0.57±0.03	0.59±0.12	0.52
	+ DPPC	1.54±0.01	1.66±0.01	0.55
V _{max} (μM.s ⁻¹)	- DPPC	1.20±0.13	0.71±0.07	0.66
	+ DPPC	4.22±0.01	3.68±0.01	2.93

When DPPC vesicles were added to the incubation mixture, the affinity of PL towards 4-NPB was slightly decreased as represented by the increase of the K_m value (3 fold) and the reaction rate was slightly increased (4-5 fold). In Shirai's work [83], the affinity of 4-NPB to LPL was unchanged and the reaction rate was similarly increased upon addition of DPPC (Table 3). Moreover, the relative standard deviation (RSD) of the migration times and CPA were calculated as 1% and 3% for 4-NP and 2% and 1% for butyrate, respectively. This confirms that the offline method can detect and quantify the PL catalyzed hydrolysis reaction with excellent repeatabilities.

To recapitulate, the influence of DPPC on the PL hydrolysis of 4-NPB remain small. For this reason, no vesicles were used in the remainder of the study, neither for the offline nor for the online assays. Furthermore, the results obtained confirms the choice of the 4-NPB as a substrate for the PL. The good repeatability ($n=3$) and the concordance of the results obtained using the dual detection system confirms the reliability of the developed offline CE-UV/ C^4D based lipase assay.

PL inhibition using the reference inhibitor orlistat

The influence of the reference inhibitor orlistat (Table 1, entry **12**) on PL using the developed CE-UV/ C^4D method was first evaluated to develop and validate the offline and online inhibition assays. Due to its limited solubility, orlistat was tested at a maximum concentration of 0.3 mg mL^{-1} in 7 % DMSO using the offline assay. To assess any non-enzymatic degradation of 4-NPB, blank assays were conducted in the absence of the enzyme, as previously detailed. Control reactions, where no modulatory molecules are involved, were conducted for comparison (Fig. 4).

Offline CE-UV/ C^4D assay to evaluate the effect of orlistat on PL activity: The offline assay consists of 4 steps: pre-incubation, incubation, termination and centrifugation. The pre-incubation step involves incubating PL with the orlistat. It was found to be essential to allow the establishment of any enzyme-inhibitor interaction as well as for thermal equilibrium of the reaction mixture at $37 \text{ }^\circ\text{C}$ [84]. In these conditions, the orlistat at 0.3 mg mL^{-1} was found to inhibit the PL activity by $24 \pm 1 \%$ ($n = 3$). Both UV and C^4D results were obtained with excellent reproducibility. This assay was easy to optimize and the quantification could be assessed in a very straightforward manner.

Online CE-UV/ C^4D assay to evaluate the effect of orlistat on PL activity: The online mode combines the enzymatic reaction and the CE analysis into a single automated and very economic process since only few nanoliters of reactants are required. The temperature of the capillary in which the PL reaction takes place was fixed at $37 \text{ }^\circ\text{C}$ (as for offline assays). A plug of the IB is typically injected first

into the BGE filled capillary to avoid any denaturation of the enzyme by the BGE. This is known as partial filling [85]. However, since we chose similar BGE and IB (Tris/MOPS 10 mM, pH 6.6), no such precaution was necessary. Furthermore, an IB plug needs to be injected at the end of the injection sequence to improve plugs' overlapping by increasing the lengths of their longitudinal interfaces. Firstly, an IB plug as long as the sum of the lengths of all the other reactant plugs was used [47]. In these conditions, the injection sequence was as follows: PL (0.5 psi × 3 sec, 4 nL), **M** (0.5 psi × 3 sec, 4 nL), S (0.5 psi × 6 sec, 8 nL), **M** (0.5 psi × 3 sec, 4 nL), PL (0.5 psi × 3 sec, 4 nL) and IB (0.5 psi × 18 sec, 24 nL). In the case of control reactions, **M** plugs were substituted with plugs of the IB. While the simulations using Krylov's program showed good reactant mixing, low detected quantities of 4-NP and of butyrate were indicative of limited PL activity. Thus, the influence of the homogeneity of the temperature along the capillary was addressed. In fact, when conducting online reactions, incubation takes place at the inlet of the capillary, in a non-thermostated part [86]. More precisely, we measured a drop of 10 °C of the temperature of the solutions in which the entry of the capillary is dipped after 5 min (corresponding to the time used for enzymatic reactions). This means that PL reactions conducted at the capillary inlet take place roughly at 37 °C at the beginning and at 27 °C at the end of the incubation time. This important decrease in temperature during the PL reaction may explain the very low enzymatic activity detected [83,86]. To overcome this, the length of the IB terminal plug was increased (injection at 0.5 psi for 90 sec, 120 nL) to push all the plugs at least 5 cm into the thermostated region of the capillary (Fig. 2). It is important to mention that the longer the terminal IB plug, the better the reactant mixing but the higher is the dilution of the reactant. This dilution is particularly difficult to measure, which makes quantification and comparison with offline assays challenging [47,87,88]. In these conditions, good enzymatic activity was obtained with the control assay as observed by a 207 % increase in the area and 167 % increase in the height of the 4-NP product peak relative to the blank assay. Additionally, the RSD of the migration time and CPA were determined as 1 % and 5 % for 4-NP and 1 % and 2 % for butyrate, respectively.

