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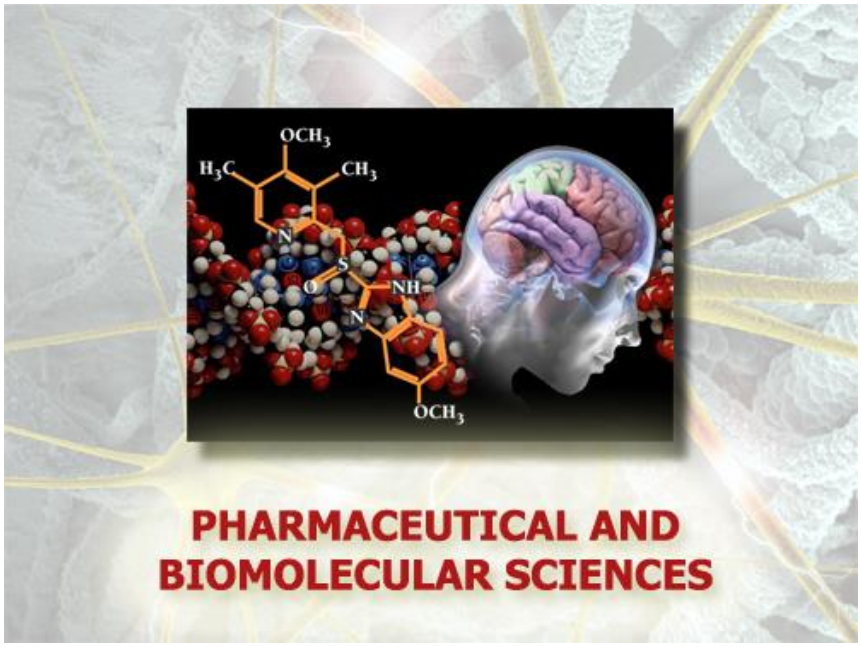

Enabling technologies in food processing and plant extraction

Candidata: Veronika Gunjević **Tutor: Prof. Giancarlo Cravotto**

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TESI PRESENTATA DA: Veronika Gunjević

TUTOR: Prof. Giancarlo Cravotto

COORDINATORE DEL DOTTORATO: Prof.ssa Roberta Cavalli

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Table of content:

Acronyms and abbreviations

7-AAD – 7-aminoactinomycin D

ANOVA – analysis of variance

ATEX – explosive atmospheres

B_0 – extraction rate at t = t_0

BBCH – Biologische Bundesanstalt, Bundessortenamt and CHemical industry

BP – blueberry peels

BV – bead volume

CAGR – compound annual growth rate

CAR – cannabimimetic activity receptor

CAT – Alcaloides Totales de Corteza

CB – cotton bag

CBD – cannabidiol

CBDA – cannabidiolic acid

CBS – cocoa bean shell

CE – circular economy

ChCl:CA – choline chloride: citric acid

ChCl:Glc – choline chloride: glucose

ChCl:Gly – choline chloride: glycerol

ChCl:LA – choline chloride: lactic acid

ChCl:MA – choline chloride: malic acid

ChCl:OA – choline chloride: oxalic acid

ChCl:U – choline chloride: urea

CHD – conventional hydrodistillation

$c_{t\to\infty}$ – maximum yield at $t_{\to\infty}$

df – degrees of freedom

DiOP – dichloromethane phase in liquid-liquid extraction

EAOP – ethyl acetate phase in liquid-liquid extraction

EC – energy consumption

EtOP – ether phase in liquid-liquid extraction

Acronyms and abbreviations

GAE – gallic acid equivalents

GLC – glucose

GRAS – generally recognised as safe

GS – grape stalks

HaCaT – human keratinocyte cells

HBD – hydrogen bond donor

IC_{50} - half maximal inhibitory concentration

K_1 – Peleg's rate constant

K_2 – Peleg's capacity constant

LD_{50} – half maximal lethal dose

LOD – limit of detection

LOQ – limit of quantification

MAE – microwave assisted extraction

MAHD – microwave assisted hydrodistillation

MAHG – microwave assisted hydrodiffusion and gravity

MASWE – microwave assisted subcritical water extraction

MS – mean square

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MW – microwave

NADES – natural deep eutectic solvent

OP – organic phase

PEEK – Polyether ether ketone

PPC – percent polymeric colour

RAW – murine macrophage cells

RSM – response surface methodology

RT – room temperature

S/L – solid/liquid ratio

SCO_2E – supercritical CO_2 extraction

SEC – size exclusion chromatography

SGC – silica gel column chromatography

SI – selective index

SPE – solid phase extraction

SS – sum of squares

TAC – total anthocyanins content

TCC – total carbohydrate content

THC – Δ^9-tetrahydrocannabinol

THCA – Δ^9-tetrahydrocannabinolic acid

TLC – thin layer chromatography

TPC – total polyphenolic content

UAE – ultrasound assisted extraction

UMAE – combined ultrasound and microwave assisted extraction

US – ultrasound

VOC – volatile organic compound

Aim and preface

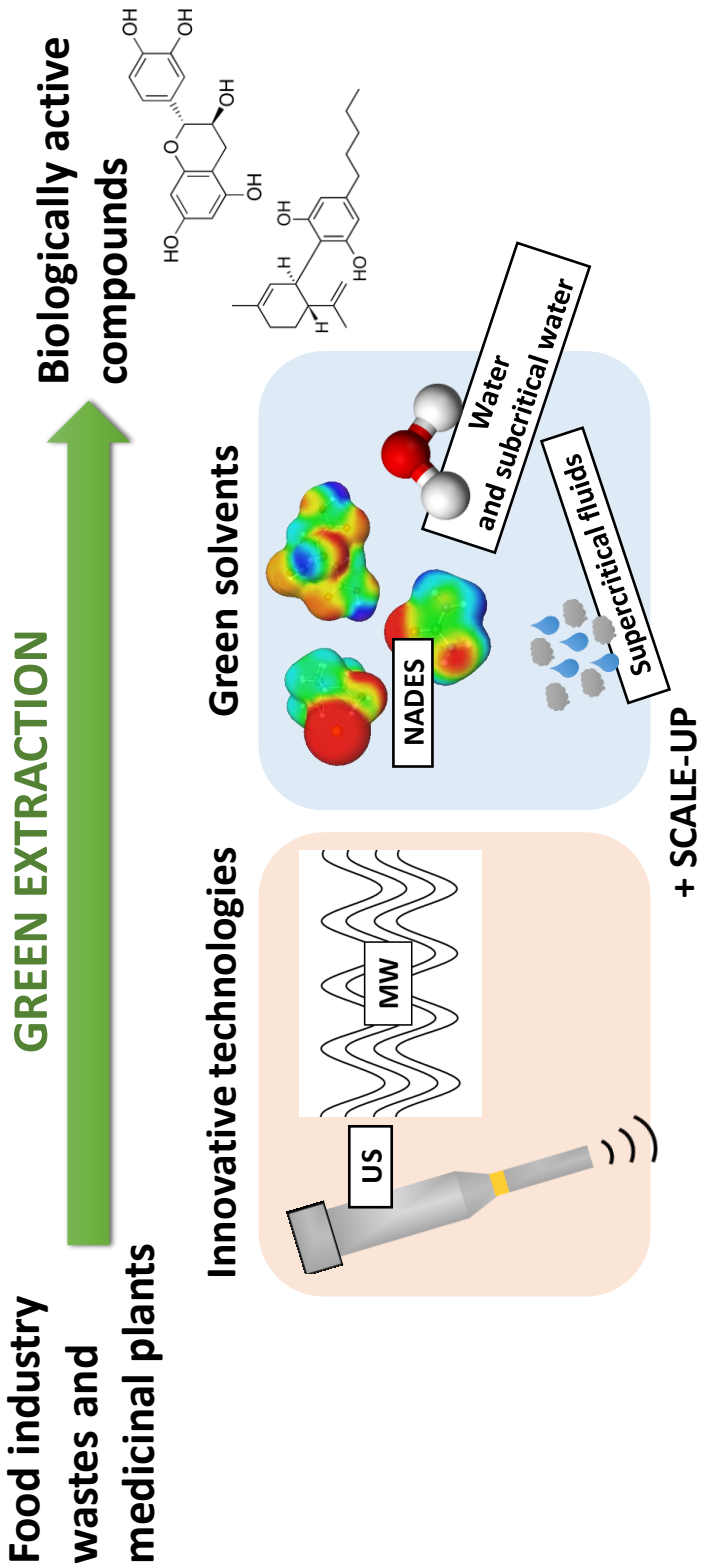
With the current advances in medicine, nutrition and chemistry, synthetic artificial pharmaceutical and cosmetic product are gaining a negative connotation by the general public. Consequently, there is a growing demand for functional foods and products of natural origin that contain phytochemicals. Phytochemicals exert numerous biological activities beneficial for human health such as anti-tumour, anti-inflammatory, anti-viral, anti-fungal, anti-bacterial, anti-parasitic and cardio-protective effects.

Traditionally, phytochemicals are recovered in long-lasting processes with conventional heating sources, where volatile organic compounds are used as solvents and often the extraction yields and selectivity are unsatisfying. Therefore, due to the need for process intensification, increasing energy prices and obligation to reduce CO_2 emission, it is necessary to find new solutions for the plant bioactives recovery. Adoption of new technologies such as ultrasound (US), microwaves (MW), hydrodynamic cavitation, pulsed electric field, etc., that are recently introduced in the field of plant extraction, could be the key innovation. Moreover, the implementation of new environmental friendly solvents that could meet both technological and economical demand is crucial factor for achieving a green and efficient process. Food industry generates a large amount of organic wastes that are generally disposed unexploited. Up to date, such wastes are only partially valorised, manufacturing only low added value products (fertilizers, animal feed, composting). Nevertheless, these matrixes still show a high content of proteins, sugars, fibres, lipids and bioactive compounds. Hence in the frame of circular economy, these by-products can be considered valuable sources for functional food, cosmetics and pharmaceuticals preparation. When this approach is adopted not only environmental impact reduction is achieved; it leads to numerous economic benefits owning to the added value maximizing.