Then, the reference inhibitor orlistat was prepared in 20 % (v/v) DMSO and injected at 1 mg mL⁻¹. The exact concentration of orlistat in the capillary is difficult to determine. In fact, the dilution factor would be 18-times assuming complete overlapping of all the injected plugs (8 nL of modulator in a total volume of 144 nL). In these conditions, no significant inhibition of PL neither by UV nor by C⁴D was detected. This can be due to the direct contact between the PL's plugs and the DMSO in the inhibitor solution before dilution [89]. It can also be due to the absence of a pre-incubation period

between the tested inhibitor and enzyme [84]. Subsequently, the injection sequence was replaced by the one presented in the “experimental section” and in which the PL and the modulator plugs were combined into an individual plug by injecting both reactants from the same solution (Fig. 2.A). In these conditions, good inhibition of PL by orlistat was obtained ($32 \pm 5 \%$).

Therefore, this method was able to detect modulators of PL activity. It is not trivial to compare this result to the one obtained previously by the offline method due to the difficulty of calculating the exact concentrations of the different reactants after using the long terminal IB plug.

PL modulation potential of aqueous plant extracts

The aqueous extracts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysanthellum americanum* were screened at 1 mg mL^{-1} using the offline CE-UV/C⁴D based assay. The modulation of PL activity by these extracts is graphically represented in Fig. 6.

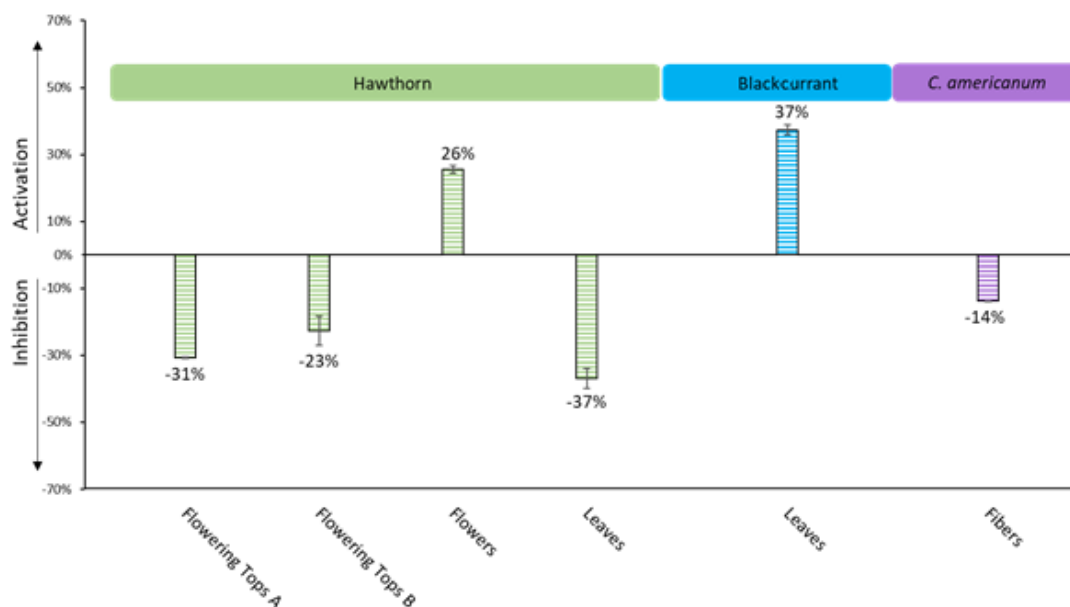


Figure 6. Modulation of PL activity by aqueous extracts of hawthorn (dry flowering tops, dry flowers, fresh flowers, fresh leaves), dry blackcurrant leaves and dry *Chrysanthellum americanum* at 1 mg mL^{-1} . The percentage of modulation is represented relative to a control reactions conducted in the absence of the extracts. Error bars indicate the standard deviation on PL activity for $n = 3$ experiments.

In a first time, we investigated hawthorn dry flowering tops (two different lots of hawthorn, lots A and B) and hawthorn dry flowers (without leaves). Surprisingly, a PL inhibition was obtained for both hawthorn dry flowering tops samples ($31 \pm 0.3 \%$ for sample A and -23% for sample B), while, an activation of PL (positive activity, superior to the control assay corresponding to 0%) was noticed

for hawthorn dry flowers. We thus suspected that hawthorn leaves and flowers act in opposite way on the PL activity. In a second series of experiments, we decided to test hawthorn leaves and flowers separately by harvesting fresh hawthorn flowering tops and by meticulously separating leaves and flowers before extraction by infusion. Results presented in Figure 6 in these fresh samples confirmed an inhibition of the PL enzymatic activity for fresh hawthorn leaves ($37 \pm 3 \%$) while the fresh flower activated the PL enzymes ($26 \pm X \%$). This finding suggests that the inhibitory potential of PL by extracts of flowering tops is mainly due to the leaves which effect is prevailing relative to the flower activity. This difference in PL activity is most likely related to the differences in chemical composition. Certain features were unique to certain parts of hawthorn [66]. For instance, ESI-FT-ICR-MS revealed that CHON species (amino acids, amino sugars) were more abundant in flower extracts of hawthorn. Difference in chemical composition was also studied by UHPLC (see Figure S.2 for UHPLC traces and Figure S.3 for the relative peak area) showing an increased presence in fresh flowers of 5-O-caffeoylquinic acid (peak 2), hyperoside (peak 10) and isoquercetin (peak 11), and lower content in vitexin-2-O-rhamnoside (peak 8) compared to fresh leaves.

Extracts of *Chrysanthellum americanum* led to slight inhibition of PL ($14 \pm 0.1 \%$), while blackcurrant leaves favored the PL activity ($37 \pm 1.5 \%$, respectively). In our recent study [64], we found that almost 16 % of the extracts' molecular features were unique to each of the three studied plants. Thus, the inhibition or activation can be related to the molecular features and type of compounds in the extracts. Concerning extracts of blackcurrant leaves, the predominant compounds were flavonoids, diterpenes, fatty acids and carbohydrates. *C. americanum* extracts had high levels of CHONS features. The *C. americanum* extracts mainly contained polyphenols, fatty acid-like compounds and terpenoids.

Moreover, the application of the online PL assay proved to be tricky to optimize when applied to screen the modulatory effect of these plant extracts. The complex nature of the extracts negatively affected the quality and the repeatability of the reactant mixing by TDLFP.

To conclude, the six aqueous extracts have shown various effects on PL activity. Interestingly, hawthorn leaves extracts induced a specific 37 % inhibition of PL, while hawthorn flower promote PL activity. Recently, [22,23] heterogeneous CE-based assay were developed to screen PL modulation by several natural extracts. For instance, at 10 mg mL^{-1} ethanolic extracts of *Fructus Crataegi* demonstrated PL inhibition of 70 % [22]. In another work, [23] methanolic extracts of *Oxytropis falcate* Bunge demonstrated 54 % and 68.6 % PL inhibition at 50 mg mL^{-1} and 100 mg mL^{-1}

¹, respectively. In comparison to these published data, only 1 mg mL⁻¹ of the aqueous hawthorn leaves extracts was sufficient to demonstrate 37 % inhibition using our developed method. This certainly reflects an interesting inhibitory effect of hawthorn leaves extracts at concentrations 10-100 times lower than that described in the literature for assaying PL inhibition by natural extracts. This also confirms the sensitivity of the CE-UV/C⁴D assay developed in this study. Moreover, it is interesting to specify that the exact same CE-based PL assay was used to monitor both the inhibition and the activation of the enzymatic activity.

PL modulation potential of natural purified compounds

The influence of **11** purified natural compounds on PL activity was evaluated (Table 1) using the CE-UV/C⁴D offline based assay. All of these compounds were extracted and purified from oak wood used for wine aging with the exception of compound **1** which was purified from grapes and wine. These compounds can be divided into three groups based on their chemical structure presented in Table 1: flavonoids (compound **1**), lignans (compounds **2** to **4**) and triterpenoids (compounds **5** to **11**). Fig. 7 represents graphically the effects of the assayed compounds at 1 mg mL⁻¹ on PL activity. Many of the investigated compounds demonstrated activation of PL reflected by a positive activity. Compounds **6** and **10** demonstrated the highest PL activation percentages (37 ± 1 % and 40 ± 0.5 %, respectively). Compounds **3**, **4** and **7** induced moderate activation of PL (between 20 and 27 %) whilst slight PL activation by compounds **1**, **2** and **8** was observed (between 5 and 13 %). On the other hand, the compounds **5**, **9** and **11**, induced considerable inhibition of PL. Compounds **5** and **11** demonstrated good inhibition of PL activity; 51 ± 1 % and 57 ± 4 %, respectively.

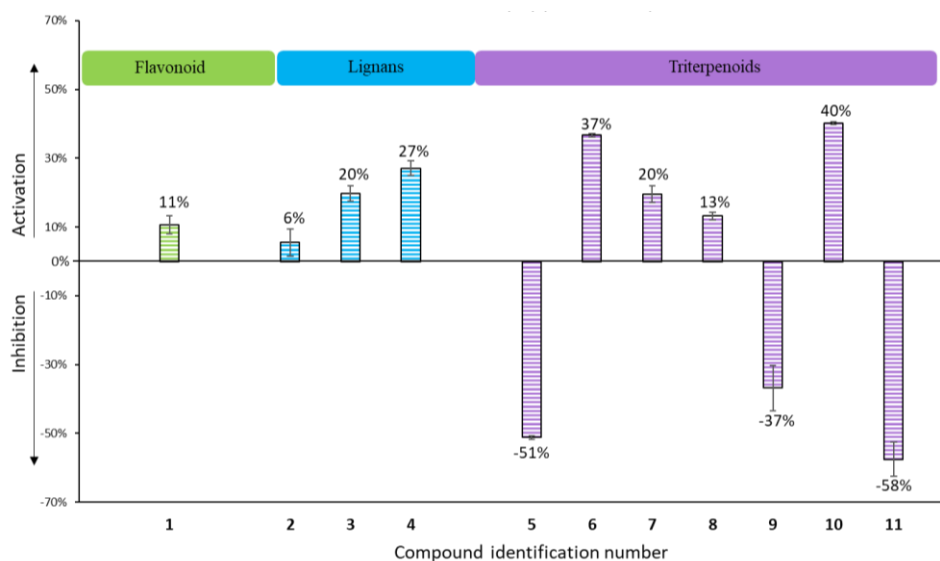


Figure 7. Modulation of PL activity by **11** compounds purified from oak wood and wine extracts (structures in Table 1). The compounds were assayed at 1 mg mL⁻¹ using the offline assay (1-11). The influence of these

compounds on PL activity was relative to control reactions conducted in the absence of the compounds. Error bars indicate the standard deviation on PL activity for $n = 3$ experiments.