In the light of the aforesaid, present thesis is focused on food industry by-products and medicinal plants biologically active compounds recovery by means of green extraction. US and MW techniques in different set ups have been exploited. Green solvents such as water, water in subcritical state, supercritical CO_2 and natural deep eutectic solvents (NADES) have been incorporated in extraction processes. In addition, solvent-free approach in a pilot scale has been evaluated. The biological activities of the obtained phytochemicals were evaluated in *in vitro* tests. This thesis is divided in 3 main chapters; first chapter is an introduction reporting green extraction processes state of the art. In the second chapter, the main focus will be placed on testing innovative technologies for phytochemicals extraction from various plant sources. The third chapter will be dedicated to anthocyanins extraction using natural deep eutectic solvents. As in chapter 3, process intensification will be experimented with different enabling technologies.

Aiming to arrange this thesis in the simplest way, Figures, Tables and references numbering is done individually for each chapter.

Thesis graphical abstract:



Chapter 1: General introduction

1.1. Circular economy

In past decades, due to the ever-growing population, lifestyle improvements and GDP increment, natural resources overexploitation has taken an increasing momentum. Hence, the traditional "take-make-consume-dispose" linear model is collapsing and should be replaced with a sustainable alternative.[1,2] Circular economy (CE) is a new paradigm that offers rather different material flow in economic system.[3,4] It aims to diminish dependence on fresh natural resources exploitation.[5] Therefore, materials can circulate in a closed loop, where the resources are kept and used for as long and as efficiently as possible. Finally, after product is used, it is regenerated and recovered (Figure 1).[6]

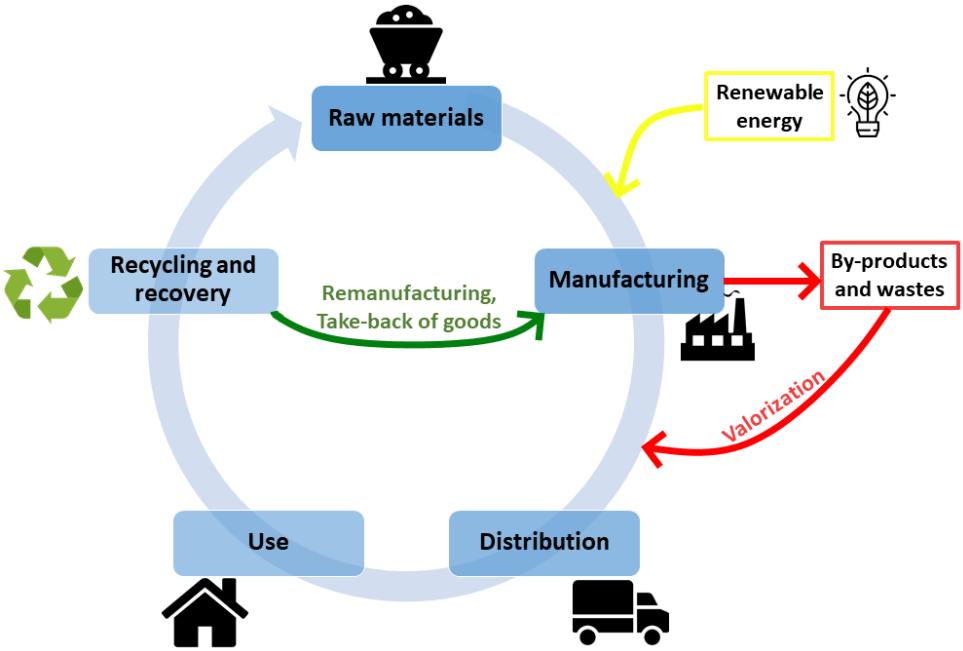


Figure 1. Schematic representation of CE concept.[3,4]

This approach implementation does not only lead to the environmental impact reduction, but it also creates additional economic benefits. Moreover, new business opportunities can be introduced by following innovative ways of creating value.[3,7] CE is currently promoted by the EU, by several national governments including China, Japan, UK, France, Canada, Netherlands, Sweden and Finland, and by various enterprises around the world.[3] These principles implementation could reduce resources needs up to 17-24% by 2030, resulting in

[1] E. Blunck, H. Werthmann, *DIEM: Dubrovnik International Economic Meeting*, **2017**, *3(1)*, 644–666.
[2] S. Yang, A. Raghavendra, J. Kaminski, H. Pepin, *Appl. Sci.*, **2018**, *8*, 1177.
[3] J. Korhonen, A. Honkasalo, J. Seppälä, *Ecol. Econ.*, **2018**, *143*, 37–46.
[4] W. R. Stahel, *Nature*, **2016**, *531*, 435–437.
[5] S. Sauvé, S. Bernard, P. Sloan, *Environ. Dev.*, **2016**, *17*, 48–56.
[6] Y. Kalmykova, M. Sadagopan, L. Rosado, *Resour. Conserv. Recycl.*, **2018**, *135*, 190–201.
[7] P. Rosa, C. Sassanelli, A. Urbinati, D. Chiaroni, S. Terzi, *Int. J. Prod. Res.*, **2020**, *58(6)*, 1662–1687.

overall savings potential of €630 billion per year for European industries.[8] In addition, the greenhouse-gas emissions could be reduced up to 70%, and 4% employment increase could be induced.[4]

As already mentioned, CE approach favours maximum added value creation for every industrial process and therefore manufacturing by-products and wastes utilization is promoted. Hence, the waste of one company can be a raw material for another.[9] Moreover, EU Waste Framework Directive[10] has placed the waste generation reduction as priority. The waste generation should be minimized, the generated waste should be recycled and recovered, while the disposal is the least desirable option.

Therefore, the presented doctoral thesis focuses on agri- and food-industry by-products valorisation to obtain valuable biologically active compounds. To enable the valorisation, emerging green extraction technologies could be key innovation. Therefore, in following sections will describe the principles of green extraction and technologies used for the aforesaid purposes.

1.2. Biologically active compounds

With the current advances in medicine, nutrition and chemistry, general public is aware of potential harmful effects of synthetic artificial products. Hence, there is a constantly growing demand for functional foods, cosmetic and pharmaceutical products of natural origin that contain phytochemicals.[11] Phytochemicals are plant secondary metabolites, organic compounds that are usually synthetized in a phase subsequent to exponential growth phase. Their presence does not affect plants growth, development or reproduction. However, these particular metabolites protect plant form the predators (bacteria, fungi, viruses, insects, large animals…), they act as symbiosis agents, reproduction hormones, differentiation factors and can be metal transporting agents.[12]

Phytochemicals possess numerous biological activities beneficial for human health, extensively reviewed in literature. These health promoting effects are anti-tumour, anti-inflammatory, anti-viral, anti-fungal, anti-bacterial, anti-parasitic and cardio-protective activities. They also act as muscle relaxants, analgesics, diuretics or anti-depressants.[13] There are different mechanisms of action by which these metabolites exhibit their effect: they can directly affect the gene expressions or may alter the essential metabolic and signal transduction pathways.[14] However, specific biological activities are ascribed to the certain group of phytochemicals. Based on their biosynthetic pathways, plant secondary metabolites

[8] European Commission, COM (2014), Brussels, **2014**.
[9] F. E. Garcia-Muiña, R. González-Sánchez, A. M. Ferrari, D. Settembre-Blundo, **2018**, *Soc. Sci.*, *7(12)*, 255.
[10] European Parliament and Council, Directive 2008/98/EC, **2008**.
[11] J. Giacometti, D. Bursać Kovačević, P. Putnik, D. Gabrić, T. Bilušić, G. Krešić, V. Stulić, F. J. Barba, F. Chemat, G. Barbosa-Cánovas, A. Režek Jambrak, *Food Res. Int.*, **2018**, *113*, 245–262.
[12] R. Tiwari, C. S. Rana, *Int. J. Eng. Res. Gen. Sci.*, **2015**, *3(5)*, 661–670.
[13] M. Wink, *Annual Plant Reviews, Vol. 39, Functions and Biotechnology of Plant Secondary Metabolites*, John Wiley & Sons, Ltd., Ames, Iowa, USA, **2010**.
[14] G. Velu, V. Palanichamy, A. P. Rajan, In: *Bioorganic Phase in Natural Food: An Overview* (ed. S. Mohana Roopan, G. Madhumitha), Springer International Publishing AG, Cham, Switzerland, **2018**, pp. 135–156.

are classified into 4 main groups: 1) polyphenolic and phenolic compounds, 2) terpenoids, 3) nitrogen-containing alkaloids and 4) sulphur-containing compounds.[15]

Natural extracts are excellent sources of aforesaid biologically active compounds and can be used as additives in food, cosmetic, perfume and pharmaceutical industry.[16] Conventional extraction methodologies can have a negative effect on extraction yield and extract's quality, whereas mainly high temperatures are applied over a long period of time.[17] Hence, the need for innovative extraction methodologies is imposed. In next chapter green extraction concept, that can ensure processes intensification and provide high-quality extracts, will be discussed.

1.3. Green extraction

Food and natural products solid-liquid extraction processes are nowadays performed in every perfume, cosmetic, pharmaceutical, food, biofuel, materials and fine chemical industry.[18] In general extraction processes consist in 3 unit operations, in particular 1) material pre-treatment such as drying and milling, 2) solid-liquid extraction and 3) downstream processing where filtration, evaporation, purification or concentration take place. If the extraction process is not optimized, it can lead to high energy consumption, prolonged extraction times, inefficiency of desired compounds recovery, extracts impurity, and even to environmental pollution if toxic and volatile organic compounds are used as solvents.[19]

On a molecular level, solid-liquid extraction is controlled by diffusion and osmosis. Several mass transfer phenomena occur when extraction is performed (Figure 2): 1) solvent diffusion form the bulk solution through the relatively stagnant boundary layer, 2) solvent penetration into the micro-/macro-porosity of the solid, 3) solute dissolution in solvent, 4) solvent and solute diffusion to the surface of solid and 5) solute and solvent diffusion to the bulk solution through the boundary layer. The solute diffuses from the higher concentration locations to the lower concentration zones and it stops when equilibrium between the solid and bulk solution is reached. The diffusion rate is governed by Fick's law. Traditional extraction processes show several limitations such as long extraction time due to the low mass transfer ascribed to the low diffusion rate. Nevertheless, by specific technologies and adequate solvent application the mass transfer can be enhanced leading to faster extraction.[20,21]

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[15] A. Crozier, M. N. Clifford, H. Ashihara, *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*, Blackwell Publishing Ltd., Oxford, UK, **2006**.