The potential of these two compounds was also evaluated by using the online assay previously developed. The results confirm the inhibition potential of both compounds **5** and **11**, however lower inhibition was evaluated (23 ± 4 % and 40 ± 5 %, respectively) due to the dilution phenomena lowering the concentration of the modulators at the time of interaction with the enzyme. The ratios of the residual activities of the online and offline assays were similar, 1.6 for **5** and 1.4 for **11**, demonstrating a constant effect of dilution in the online assay. These ratios also suggest that the dilution factor is not as high as expected probably due to incomplete mixing between all reactant plugs.

The dissimilarity in the bioactivity of the **11** compounds tested is due to the difference in their structure. [3] Compound **5**, bartogenic acid, one of the most potent tested PL inhibitors, is a penta-cyclic triterpenoid having carboxyl groups at positions 24 and 28 bound respectively to C4 and C17. Compounds **6** to **11** all have a common penta-cyclic backbone structure very similar to that of **5**. Compound **6** is carboxylated at position 23 and has a glucosyl moiety attached to position 28. Compared to the bioactivity of **5** against PL, compound **6** resulted in PL activation. Glycosylation and/or the position of the carboxyl group thus appears to play a role in attenuating any enzyme inhibition induced by the molecules. On a similar note, the structures of compounds **9** and **10** differ solely in the positions of the carboxyl groups (position 24 in **9** and position 23 in **10**). Contrasting modulations of PL activity were observed where **9** was found to be an interesting inhibitor whereas the compound **10** was amongst the most activating compounds. Relating these findings to the position of the carboxyl group shows a similar trend to that described for compounds **5** and **6**. Nonetheless, the position of the carboxyl group in the structure does not seem to be the sole contributor to PL modulation. For instance, compound **11** has the carboxyl group at position 23 yet has demonstrated the highest PL inhibition percentage amongst all screened compounds. Interestingly and in contrast to compounds **9** and **10**, compound **11** is galloylated at C3. The presence of galloyl groups in the structure of the compounds has been found to influence competitive inhibition of PL. However, this inhibition was weaker in non-flavonoid compounds compared to flavonoids [90]. Thus, the inhibitory potential of compound **11** can be due to the absence of a glucosyl moiety and/or the presence of a galloyl moiety at C3. This can be further supported by comparing the structures of compounds **7** and **8** which have respectively

demonstrated moderate and limited activation of PL despite both containing a galloyl moiety in their structures at position 23 in **7** and attached to C3 in **8**. Both compounds are however, glycosylated at position 28 thus suggesting that the glycosylation at this position results in decreased inhibition towards PL. In conclusion, glycosylation seems to negate the effects of other substituents such as the galloyl group particularly when this glycosylation is at position 28. Additionally, compounds with glucosyl-galloyl groups on C4 show a modulation of PL activity dependent on the position of the carboxyl groups, demonstrating inhibition when the latter is at position 24 and activation at position 23. In the recent work of J. Liu *et al.* [23] several natural compounds demonstrated interesting PL inhibition using a heterogeneous CE-based PL inhibition assay. Mainly, kaempferol demonstrated the highest PL inhibition; respectively 92 % and 96 % at 50 mg mL⁻¹ and 100 mg mL⁻¹. In our work, compound **11** was shown to inhibit PL by 58 % using only 1 mg mL⁻¹ (50–100 times lower compared to literature). On a similar notice to that described for the offline assay, the results described here for modulation of PL activity by purified compounds demonstrate interesting PL inhibition potential of the compounds at merely 1 mg mL⁻¹. Additionally, despite the dilution associate with online CE assays, some molecules demonstrated potent PL inhibition at final concentrations inferior to 1 mg mL⁻¹ reflecting the sensitivity of the online CE-UV/C⁴D assay developed.

Conclusion

In this study, we described the development and optimization of a CE enzymatic assay hyphenated to dual spectrophotometric and conductometric detection (CE-UV/C⁴D). A lab-made 3D printed scaffold was constructed to hold the C⁴D detection cell while avoiding any coolant liquid leakage. Tris/MOPS (10 mM, pH 6.6) was chosen as the BGE for providing good peak symmetries, low LOD and short analysis times. This buffer was also chosen as the IB as it also proved to be adequate for PL activity. It also limited spontaneous non-enzymatic 4-NPB hydrolysis observed at higher pH. Additionally, having the same buffer as both the BGE and IB facilitated the CE online assay as there was no longer need to implement the partial filling technique. In this study, we describe for the first time, an homogeneous online CE-based PL assay (using non-immobilized PL). The offline CE-UV/C⁴D assay was used to screen the aqueous extracts of various parts of different plants for PL activity modulation. The six aqueous extracts tested revealed different PL activity depending on the plant nature (hawthorn, chrysanthellum americanum, black current) and the part of plant (leaves vs

flowers). Hawthorn leaves presented a promising inhibition potential (37 %) whereas the flowers had an opposite effect on PL activity (activation by + 22-26 %). Furthermore, triterpenoid compounds **5**, **9** and **11**, purified from oak wood used for wine aging, were shown for the first time to have interesting inhibitory effect on PL (up to 57 %), which could be considered as comparable to the one induced by the reference PL inhibitor orlistat. The modulation was related to the structures of these compounds where the presence of a glucosyl, galloyl or carboxyl groups at certain positions of the molecule modulated the activity of PL. The online assay was used to evaluate the inhibition of orlistat (for validation of the assay) and of compounds **5** and **11**. Online results confirmed the inhibition obtained by the offline assay. However, the dilution of the reactants after TDLFP mixing caused an underestimation of the inhibitory effects. All these results are very interesting to further develop our structure-activity relationships (SAR) studies, helping to understand and then to predict biological activity toward PL from molecular structure of interesting compounds.

Further studies will be conducted in the near future to obtain additional insights into the binding and inhibition mechanisms of the best inhibitors identified. Mainly, microscale thermophoresis will be used as a complementary technique to determine the dissociation constant of inhibition (K_i) of such promising compounds.

Acknowledgements

The authors would like to thank the Région Centre Val de Loire and the Labex SynOrg (ANR-11- LABX-0029) for financial support, Florian Coudray for the design and construction of the 3D capillary support and Cedric Maffre for technical support.

Author contribution

R. Nehmé, G. Al Hamoui Dit Banni, R. Nasreddine and S. Fayad planned and performed CE-based enzymatic experiments and analyzed the corresponding data; S. Fayad and A. Marchal planned and performed purifications and characterization of oak wood and wine extracts; P. Cao-Ngoc, J-C. Rossi, L. Leclercq and H. Cottet performed purifications, characterization and quantification of aqueous extracts of *Crataegus oxyacantha* (hawthorn), *Ribes nigrum* (blackcurrant) and *Chrysanthellum americanum*. All authors discussed the results and contributed to the final manuscript.

Figure list

Figure 1. Hydrolysis of 4-NPB into 4-NP and butyrate catalyzed by pancreatic lipase (PL). The dashed double-sided arrow indicates the enzyme cleavage site.

Figure 2. Schematic representation of the different steps of the online enzymatic reaction **A**). Injection of the different reactants sequentially into the capillary filled with the BGE as follows; 8 mg mL⁻¹ PL + 1 mg mL⁻¹ of the modulator molecule (**M**) (0.5 psi × 3 sec, 4 nL), 1 mg mL⁻¹ 4-NPB (**S**) (0.5 psi × 6 sec, 8 nL), PL + **M** (0.5 × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL). Transverse diffusion of the different reactant plugs into each other. Plug mixing by incubation of the plugs for 5 min at 37 °C allowing them to mix to initiate the enzymatic reaction. Simulation of the concentrations of the different reactants relative to their position in the capillary in addition to the overlapping of their plugs **B**).

Figure 3. Relative peak area distributions for the main identified chromatographic peaks according to infusion extraction (grinded fresh leaves, flowers and flowering tops) of hawthorn. Same experimental conditions as in Figure S.2. The relative area was calculated by dividing the peak area of each component by the sum of the peak area of the 12 identified components. Error bars: ± 1 standard deviation calculated on n=3 repetitions. 1=Cyanidin, 2=5-O-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7= cinnamtanin A2, 8=vitexin-2-O-rhamnoside, 9=Pinatifinose A, 10=hyperoside, 11=isoquercetin, 12=apigenin C-hexoside.

Figure 4. Electropherograms obtained after monitoring 4-NPB hydrolysis by PL using online CE-UV/C⁴D assay. The injection sequence was carried out as follows: PL + Orlistat (0.5 psi × 3 sec, 4 nL), 4-NPB (0.5 psi × 6 sec, 8 nL), PL + Orlistat (0.5 psi × 3 sec, 4 nL) and IB (0.5 psi × 90 sec, 120 nL). The reactants were incubated for 5 min at 37 °C before the application of + 30 kV. BGE/IB: Tris/MOPS (10 mM, pH 6.6). For more details: see “Experimental section”. EOF: electroosmotic flow, AU: absorbance units and mV: millivolts.

Figure 5: Visual spontaneous hydrolysis of 4-NPB in the absence of PL. A) The yellow color represents the release of 4-NP as a result of spontaneous 4-NPB hydrolysis. The substrate was prepared at a stock concentration of 5 mM in either water (left) or ACN (right). 10 µL of 5 mM 4-NPB was then mixed with 40 µL of 10 mM Tris/ 40 mM MOPS (Ionic strength= 10 mM, pH 7.5 (top row) or pH 6.6 (bottom row) to have a final substrate concentration of 1 mM and a final volume of 50 µL. The mixtures were incubated at 37 °C for 5 min and then at 95 °C for another 5 min before the color of each tube was observed. B) CE injection at different time points of 4-NPB solution prepared in ACN and diluted in Tris/MOPS (10 mM, pH 6.6) to estimate time dependent spontaneous hydrolysis.