[16] R. N. Cavalcanti, T. Forster-Carneiro, M. T. M. S. Gomes, M. A. Rostagno, J. M. Prado, M. A. A. Meireles, In: *Natural products extraction: principles and application* (ed. M. A. Rostagno, J. M. Prado), Royal Society of Chemistry, Cambridge, UK, **2013**, pp. 3–46.

[17] P. Putnik, J. M. Lorenzo, F. J. Barba, S. Roohinejad, A. R. Jambrak, D. Granato, D. Montesano, D. Bursać Kovačević, *Foods*, **2018**, *7*, 106.

[18] F. Chemat, M. Abert Vian, A. S. Fabiano-Tixier, M. Nutrizio, A. Režek Jambrak, P. E. S. Munekata, J. M. Lorenzo, F. J. Barba, A. Binello, G. Cravotto, *Green Chem.*, **2020**, *22*, 2325–2353.

[19] F. Chemat, M. Abert-Vian, A. S. Fabiano-Tixier, J. Strube, L. Uhlenbrock, V. Gunjevic, G. Cravotto, *Trends Anal. Chem.*, **2019**, *118*, 248–263.

[20] G. Cravotto, F. Mariatti, V. Gunjevic, M. Secondo, M. Villa, J. Parolin, G. Cavaglià, *Foods*, **2018**, *7*, 130.

[21] P. C. Veggi, J. Martinez, M. A. A. Meireles, In: *Microwave-assisted Extraction for Bioactive Compounds* (ed. F. Chemat, G. Cravotto), Royal Society of Chemistry, Cambridge, UK, **2013**, pp. 15–52.

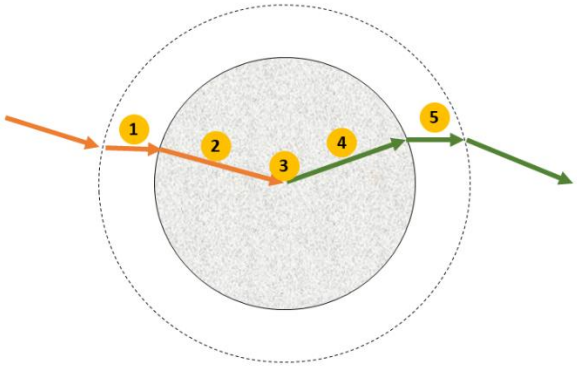

Figure 2. Solid-liquid extraction mechanism.

Therefore, driven by the need for process intensification, increasing energy prices and obligation to reduce CO_2 emission, scientific and academic community was challenged to find new solutions for the plant compounds recovery.[11] Green extraction concept was first introduced in 2012 by Chemat et al.[22] and was defined as *"a concept based on the discovery and design of extraction processes which will reduce energy consumption, allow use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product"*. It is based on 6 principles shown in Figure 3.

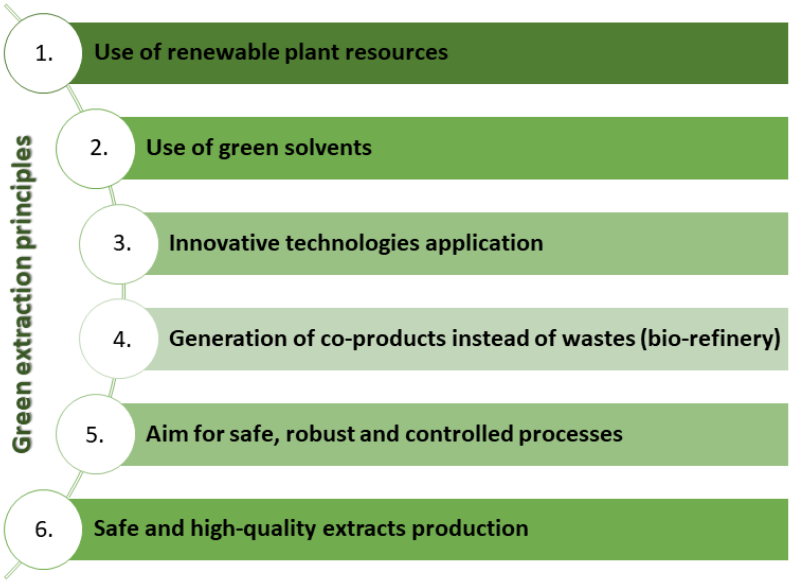

Figure 3. Six green extraction principles.[19]

Firstly, renewable plant matrices are preferable due to the natural plant resources uncontrolled harvesting and overexploitation in last decades. Alternative sources for certain bioactive compounds could be introduced. Moreover, plants could be designed to synthetize larger amounts of desired compounds by natural selection. Secondly, solvents that are toxic and generally have a negative environmental impact should be replaced with greener alternatives. Principally water and agro-solvents should be used. Nevertheless, solvent-free processes can be considered. Thirdly, conventional extraction technologies are to be replaced with the emerging enabling technologies to intensify the extraction process. By these technologies application overall energy consumption would be significantly reduced,

[22] F. Chemat, M. Abert Vian, G. Cravotto, *Int. J. Mol. Sci.*, **2012**, *13*, 8615–8627.

rendering extraction processes profitable and environmental-friendly. In addition, process optimization and continuous systems could bring further benefits. Forth principle is focused on bio-refinery approach, in particular development of valorisation pathways for complete by-products exploitation. In addition, according to the fifth principle, the industries should aim for safe and robust processes that have a low number of unit operations. The final products should have a low environmental footprint, while their quality, functionality and safety should be highly pronounced (principle 6).[19]

Extract obtained by following these principles would have the lowest environmental impact, since low energy consumption, green solvents and avoidance of toxic compounds are ensured. Green extraction of natural products is a paradigm that could offer solutions for today's challenges that involve both environmental and consumers' safety, and simultaneously enable the economic profit and competition between the industries. Moreover, green extraction is devoted to process intensification, since it favours low extraction times, solvent and energy consumption reduction, innovative technologies application and unit operations reduction.[23]

Following subchapters will thoroughly describe main green extraction principles applied in preparation of this thesis.

1.3.1. Enabling technologies for extraction process intensification

Traditional techniques that are used for bioactive compounds extraction are maceration, Soxhlet extraction and hydrodistillation. Major drawbacks for the application of these technologies are long extraction time, thermo-labile compounds degradation, requirement for high purity solvents application, low extraction selectivity and huge solvent volume evaporation in downstream processing.[24] Therefore, to overcome these drawbacks, novel extraction technologies have been introduced in recent years. Such technologies are ultrasound (US), hydrodynamic cavitation, microwaves (MW), supercritical CO_2 extraction (SCO_2E), pulsed electric field and high voltage electric discharge.[19] In this thesis, mainly ultrasound (UAE), microwave assisted extractions (MAE) and SCO_2E were studied.

Ultrasound assisted extraction (UAE)

US are mechanical oscillating sound waves that have a frequency ranging from 20 kHz to 10 MHz. Based on the intensity and frequency, US can be categorized in 2 classes: 1) diagnostic US having low intensity (<1 W/cm^2) but high frequency (1 – 10 MHz) and 2) power US that has high intensity (10 – 1000 W/cm^2) and low frequency (20 kHz – 1 MHz). Latter is used in extraction since it promotes cavitation.[25,26] The sonication effect on extraction processes mainly derives form aforesaid cavitation phenomenon (Figure 4). The bubbles are formed by US propagation in the elastic medium when the US power reaches a certain threshold and are

[23] F. Chemat, N. Rombaut, A. S. Fabiano-Tixier, J. T. Pierson, A. Bily, In: *Green Extraction of Natural Products: Theory and Practice* (ed. F. Chemat, J. Strube), Wile - VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, **2015**, pp. 1–30.
[24] J. Azmir, I.S.M. Zaidul, M.M. Rahman, K.M. Sharif, A. Mohamed, F. Sahena, M.H.A. Jahurul, K. Ghafoor, N.A.N. Norulaini, A.K.M. Omar, *J. Food Eng.*, **2013**, *117*, 426–436.
[25] B. K. Tiwari, *Trends Anal. Chem.*, **2015**, *71*, 100–109.
[26] L. Vernes, M. Vian, F. Chemat, In: *Liquid-Phase Extraction*, (ed. C. F. Pool), Elsevier Inc., Amsterdam, Netherlands, **2020** pp. 355–374.

constantly compressed and expanded. The compression and expansion oscillation depends on the frequency. The bubbles grow in size and finally, they reach their critical diameter and implode. Violent bubbles collapse generates temperatures of about 5000 K and pressures of the order of 50 MPa.[26]

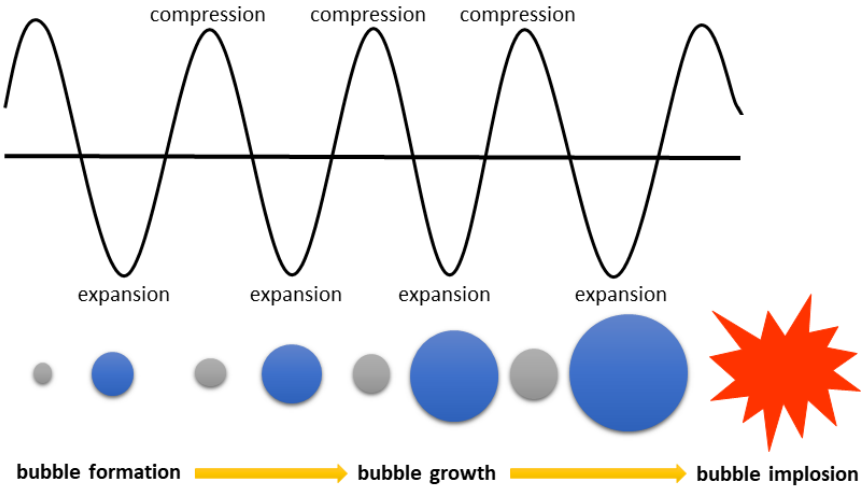

Figure 4. Acoustic cavitation phenomenon depicted.