Figure 6. Modulation of PL activity by aqueous extracts of hawthorn flowering tops (flowers and leaves, 2 lots A and B), flowers only and leaves only, blackcurrant leaves and Chrysanthellum americanum at 1 mg mL⁻¹. The percentage of modulation is represented relative to a control reactions conducted in the absence of the extracts. Error bars indicate the standard deviation on PL activity for n = 3 experiments.

Figure 7: Modulation of PL activity by **11** compounds purified from oak wood, grapes and wine extracts (structures in **Table 1**). The compounds were assayed at 1 mg mL⁻¹ using the offline assay (**1-11**). The influence of these compounds on PL activity was expressed relative to control reactions in the absence of the compounds. Error bars indicate the standard deviation on PL activity for n = 3 experiments.

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SUPPORTING INFORMATION OF APPENDIX C

Screening for pancreatic lipase natural modulators by capillary electrophoresis hyphenated to spectrophotometric and conductometric dual detection

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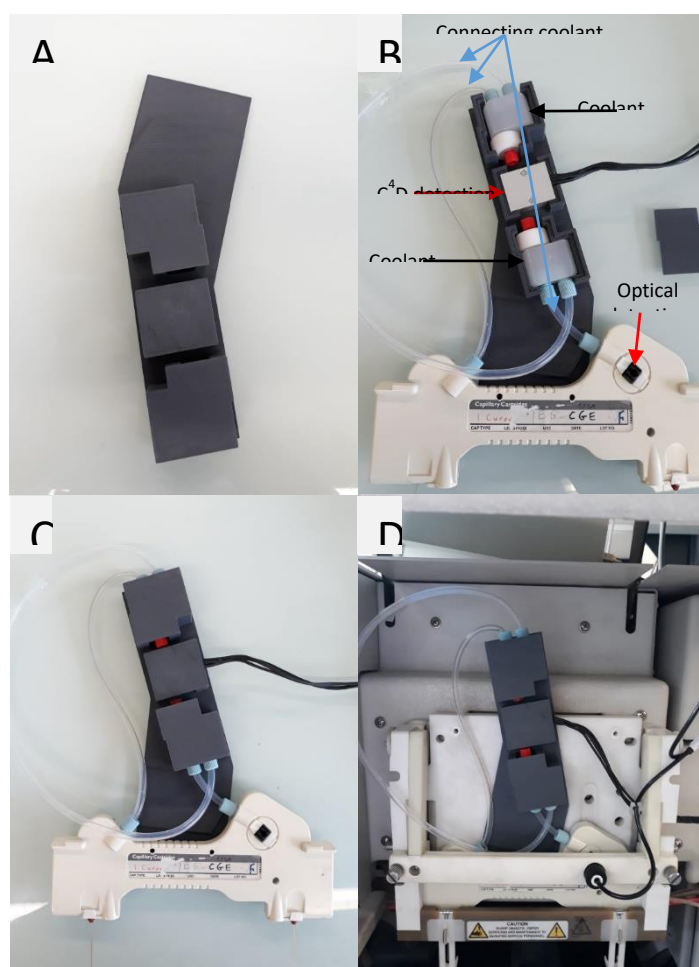


Figure S.1: Capillary cartridge with the connecting tubings and reservoirs supported by a 3D printed scaffold. A) The 3D printed scaffold, B) Insertion of all components (two reservoirs, C⁴D detection cell) into their designated positions in the scaffold, C) Addition of 3D printed clips to fix loose parts of the system in place and D) installation of the cartridge into the CE instrument.

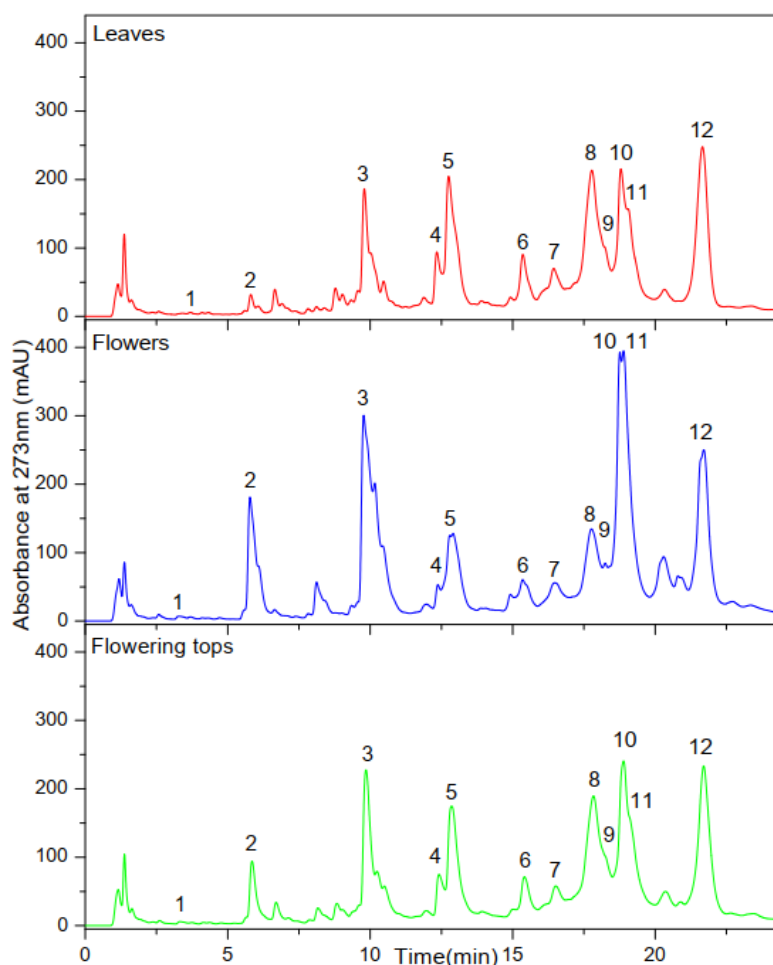


Figure S.2 UHPLC profiles of different parts of hawthorn extracts obtained from infusion extraction modes for ground fresh leaves, flowers and flowering tops (fresh materials harvested in April 2020). Experimental conditions: A Kinetex C18 100A 100×2.1 mm, 2.6 μm column, binary solvent system: water/formic acid (1%, v/v) as solvent A and acetonitrile/formic acid (1%, v/v) as solvent B. Gradient program: 5 % B, then increase of B to 100 % in 35 min with a convex increase, flow rate: 0.3 mL.min⁻¹, injection volume: 20 μL . Column temperature: 20°C, UV monitoring at 273 nm. UV-Vis spectra recorded between 200 and 550 nm. Peak identification: 1=Cyanidin, 2=5-O-caffeoylquinic acid, 3=chlorogenic acid, 4=procyanidin B2, 5=epicatechin, 6=procyanidin C1, 7=cinnamtanin A2, 8=vitexin-2-O-rhamnoside, 9=pinnatifinose A, 10=hyperoside, 11=isoquercetin, 12=apigenin C-hexoside.

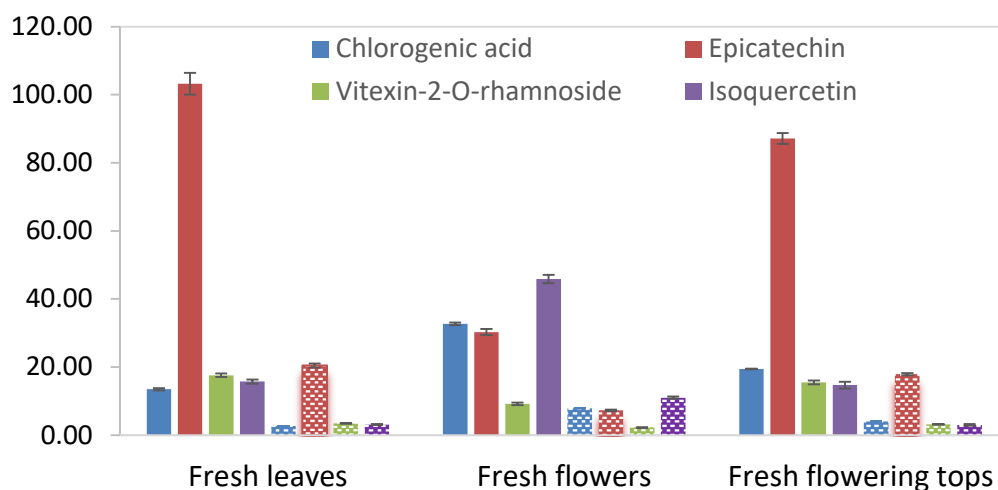


Figure S.3. Quantification of chlorogenic acid (peak 3), epicatechin (peak 5), vitexin-2-O-rhamnoside (peak 8), isoquercetin (11) were determined by external calibration, using commercially available standards. Error bars: ± 1 standard deviation calculated on $n=3$ repetitions. ■ ■ ■ ■, in mg g^{-1} dry extract. ■ ■ ■ ■, in mg g^{-1} fresh plant.

Table S.1: Comparison of the conductivities and effective mobilities of both BGEs using PeakMaster v5.3

BGE	Tris/CHES (12 mM, pH 9.0)	Tris/MOPS (10 mM, pH 6.6)
Conductivity (S m^{-1})	0.054	0.046
Effective mobility ($\text{m}^2 \text{V}^{-1} \text{s}^{-1}$)	Co-ion CHES: -5.1	Co-ion MOPS: -5.7
	4-NP: -29.3	4-NP: -7.9
	Butyrate: -30.0	Butyrate: -30.0