The cavitation phenomenon drastically improves the mass transfer. However, this is not only benefit that US offers when applied in vegetal matrixes extraction. Sonication can lead to several other effects that could occur simultaneously or individually: matrix fragmentation, erosion, detexturation, sonocapillary effect and sonoporation.[27] Nevertheless, high speed jets of solvent targeting the vegetal material created by cavitation play the crucial role in plant extraction.[28] Due to all the aforementioned effects, US provides shorter processing time, energy and solvent saving, low wastes and by-products generation and improved extracts quality.[29]

Microwave assisted extraction

Like US, MW provides significant benefits to the extraction processes, however with a different mechanism of action. MW is a nonionizing electromagnetic irradiation whit a frequency from 300 MHz to 300 GHz. It heats the matter and solvent in a selective manner. In particular, there are two mechanisms by which MW causes heating: ionic conduction and dipole rotation. Ionic conduction is electrophoretic ions and electrons migration due to presence of electric field. These migrations create frictions between the moving compounds and the medium, leading finally to temperature increase. Dipole rotation likewise occurs due to the MW's electric filed. MW irradiation is characterised by a constantly changing electric field. Therefore, dipoles aim to align to the electric filed and finally oscillate creating a friction between them and the surrounding molecules which eventually creates heat (Figure 5).[26,30] Consequently, the heating provided by MW is selective and volumetric, dispersed uniformly trough the medium. Unlike the conduction and convection heating, where the heat transfer

[27] F. Chemat, N. Rombaut, A. G. Sicaire, A. Meullemiestre, A. S. Fabiano-Tixier, M. Abert-Vian, *Ultrason. Sonochem.*, **2017**, *34*, 540–560.
[28] M. Vinatoru, T.J. Mason, I. Calinescu, *Trends Anal. Chem.*, **2017**, *97*, 159–178.
[29] X. Fu, T. Belwal, G. Cravotto, Z. Luo, *Ultrason. Sonochem.*, **2020**, *60*, 104726.
[30] S. B. Bagade, M. Patil, *Crit. Rev. Anal. Chem.* [online], **2019**, https://doi.org/10.1080/10408347.2019.1686966.

occurs form the outer surface into the bulk fluid, in the case of MW heating no heat loss into the environment occurs. The mass and heat transfer, working in synergy, significantly enable the extraction process.[31]

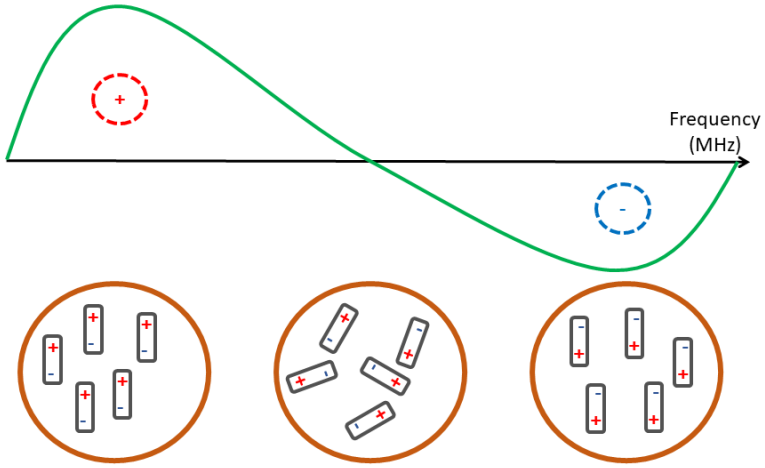

Figure 5. MW effect on dipoles in vegetal cells.

Based on the interaction with MW, there are 3 types of materials: transparent, absorbing and opaque materials. Transparent materials are those who absorb the electromagnetic waves to a negligible extent and allow the MW to easily pass through with no attenuation. On the other hand, absorbing materials are those that absorb the electromagnetic waves and convert it into heat, and are generally the subjects of MAE. Only this class of materials can be heated by MW. Opaque materials reflect the electromagnetic waves and do not allow them to pass through. They are therefore used in MW reactors fabrication.[28] Hence, when MAE is performed, particular attention should be paid on solvent selection depending on the compound of interest.

However, MAE could be performed also without the addition of any solvent. Such technologies are microwave assisted hydrodistillation (MAHD) and microwave assisted hydrodiffusion and gravity (MAHG). MAHD was developed by Stashenko et al. in 2004 and is used for volatile compounds recovery.[32] It is based on the classic hydrodistillation process, however the vegetal matrix is placed into the cavity of MW reactor.[33] Nevertheless, MAHD is much more efficient, as the irradiation heats the plant material evenly. Essential oils obtained *via* MAHD are of higher quality since the extraction time is significantly reduced.[34] MAHG was patented in 2008 by Chemat et al.[35] It is first designed for essential oils extraction, but can also be used for other phytochemicals extraction. Matrix is placed in the MW reactor and the extract is recovered in a flask under the reactor. MW heats the intracellular water causing cell walls rapture. Consequently, the compounds and internal water are released, which is called hydrodiffusion effect. Driven by gravity, the released compounds fall into the receiving flask.[36]

[31] F. G. Chizoba Ekezie, D. W. Sun, J. H. Cheng, *Trends Food Sci. Tech.*, **2017**, *67*, 160–172.
[32] E. E. Stashenko, B. E. Jaramillo, J. R. Martínez, *J. Chromatogr. A*, **2004**, *1025*, 93–103.
[33] F. Chemat, M. Abert-Vian, X. Fernandez, In: *Microwave-assisted Extraction for Bioactive Compounds: Theory and Practice* (ed. F. Chemat, G. Cravotto), Springer Nature, Cham, Switzerland, **2013**.
[34] S. Markle, *Cannabis Sci. Technol.*, **2019**, *2*, 50–76.
[35] F. Chemat, M. Vian, F. Visioni, European Patent EP 1,955,749 A1, **2008**.
[36] M. A. Vian, X. Fernandez, F. Visinoni, F. Chemat, *J. Chromatogr. A*, **2008**, *1190*, 14–17.

When compared to the conventional extraction methodologies, MAE in any set-up, offers numerous advantages such as rapid heating, shorter process time, reduction in solvent usage, higher reproducibility, higher extraction rates and yield increment.[31]

Supercritical CO_2 extraction (SCO_2E)

Carbon dioxide (CO_2) is the most commonly used solvent in supercritical fluid extraction. This is due to its remarkable properties such as reusability, low critical temperature (31 °C), relatively low critical pressure (72.8 bar) and great natural abundance and therefore low cost. Low critical temperature ensures the thermo-labile compounds stability. In addition, it has a relatively high density (467.6 kg/m^3) indicating its high solvatation power, when compared to other supercritical fluids. However, its density could be tuned by temperature and pressure modifications to enable the desired compounds recovery. Moreover, SCO_2E can be controlled by co-solvents addition, ensuring possibility of polar compounds extraction. Typical SCO_2E system consists in CO_2 pump or compressor, CO_2 working tank, a modifier pump where a co-solvent is required, extraction reactor, and fractionation/collection vessel. A separator could be added to the equipment, enabling CO_2 recycling. SCO_2E ensures selective and fast extraction, safe extract with no residual solvent and it is a quite inexpensive scalable extraction method.[37,38,39]

1.3.2. Green solvents in plant extraction

Organic solvents, mostly belonging to the group of volatile organic compounds (VOCs), have a serious of well-known advantages for phytochemicals extraction. However, due to their toxicity for human and environment and their flammability, greener alternatives should be considered.[19] The hazardous solvents use reduction is also one of the priorities of EU environmental policy and legislation for 2010-2050 period.[40] Therefore, the implementation of new environmental friendly solvents that could meet both technological and economical demand is considered; moreover, solvent-free processes are emerging (Figure 6).[41]

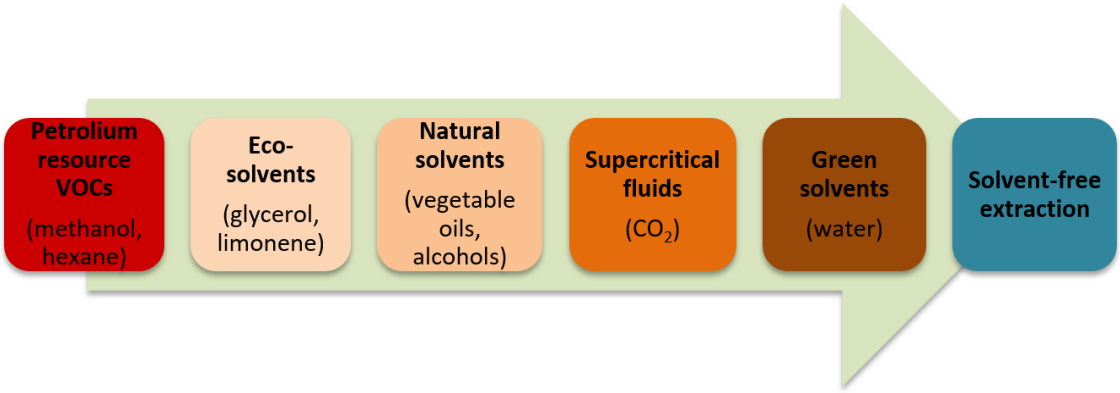

Figure 6. Towards green solvents.

[37] K. Y. Khaw, M. O. Parat, P. N. Shaw, J. R. Falconer, *Molecules*, **2017**, *22*, 1186.
[38] N. Polikhronidi, R. Batyrova, A. Aliev, I. Abdulagatov, *J. Therm. Sci.*, **2019**, *28*, 394–430.
[39] E. Ramsey, Q. Sun, Z. Zhang, C. Zhang, W. Gou, *J. Environ. Sci.*, **2009**, *21*, 720–726.
[40] EEA, EU environmental policy targets and objectives 2010–2050, EEA Report No 8/2013, **2013**.
[41] M. Cvjetko Bubalo, S. Vidović, I. Radojčić Redovniković, S. Jokić, *Food Bioprod. Process.*, **2018**, *109*, 52–73.

Herein will be in detail described the benefits of green solvents used in preparation of this thesis, in particular, water, subcritical water, natural deep eutectic solvents and moreover, the advantages of solvent-free techniques. Supercritical CO_2 was described in previous subchapter.