RESUME en Français

Ce travail aborde la question de la standardisation, de la répétabilité et de l'optimisation de l'extraction des plantes médicinales dans l'eau. Trois plantes ont été sélectionnées, pour lesquelles les activités pharmacologiques complémentaires reposent sur les différents flavonoïdes, dont deux sont bien documentées (sommités fleuries d'aubépine et feuilles de cassis) avec des propriétés bien connues, et la troisième a été peu étudiée (*Chrysanthellum americanum*). Nous avons établi un protocole d'extraction général dans l'eau basé sur l'infusion pour ces trois plantes, utilisable par chacun de nous, qui peut permettre une absorption quotidienne standardisée et reproductible de composants bioactifs (phénols, flavonoïdes, oligomères proanthocyanidines) à une température buvable. La granulométrie est le facteur le plus important pour obtenir les meilleurs rendements d'extraction (environ 22% pour l'aubépine, 26% pour le *Chrysanthellum americanum* et 28,5% pour le cassis). La composition chimique de ces plantes a été étudiée par des méthodes colorimétriques, ainsi que par des instruments analytiques performants et complémentaires (UHPLC-ESI-MS et FT-ICR MS). Les extraits de cassis contiennent beaucoup plus de composés phénoliques (les principaux composants détectés en UV étant les flavonols) que les deux autres plantes. Les extraits d'aubépine contiennent beaucoup plus d'oligomères de proanthocyanidines (les principaux composants détectés en UV étant les flavanols, les flavonols et les flavones) que les deux autres plantes. Les extraits de *Chrysanthellum americanum* et de cassis contiennent des quantités similaires de flavonoïdes, le premier contenant essentiellement des dérivés d'acide hydrocinnamique, des flavones, des flavanones et des aurones comme composants détectés en UV. Environ 2500 molécules ont été détectées pour chaque plante, parmi lesquelles environ 25% sont communes aux 3 plantes et environ 15% sont spécifiques à chaque plante. Des dérivés de quercétine et de kaempférol ont été identifiés dans les extraits de feuilles de cassis, tandis que la vitexine-2-O-rhamnoside, l'hyperoside et l'isoquercétine ont été identifiés dans les extraits de sommités fleuries d'aubépine, et des dérivés de flavanomaréine et de martitimeine, ainsi que l'acide oléanolique ou ursolique ont été identifiés dans les extraits de *Chrysanthellum americanum*. Une inhibition intéressante de la hyaluronidase ($\geq 90\%$) a été rapportée pour les extraits d'aubépine, bien supérieure à celle des deux autres extraits de plante. Quant à l'activité anti-hypertensive, les extraits de *Chrysanthellum americanum* ont démontré une inhibition de l'ECA plus élevée que les deux autres extraits de plante. Concernant l'activité antioxydante, les extraits de feuilles de cassis ont montré la capacité antioxydante la plus élevée. Enfin, la formation de nanoparticules dans les infusions (appelées teacreaming) a été étudiée d'un point de vue cinétique et rayon hydrodynamique en fonction de la température.

Mots-clés: aubépine; cassis; *chrysanthellum americanum*; standardisation; mode d'extraction; infusion, extraction dans l'eau; proanthocyanidine; polyphénol; flavonoïde; granulométrie; activité enzymatique, tea creaming

TITRE en Anglais

Analysis and biological activity of water-based extracts of herbal tea plants (hawthorn, blackcurrant, *chrysanthellum americanum*)

RESUME en Anglais

This work deals with the question of standardization, repeatability and optimization of medicinal plant extraction in water. Three plants were selected, for which the complementary pharmacological activities are based on different flavonoids, two of which are well documented (hawthorn flowering tops and blackcurrant leaves) with well-known properties, and the third one has been little studied (*Chrysanthellum americanum*). We established a general extraction protocol in water for these three plants that can be used by each of us, based on infusion that can afford a reproducible daily uptake of bioactive components (phenols, flavonoids, proanthocyanidin oligomers) at drinkable temperature. Granulometry was the most important factor to get the best extraction yields (about 22% for hawthorn, 26% for *Chrysanthellum americanum* and 28.5% for blackcurrant). Chemical composition of these plants was investigated by colorimetric methods, and also using performant and complementary analytical instrumentations (UHPLC-ESI-MS and FT-ICR MS). Blackcurrant extracts contained much more phenolic compounds (the main UV-detected components detected in UHPLC being flavonols) than the two other plants. Hawthorn extracts contained much more proanthocyanidin oligomers (the main UV-detected components in UHPLC being flavanols, flavonols and flavones) than the two other plants. *Chrysanthellum americanum* and blackcurrant extracts contained similar amounts of flavonoids, the former one containing essentially hydrocinnamic acid derivatives, flavones, flavanones and aurones as UV-detected components. About 2500 hints were obtained for each plant, among which about 1100 are common to all 3 plants and about 700 are specific to each plant. Quercetin and kaempferol derivatives were identified in blackcurrant leaves extracts, while vitexin-2-O-rhamnoside, hyperoside and isoquercetin were identified in hawthorn flowering tops extracts and flavanomorein and martitimein derivatives, and Oleanolic or Ursolic acid were identified in *Chrysanthellum americanum* extracts. A significant inhibition of hyaluronidase ($\geq 90\%$) was reported for hawthorn extracts, much higher than that of the other two plant extracts. As for the anti-hypertensive activity, the *Chrysanthellum americanum* extracts demonstrated higher ACE inhibition than the other two plant extracts. Regarding antioxidant activity, blackcurrant leaf extracts showed the highest antioxidant capacity. Finally, the formation of nanoparticles in the herbal tea infusions (also known as tea creaming), was studied from a kinetic and size-distribution point of view as a function of temperature.

Keywords: hawthorn; blackcurrant; *chrysanthellum americanum*; standardization; extraction mode; infusion, water-based extraction; proanthocyanidin; polyphenol; flavonoid; granulometry; enzymatic activity, tea creaming

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