Solvent-free methodologies

Solventless extraction displays numerous benefits and these are: large volumes of solvent that contribute to economic and environmental issues are avoided, scale-up is facilitated, process safety is enhanced since there is no risk of explosion or fire, and extracts safety and purity are improved. Moreover, extraction processes are accelerated whereas there is no need for solvent evaporation which is usually the bottleneck in the work-up process. Several setups have been developed for solvent-free extraction, i.e. solvent-free MW extraction, MAHG, pulsed electric field and instantaneous controlled pressure drop.[42,43]

Water and subcritical water

If the solvent use cannot be avoided, water could be used as the greenest option, since it is non-toxic, noncorrosive, non-flammable, environmentally benign, naturally abundant, and available at low cost. Still, water has some drawbacks which limit its use as universal sustainable solvent for extraction processes, such as low apolar compounds solubility and high energy consumption required to concentrate the product.[44] However, water in subcritical state, or so called pressurized hot water, could improve the moderately apolar compounds extraction. Subcritical water state occurs between 100 and 374 °C, while required quantity of pressure is applied. It shows many advantages with regard to extraction efficiency and selectivity. Increased temperature leads to permittivity and viscosity decrease and an increase in the diffusion rate. In spite of the improvements in all these properties, the most important effect of the water temperature increase is undoubtedly the weakening of hydrogen bonds, resulting in a lower dielectric constant. Moreover, by temperature modulation that can favour specific compound extraction, increased selectivity could be achieved. Hence, moderately polar and non-polar targets could be recovered by subcritical water extraction, whereas more polar target compounds with high solubility in water at ambient conditions are extracted most efficiently at lower temperatures.[45,46,47]

Natural deep eutectic solvents (NADES)

NADES emerged as promising extraction media for being environmentally benign (non-flammable, non-volatile, stable, non-toxic), and composed of low-cost compounds. These solvents are a new generation of ionic liquids, formed by complexation between two or more naturally occurring compounds; in particular, between hydrogen bound acceptor, most commonly cholinum chloride, and hydrogen bound donors (sugars, carboxylic acids, amines, alcohols, etc.). This wide range of compounds that can be used in NADES synthesis suggests that solvents with particular physicochemical properties could be obtained and thus, facilitate

[42] F. Chemat, A. S. Fabiano-Tixier, M. Abert Vian, T. Allaf, E. Vorobiev, *Trends Anal. Chem.*, **2015**, *71*, 157–168.
[43] M. Abert Vian, T. Allaf, E. Vorobiev, F. Chemat, In: *Alternative Solvents for Natural Products Extraction* (ed. F. Chemat, M. Abert Vian), Springer, Heidelberg, Germany, **2014**, pp. 25–38.
[44] N. Florez, E. Conde, H. Domínguez, *J. Chem. Technol. Biotechnol.*, **2014**, *90*, 590–607.
[45] M. Plaza, C. Turner, *TrAC Trends Anal. Chem.*, **2015**, *71*, 39–54.
[46] M. Herrero, M. Castro-Puyana, J.A. Mendiola, E. Ibanez, *TrAC Trends Anal. Chem.*, **2013**, *43*, 67–83.
[47] M. Plaza, M. L. Marina, *TrAC Trends Anal. Chem.*, **2019**, *116*, 236–247.

the target-compound extraction and enhancing the selectivity. Moreover, NADES can be in most cases easily recycled. Nevertheless, their natural origin implies that the incorporation of NADES into food formulations without additional purification and separation steps is feasible.[48,49,50]

1.3.3. Agricultural and food industry by-products exploitation according to the bio-refinery approach

Biorefinery concept is defined by the International Energy Agency as "*the sustainable processing of biomass into a spectrum of marketable products*".[51] Biorefinery is basically an industrial plant or network of plants that take biomass wastes and by-products and using various technologies processes them to obtain bioenergy and a wide range of products such as chemicals, biofuels, food and feed ingredients, biomaterial and fibres.[52] By implementing this approach, the food processing industry can achieve zero-waste production. When this concept is taken into account not only environmental impact reduction is achieved; it leads to numerous economic benefits owning to the added value maximizing.[53]

Numerous food companies are paying for the generated wastes disposal. Annually, European food sector generates 250 Mt of solid by-products and wastes. Up to date, such wastes are only partially valorised, manufacturing only low added value products (fertilizers, animal feed, composting), while the largest fraction remains unexploited. Nevertheless, these matrixes still contain a considerable amount of primary and secondary metabolites. Hence, they are cheap source for functional food, cosmetic and pharmaceutical formulations.[52,54]

Aiming to harvest the maximal value, it is crucial to be aware of the matrix composition, thus knowing which products could be obtained. Moreover, the waste biomass should be processed in a cascade manner (Figure 7). Valuable extractants such as essential oils, polyphenols, alkaloids and other secondary metabolites, should be the first choice for valorisation, since such approach yields in the biggest economic benefits. Subsequently, primary metabolites such as carbohydrates, lipids, pectin, starch extraction is the next opportunity for added value creation, followed by platform chemicals conversions. Next options should be biomaterials and biofuels production. Whereas the added value brought by

[48] H. Vanda, R. Verpoorte, P. G. L. Klinkhamer, Y. H. Choi, In: *Deep Eutectic Solvents: Synthesis, Properties, and Applications* (ed. D. J. Ramon, G. Guillena), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, **2020**; pp. 61–82.
[49] M. Cvjetko Bubalo, S. Vidović, I. Radojčić Redovniković, S. Jokić, *J. Chem. Technol. Biotechnol.*, **2015**, *90*, 1631–1639.
[50] Y. Dai, J. van Spronsen, G. J. Witkamp, R. Verpoorte, Y. Hae Choi, *Anal. Chim. Acta*, **2013**, *766*, 61–68.
[51] N. Rombaut, A. S. Tixier, A. Bily, F. Chemat, Biofuels, *Bioprod. Bioref.*, **2014**, *8*, 530–544.
[52] F. Fava, G. Totaro, L. Diels, M. Reis, J. Duarte, O. Beserra Carioca, H. M. Poggi-Varaldo, B. Sommer Ferreira, *New Biotechnol.*, **2015**, *32*, 100–108.
[53] A. S. Matharu, E. M. de Melo, J. A. Houghton, *Bioresour. Technol.* **2016**, *215*, 123–130.
[54] L. A. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin, J. H. Clark, *Green Chem.*, **2013**, *15*, 307-314.

these valorisation techniques is quite low, energy production, biomass utilization as animal feed and fertilizers should be least favourable valorisation approaches.[55,56]

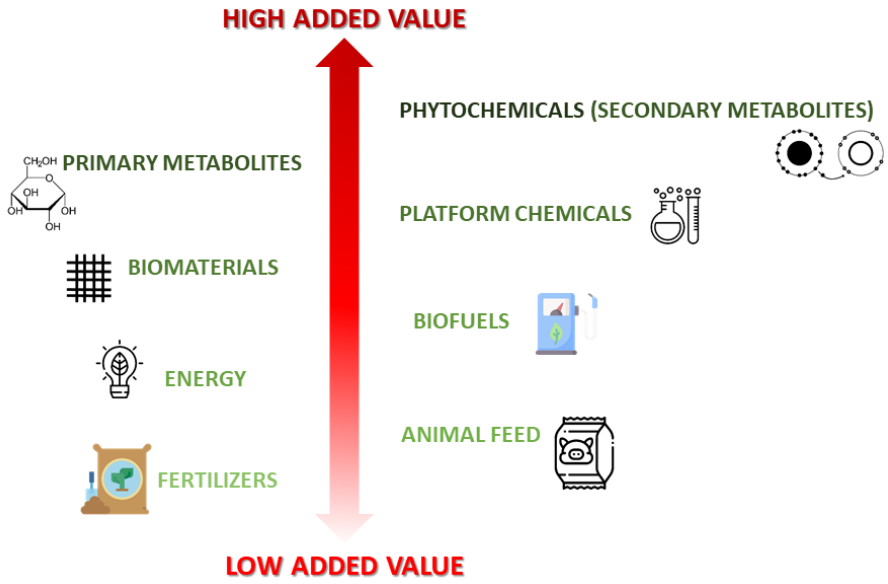

Figure 7. Food industry wastes valorisation strategies from low to high added value.

Therefore, this PhD project had an aim to valorise food industries' by-products and wastes according to the biorefinery concept. The major focus was paid on the recovery of the compounds that can give the highest added value.

[55] L. Garcia-Gonzalez, S. Bijttebier, S. Voorspoels, M. Uyttebroek, K. Elst, W. Dejonghe, Y. Satyawali, D. Pant, K. Vanbroekhoven, H. De Wever, In: *Advances in Food Biotechnology* (ed. R. Rai V.), John Wiley & Sons Ltd., Weinheim, Germany, **2016**, pp. 427–442.

[56] C. Fritsch, A. Staebler, A. Happel, M. A. Cubero Márquez, I. Aguiló-Aguayo, M. Abadias, M. Gallur, I. M. Cigognini, A. Montanari, M. J. López, F. Suárez-Estrella, N. Brunton, E. Luengo, L. Sisti, M. Ferri, G. Belotti, *Sustainability* **2017**, *9*, 1492.

Chapter 2: Enabling technologies for food industry by-products and medicinal plants bioactive compounds extraction

As already stated in *Chapter 1*, green extraction processes are pushed in the spotlight as an alternative for traditional time consuming and hazardous extraction methodologies. Crucial element of green extraction processes are innovative enabling technologies. These emerging extraction tools could offer a cut down in extraction time, extraction selectivity improvements, energy consumption reduction and overall process efficiency increment.[1,2] Therefore, enabling extraction methodologies were thoroughly studied. In particular, US, MW and SCO_2E were exploited.

In this Chapter, focus was pointed on utilization of the aforementioned technologies for biologically active compounds recovery form food industry wastes and medicinal plants. Phytochemicals closely studied were polyphenols, terpenoids and cannabinoids.

Polyphenols and terpenoids are prominent class of secondary metabolites widespread in plant kingdom, and in past decade have been thoroughly studied for their possible positive effects on human health.[3,4] Polyphenols are characterized by a wide range of complex structures and, up to date, several thousand plant polyphenols have been identified. The main structural unit in polyphenols is the phenolic ring. This phytochemicals class can be categorised into several groups according to the number of phenolic units and the structural moieties that bind them: a) phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), b) flavonoids (flavonols, flavones, flavanols, flavanones, isoflavones, proanthocyanidins), c) stilbenes and d) lignans.[5,6,7] The global polyphenols market is constantly growing. In 2018, it was valued at USD 1.28 billion and is expected to undergo an estimated compound annual growth rate (CAGR) of 7.2% from 2019 to 2025. Functional foods and beverages have emerged as the largest application sectors in the polyphenol market.[8] In addition, their applications in cosmetic and pharmaceutical formulations have increased in recent years. Nevertheless, these bioactive compounds are currently recognized as an essential part of the human diet by the scientific community and general public.[9,10]

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[1] F. Chemat, M. Abert Vian, A. S. Fabiano-Tixier, M. Nutrizio, A. Režek Jambrak, P. E. S. Munekata, J. M. Lorenzo, F. J. Barba, A. Binello, G. Cravotto, *Green Chem.*, **2020**, *22*, 2325–2353.
[2] T. Belwal, F. Chemat, P. R. Venskutonis, G. Cravotto, D. K. Jaiswal, I. D. Bhatt, H. P. Devkota, Z. Luo, *Trends Anal. Chem.*, **2020**, *127*, 1158952.
[3] C. G. Fraga, K. D. Croft, D. O. Kennedy, F. A. Tomás-Barberán, *Food Funct.*, **2019**, *10*, 514-528.
[4] C. Gupta, D. Prakash, *J. Complement Integr. Med.*, **2014**, *11(3)*, 151–169.
[5] M. Abbas, F. Saeed, F. M. Anjum, M. Afzaal, T. Tufail, M. S. Bashir, A. Ishtiaq, S. Hussain, H. A. R. Suleria, *Int. J. Food Prop.*, **2017**, *20(8)*, 1689–1699.
[6] H. El Gharras, *Int. J. Food Sci. Tech.*, **2009**, *44(12)*, 2512–2518.
[7] S. Petti, C. Scully, *J. Dent.*, **2009**, *37(6)*, 413–423.
[8] Grand View Research, **2019**, https://www.grandviewresearch.com/industry-analysis/cocoa-beans-market.
[9] G. F. Ferrazzano, I. Amato, A. Ingenito, A. Zarrelli, G. Pinto, G. Pollio, *Molecules*, **2011**, *16(2)*, 1486–1507.
[10] H. Rasouli, M. H. Farzaei, R. Khodarahmi, *Int. J. Food Prop.*, **2017**, *20(2)*, 1700–1741.

Numerous biological effects that polyphenols exert on human health have been up to date extensively reviewed,[6] and are reported in Figure 1.

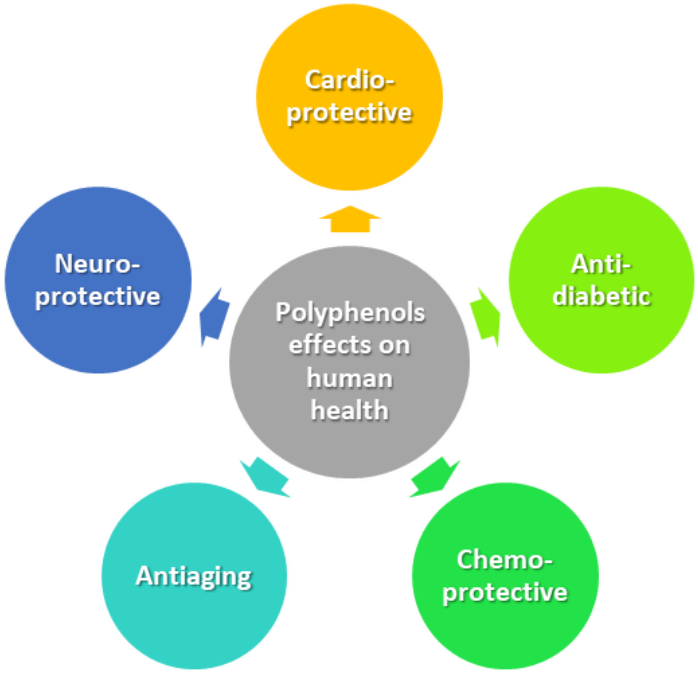


Figure 1. Main health beneficial effects attributed to polyphenols.[10,11]

Polyphenols possess strong antioxidant activity and are able to scavenge various types of oxygen, nitrogen, and chlorine species, thus lowering oxidative-stress damage.[7,10] Reactive oxygen species form during physiological processes such as respiration and metabolism. Their generation increases under situations of stress including exposure to environmental pollution and in aging. These free oxygen radicals are highly reactive oxidisers that can bind DNA, lipids and proteins, and hence, turn a physiological condition into a pathological state.[12] While substantial amounts of research have associated oxidative damage with the development of most age-related degenerative diseases, it is assumed that polyphenols can be used in the prevention of such conditions.[7]

Numerous epidemiological studies and human trials have suggested that a polyphenolic-rich diet reduces the risks of cardiovascular diseases.[5] Their cardio-protective effect derives from improvements in endothelial function, increase in high-density-lipoprotein amounts and their antioxidant, anti-platelet and anti-inflammatory properties. Moreover, the prevention of atherosclerosis has been related to the low-density-lipoprotein oxidation inhibition by polyphenols.[11] The polyphenols anti-diabetic action has also been studied. These phytochemicals can reduce and inhibit enzyme activity that is involved in the release of glucose into the gastrointestinal tract and can delay glucose transfer from the stomach to the small intestine.[5,11] Polyphenols are able to interact with neuronal and glial pathways, modulate gene expression and arrest cell-apoptosis mechanisms. In such a way, neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, neuro-inflammation and glutamatergic excitotoxicity are prevented.[13] Furthermore, numerous

[11] K. B. Pandey, S. I. Rizvi, *Oxid. Med. Cell. Longev.*, **2009**, *2(5)*, 270–278.
[12] S. Khurana, K. Venkataraman, A. Hollingsworth, M. Piche, T. C. Tai, *Nutrients*, **2013**, *5(10)*, 3779–3827.
[13] R. Sharma, In: *Polyphenols in Human Health and Disease*, Vol. 1 (ed. R. R. Watson, V. R. Preedy, S. Zibadi), Elsevier Inc., London, UK, **2013**, pp. 757–778.

studies have indicated that polyphenols exert chemo-protective effects, especially in the gastrointestinal system, thanks to their interactions with nutrients, reactive metabolites, activated carcinogens and mutagens. They can influence the expression of many genes that are associated with cancer, and modulate the activity of key proteins that control cell proliferation.[5] In addition to the above-mentioned biological activities, polyphenols can also act as anti-inflammatory, anti-microbial and antiviral agents, and as fungicides.[7,11,14]

Terpenoids are the most numerous and structurally diverse class of bioactive compounds broadly occurring in plant, microbial, fungi and animal kingdom.[15,16] In particular, terpenoids class consists in approximately 25 000 different compounds with wide range of chemical structures. This broad diversity suggests that there is a possibility for terpenoids application in many industrial sectors, such as cosmetic, pharmaceutical and chemical industry.[17] These compounds consist in isoprene units (C_5H_8) and are therefore also called isoprenoids. Based on the number of these building blocks, terpenes can be categorized in four most commonly occurring groups: monoterpenes, sesquiterpenes, diterpenes and triterpenes.[18] Up to date, terpenoids biological activities have been extensively reviewed in literature.[16] These phytochemicals possess pronounced antioxidant activity and therefore can improve tissue redox system. Nevertheless, other notable activities of great importance for human health as anti-bacterial, anti-fungal, anti-inflammatory, anti-leishmanial and anti-tumour activities have been observed.[19] Even in history, when these activities were not scientifically confirmed, essential oils containing terpenoids were used for various diseases treatment.[16]

One of the plants extremely rich in terpenoids is *Cannabis sativa* L. Its characteristic fragrance is attributed to approximately 140 different terpenoids.[20] In particular, the volatile and semivolatile fractions in *Cannabis* are composed of two terpenoids classes, namely monoterpenes and sequiterpenes, where the larger are present in the form of waxes and resins. Additionally, oxygenated terpenoids can also be found.[21] In forthcoming years, *Cannabis* derived terpenoids have received great attention because of their sensorial properties, with peculiar chemical fingerprinting for various *Cannabis* cultivars, and investigations concerning their synergism with phytocannabinoids.[22]

Cannabinoids are particular *Cannabis* plant secondary metabolites. The term phytocannabinoids was proposed for specific *Cannabis* plant products due to the occurrence of synthetic cannabinoids and endocannabinoids.[20] One of the most interesting phytocannabinoids in hemp is the non-psychoactive cannabidiol (CBD)[23] whose global market increased to a value of USD 1.90 billion in 2018, and it is estimated that it will grow by a further

[14] J. Wollgast, E. Anklam, *Food Res. Int.*, **2000**, *33(6)*, 423–447.
[15] A. Ludwiczuk, K. Skalicka-Wozniak, M. I. Georgiev, In: *Pharmacognosy* (ed. S. Badal, R. Delgoda), Elsevier Inc., London, UK, **2017**, pp. 233–265.
[16] D. N. Roy, *Terpenoids Against Human Diseases*, Taylor & Francis Group, Boca Raton, FL, USA, **2019**.
[17] M. Huang, J. J. Lu, M. Q. Huang, J. L. Bao, X. P. Chen, Y. T. Wang, *Expert Opin. Investig. Drugs*, **2012**, *21(12)*, 1801–1818.
[18] G. Kuttan, P. Pratheeshkumar, K. Aryan Manu, R. Kuttan, *Pharm. Biol.*, **2011**, *49(10)*, 995-1007.
[19] C. Gupta, D. Prakash, *J. Complement Integr. Med.*, **2014**, *11(3)*, 151–169.
[20] R. Brenneisen, In: *Marijuana and the Cannabinoids* (ed. M. A. El Sohly), Springer Nature, Cham, Swizerland, **2007**, pp. 17–49.
[21] A. Leghissa, Z. L. Hildenbrand, K. A. Schug, *J. Sep. Sci.*, **2018**, *41*, 398–415.
[22] M. W. Giese, M. A. Lewis, L. Giese, K. M. Smith, *J. AOAC Int.*, **2015**, *98*, 1503–1522.
[23] D. De Vita, V. N. Madia, V. Tudino, F. Saccoliti, A. De Leo, A. Messore, P. Roscilli, A. Botto, I. Pindinello, G. Santilli, L. Scipione, R. Costi, R. Di Santo, *Nat. Prod. Res.*, **2019**, *0*, 1–7.

49% by 2024.[24] Besides CBD, other notable phytocannabinoids that possess no or low psychotropic activity are cannabigerol, cannabichromene, cannabinol, cannabicyclol, cannabinodiol, and there is the psychoactive Δ^9-tetrahydrocannabinol (THC).[25,26] However, up to date, more than 100 cannabinoids have been identified.[27] They possess numerous biological activates and act as antiepileptic, anticonvulsive, anti-neurodegenerative, antiemetic and analgesic agents. Moreover, antibacterial and anti-inflammatory properties have been reported.[25] A positive effect on HIV and multiple sclerosis has been noted.[23] Most of these metabolites present in fresh hemp carry a carboxylic acid moiety.[23,27] Acid cannabinoids show low potency for cannabimimetic activity receptor (CAR) binding. However, their decarboxylated homologues forms, usually called neutral cannabinoids, display high affinities for CAR and psychological activities. The decarboxylation step is therefore crucial for the strengthening of *Cannabis* pharmacological activity,[27] and easily occurs when the acid metabolites are exposed to heat and light, due to their instability.[28,29] Many studies have proposed the application of extracts, so-called phytocomplexes, containing a mixture of phytocannabinoids and terpenoids, rather than pure synthetic molecules, thus suggesting the existence of complementary or synergistic interactions, often called entourage effects.[23,30] Relative evidences are still to be clarified.

Traditionally, the aforesaid phytochemicals are extracted using conventional technologies. More precisely, conventional conductive heating is applied, together with organic solvents. Nevertheless, green extraction principles suggest other technologies and solvents that could increase the extraction efficiency and lower the environmental impact. In this chapter, green extraction technologies will be thoroughly studied on various plant matrices. In particular, UAE, MAE and SCO_2E were tested. Nevertheless, in the light of green extraction principles, water was used as the solvent in several processes and solvent-free methodologies were as well effectuated.

[24] BDS Analytics, **2019**, https://bdsanalytics.com/will-cbd-overtake-thc-white-paper/.
[25] F. Fathordoobady, A. Singh, D. D. Kitts, A. Pratap Singh, *Food Rev. Int.*, **2019**, 35, 664–684.
[26] S. D. McAllister, L. Soroceanu, P. Y. Desprez, *J. Neuroimmune Pharmacol.*, **2015**, 10, 255–267.
[27] M. M. Lewis-Bakker, Y. Yang, R. Vyawahare, L. P. Kotra, *Cannabis Cannabinoid Res.*, **2019**, *4*, 183–194.
[28] V. Brighenti, F. Pellati, M. Steinbach, D. Maran, S. Benvenuti, *J. Pharm. Biomed. Anal.*, **2017**, *143*, 228–236.
[29] M. Wang, Y. H. Wang, B. Avula, M. M. Radwan, A. S. Wanas, J. Van Antwerp, J. F. Parcher, M. A. Elsohly, I. A. Khan, *Cannabis Cannabinoid Res.*, **2016**, *1*, 262–271.
[30] S. Elzinga, J. Fischedick, R. Podkolinski, J. Raber, *Nat. Prod. Chem. Res.*, **2015**, *3(4)*, 181.

2.1. Green extraction technologies for cocoa been shell polyphenols recovery

Cocoa industry has been facing sustainability issues for decades, since the cocoa products demand is increasing year by year. Cocoa bean, only coca fruit part used in food industry, barely makes up to 33% of fruit weight,[31] indicating generation of large amount of by-products making waste management extremely challenging.[32] Whereas cocoa industry by-products are considered undesirable, they are simply disposed unexploited on the cocoa plantation fields.[33] This approach can have a considerable impact on environmental pollution. Due to the intrinsic high moisture and organic content, bacterial degradation occurs creating methane and carbon dioxide that contribute to the global warming.[34] Moreover, disposed by-products can propagate diseases such as pod rot, black pod and cocoa pod borer, resulting in significant crop loss.[33,35] Pod rot disease can lead to global annual crop loss from 20% to 30%, while for individual farmers this value can vary from 30% to even 90%.[36]

Cocoa bean shell (CBS) is one of the aforementioned cocoa industry by-products. Given CBS chemical compositions reported in Table 1, several studies proposed valorisation strategies: from simple low-value application as animal feed, to top-value exploitation as a source of dietary fibres and polyphenols.[35,36,37]

[31] E. O. K. Oddoye, C. K. Agyente-Badu, E. Gyedu-Akoto, In: *Chocolate in Health and Nutrition* (ed. R. Watson, V. Preedy, S. Zibadi), Springer, Cham, Switzerland, **2013**, pp. 23–37.
[32] K. H. N. Figueroa, N. V. Mendoza Garcia, R. Campos Vega, *Cocoa By-products*, In: *Food wastes and by-products: nutraceutical and health potential* (ed. R. Campos Vega, B. D. Oomah, H. A. Vergara-Castañeda), John Wiley & Sons Ltd., Hoboken, NJ, USA, **2020**, pp. 373–411.
[33] R. Martínez, P. Torres, M. A. Meneses, J. G. Figueroa, J. A. Pérez-Álvarez, M. Viuda-Martos, *Food Res. Int.*, **2012**, *49(1)*, 39–45.
[34] C. L. Hansen, D. Y. Cheong, In: *Handbook of Farm, Dairy and Food Machinery Engineering* (ed. M. Kutz), Academic Press, Cambridge, MA, USA, **2019**, pp. 673–716.
[35] R. Campos-Vega, K. H. Nieto-Figueroa, B. D. Oomah, *Trends Food Sci. Tech.*, **2018**, *81*, 172–184.
[36] F. Lu, J. Rodriguez-Garcia, I. van Damme, N. J. Westwood, L. Shaw, J. S. Robinson, G. Warren, A. Chatzifragkou, S. McQueen Mason, L. Gomez, L. Faas, K. Balcombe, C. Srinivasan, F. Picchioni, P. Hadley, D. Charalampopoulos, *Curr. Opin. Green Sustain. Chem.*, **2018**, *14*, 80–88.
[37] J. Panak Balentić, Đ. Ačkar, S. Jokić, A. Jozinović, J. Babić, B. Miličević, D. Šubarić, N. Pavlović, *Molecules*, **2018**, *23(6)*, 1–14.

Table 1. CBS chemical composition.[38,39,40]

Compounds	g/100g dry shell
Protein	6.20 – 18.1
Ash	5.96 – 11.42
Lipid	1.80 – 6.87
Total carbohydrates	17.8 – 23.2
Lignin	32.41
Fibers	18.6–60.6
Pectin	4.7–6.0
Polyphenols	1.32–5.78

As already mentioned, in forthcoming years, agri-food waste valorisation has received great attention owning to the bioactive compounds recovery possibilities that can give an added value to the unexploited by-products.[39] Moreover, new food and beverages ingredients that derive from natural sources show a constantly increasing demand.[33] From this perspective, CBS can be considered a cheap source for valuable secondary metabolites recovery.[41]

Hence, this study focused on CBS valorisation aiming to obtain polyphenols rich extracts by means of green extraction technologies. In particular, US and MW were exploited. The extracts were additionally purified yielding in polyphenols enriched fractions which were analysed by HPLC to determine individual polyphenols.

2.1.1. Results and discussion

Exhaustive conventional CBS polyphenols extraction

The conventional exhaustive CBS polyphenols extraction was performed as described in the work by Guyot.[42] To achieve complete proanthocyanidins recovery, extraction with 70% aqueous acetone was carried out for total of 16 h. Afterwards, an extraction with 80% aqueous ethanol was performed to recover monomeric flavonoids. The extracts were analysed in terms of total polyphenolic content (TPC). The results are reported in Table 2.

[38] Z. S. Vásquez, D. P. de Carvalho Neto, G. V. M. Pereira, L. P. S. Vandenberghe, P. Z. de Oliveira, P. B. Tiburcio, H. L. G. Rogez, A. G. Neto, C. R. Soccol, *Waste Manage.*, **2019**, *90*, 72–83.
[39] A. C. Mellinas, A. Jiménez, M. C. Garrigós, *Lwt*, **2020**, *127*, 109361.
[40] D. C. G. Okiyama, I. D. Soares, T. A. Toda, A. L. Oliveira, C. E. C. Rodrigues, Ind. Crop Prod., **2019**, *130*, 96–103.
[41] G. Grillo, L. Boffa, A. Binello, S. Mantegna, G. Cravotto, F. Chemat, T. Dizhbite, L. Lauberte, G. Telysheva, *Food Res. Int.*, **2019**, *115*, 200–208.
[42] S. Guyot, In: *Handbook of Analysis of Active Compounds in Functional Foods* (ed. L. M. L. Nollet, F. Toldrá) CRC Press Taylor & Francis Group, Boca Raton, FL, USA, **2012**, pp. 317–348.

Table 2. Conventional exhaustive extraction yields and TPC expressed on extract and CBS.

Solvent	Yield (%, g/g)	TPC (mg/g CBS)	TPC (mg/g extract)
70% acetone	16.67	20.51	123.09
80% EtOH	2.93	3.85	131.43

In total, the TPC yield obtained in conventional exhaustive extraction was 24.36 mg/g CBS.

Ultrasound assisted extraction (UAE)

Solvent and conditions screening

UAE was performed using two immersion horns made in titanium; one operating at 250 W and other working at 500 W. Two solvents were tested for CBS polyphenols extraction, in particular hydroalcoholic solution (70% aqueous EtOH) and water. The extracts were analysed for TPC and the results are depicted in Figure 2.

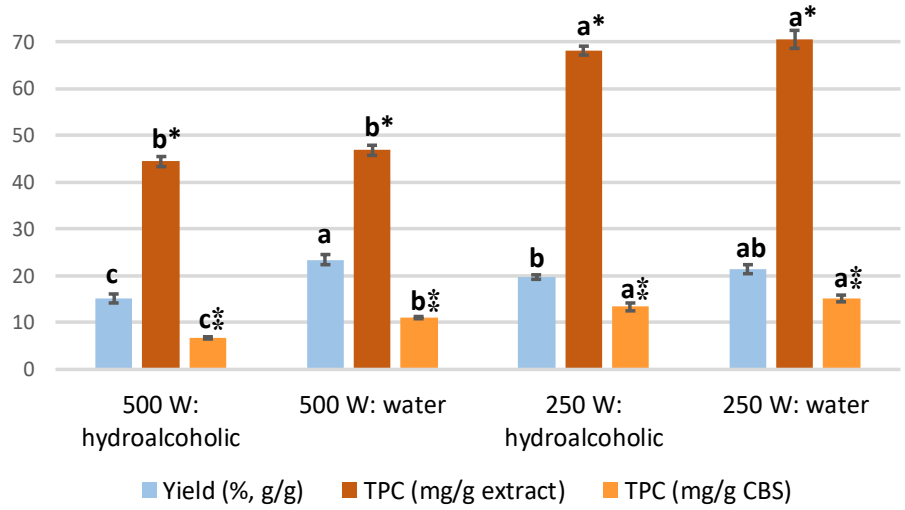


Figure 2. Yield and TPC (expressed on the mass of extract or mass of CBS) obtained from solvent and US power screening. The presented values followed by different lower-case letters (a–c, a*–b*, a⁂–c⁂) are significantly different from each other ($p < 0.05$), compared according to extraction yield, TPC mg/g extract and TPC mg/g CBS.

Horn operating at 500 W gave lower polyphenols yield in respect to the 250 W system, both when water and hydroalcoholic solution are used. At the optimal power, water and hydroalcoholic solution gave comparable yields, statistically indifferent. Therefore, aiming to find the optimal solvent and extraction time, polyphenols extraction kinetics under UAE was monitored.

Polyphenols UAE kinetics

Whereas water and hydroalcoholic solution gave a similar extraction yield, polyphenols UAE kinetics was monitored. The extraction kinetics was described using Peleg's model. Peleg's model is a widely used semi-empirical kinetic model. It was first intended for describing moisture sorption and desorption of food materials.[43] Nevertheless, owning to the shape of

[43] M. Peleg, *J. Food Sci.*, **1988**, *53*, 1216-1217.

the curve, it is widely used for evaluation of plant material compounds extraction.[44] The Peleg's equation is described as follows:

$$c(t) = c_0 \times \frac{t}{K_1 + K_2 \times t} \quad (1)$$

where $c(t)$ is solute concentration in time t, c_0 is starting solute concentration in solvent, K_1 is Peleg's rate constant and K_2 Peleg's capacity constant. When the extraction is performed with a fresh solvent, c_0 parameter can be put out, resulting in following equation:

$$c(t) = \frac{t}{K_1 + K_2 \times t} \quad (2)$$

K_1 and K_2 parameters, that can be obtained by solute concertation linearization, are used to calculate extraction rate (B_0) at $t = t_0$ and maximum solute yield ($c_{t\to\infty}$) at $t_{\to\infty}$. These values are applied to characterise the extraction kinetics. To determine the extraction kinetics, CBS polyphenols UAE at 250 W in both water and 70% EtOH were performed for 2 h. During the extraction, periodical sampling was done and samples TPC expressed as mg polyphenols/g CBS was measured.

Finally, the extraction kinetics when water is used as the solvent was described as follows:

$$TPC = \frac{t}{0.3572 + 0.0666 \times t} \quad (3)$$

B_0 at $t = t_0$ was 2.80 mg/g min and $c_{t\to\infty}$15.02 mg/g.

In the case of 70% EtOH used, Peleg's extraction kinetics was expressed as:

$$TPC = \frac{t}{1.0138 + 0.0541 \times t} \quad (4)$$

B_0 at $t = t_0$ was 0.99 mg/g min and $c_{t\to\infty}$18.48 mg/g.

The defined theoretical Peleg curves and the experimental data are shown on Figure 3 for both extraction processes.

[44] E. Karacabey, L. Bayindirli, N. Artik, G. Mazza, *J. Food Process Eng.*, **2013**, *36*, 103–112.

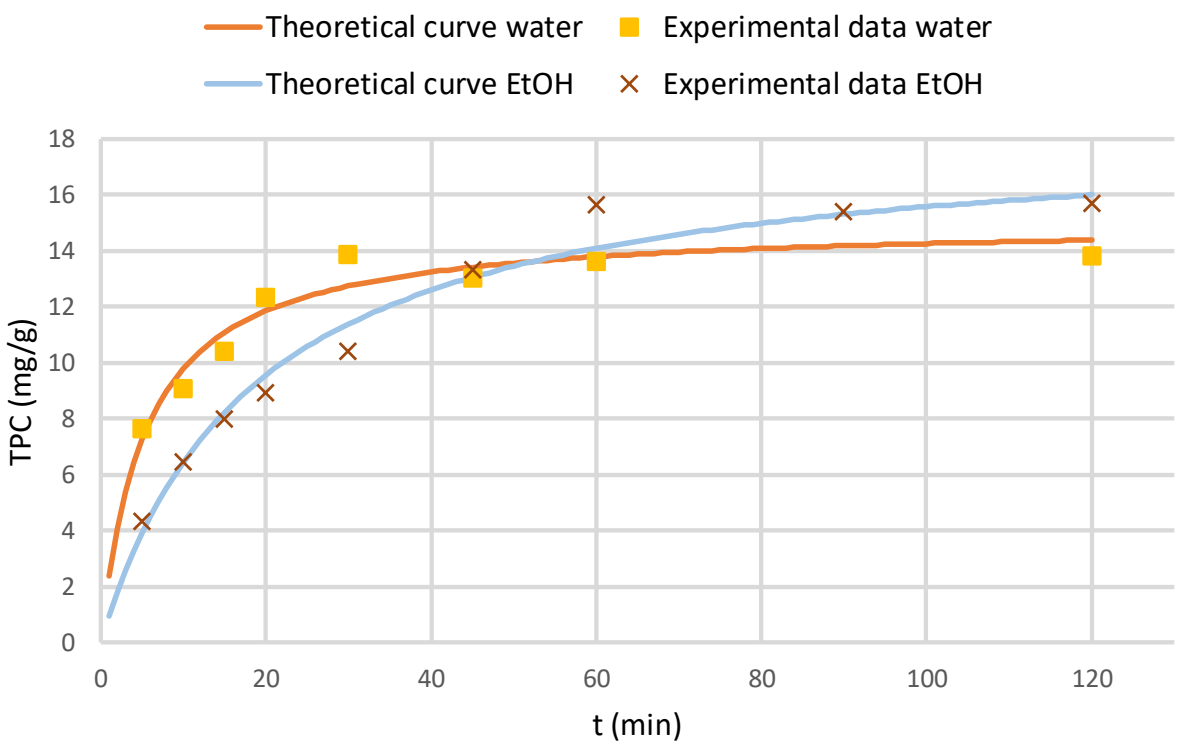


Figure 3. The experimental data on CBS polyphenols extraction and the data elaborated by Peleg's model both for water and 70% EtOH. TPC is expressed as mg polyphenols/g CBS.

The theoretical Peleg's model curve shown a good fit with the experimentally determined data. The maximum yield defined by the Peleg's model indicate that the extraction in hydroalcoholic solution can give a higher polyphenols yield. Nevertheless, the extraction rate is much lower than in the case of water use, in particular 0.99 instead of 2.80, respectively. Long extraction times are in general less preferable in the industrial extraction processes due to the high energy consumption. In addition, lower extraction times can prevent thermos-labile compounds degradation.[45] At 30 min, UAE performed in water reaches it maximum, where TPC yield is 13.84 mg/g CBS, while the theoretical value is 12.74 mg/g CBS. Regarding UAE in hydroalcoholic solution, at 30 min 10.38 mg/g CBS can be obtained, corresponding to theoretical 11.54 mg/g CBS. Owning to all the aforesaid, water is selected as the optimal solvent, and optimal extraction time is 30 min. Thanks to its safety and low price, water is generally preferable solvent in industrial-scale processes. Moreover, it is not a taxed solvent as EtOH and no dedicated equipment is necessary for its use.[46]

Nevertheless, when compared to the conventional exhaustive extraction, UAE offers recovery of 52.30% polyphenols present in raw CBS. However, UAE is performed for only 30 min instead of 32 h that are necessary for exhaustive extraction. In addition, the use of organic flammable solvents is avoided in the case of UAE.

[45] P. Garcia-Salas, A. Morales-Soto, A. Segura-Carretero, A. Fernández-Gutiérrez, *Molecules*, **2010**, *15*, 8813–8826.
[46] F. Chemat, M. Abert-Vian, A. S. Fabiano-Tixier, J. Strube, L. Uhlenbrock, V. Gunjevic, G. Cravotto, *TrAC - Trends Anal. Chem.*, **2019**, *118*, 248–263.

UAE conventional comparison

To evaluate the intensification that US offers to the CBS polyphenols extraction process, a silent conventional extraction was performed in a conventional set-up. Optimal UAE extraction conditions were transposed in a heating and stirring equipment. Temperature was set at 40 °C, for being the medium temperature during UAE.

The extraction yield was 23.82%, g/g. The extract had a TPC of 26.80 mg/g, therefore only 6.38 mg/g CBS were recovered in this process. The obtained TPC is 46.10% lower than the result obtained in optimal UAE. This result emphasises effectiveness of US technology when applied for polyphenols extraction. Nevertheless, numerous research papers likewise suggest so. For example, Both et al.[47] have compared black tea polyphenols extraction by maceration and UAE. Use of UAE enabled higher polyphenols equilibrium concentration in the solvent, demonstrating the efficiency of the applied technology.

Detailed optimal UAE extract characterisation

The CBS polyphenols-rich extract obtained in UAE was thoroughly analysed, aiming to fully characterize the extract, due to its potential application in food industry. Lipidic, total carbohydrate (TCC), water and ash contents were determined.

Firstly, to evaluate the lipidic content, a solubilisation of extracts lipophilic compounds in CH_2Cl_2 was performed and the mass of the obtained fraction was noted. Total carbohydrate content was determined by a phenol-sulphuric acid test. Moreover, whereas the extract was quite hygroscopic due to the high carbohydrates content, its water content was also determined by a gravimetric method. The inorganic compounds content (ash) was likewise evaluated by a gravimetric method. The obtained data are reported in Table 3.

Table 3. TPC, TCC, lipidic, water and ash content in optimal UAE extract.

	%, g/g extract	mg/g extract	mg/g CBS
TPC	6.92	69.20	13.84
TCC	40.51	405.09	86.62
Lipids content	0.28	2.80	0.60
Water content	22.84	/	/
Ash content	16.52	165.22	35.32

By the analysis performed, overall, 87.07% of the obtained extract was characterised. The prevailing class of the compounds was found to be carbohydrates, while the lipids content was quite negligible. Nevertheless, the obtained result was expected since the solvent used for UAE was water.

To analysed polyphenols, a dedicated extraction was conducted. Firstly, raw CBS extraction using CH_2Cl_2 was performed. This step enabled lipids, but most importantly, methylxanthines recovery. This extraction yield was 2.41%, g/g. The methylxanthines found in cocoa plant are theobromine and caffeine, with theobromine being the abundant one.[48] This step is crucial for individual polyphenols identification and quantification. Namely, when polyphenols enrichment is performed, the methylxantines due to their structure, can still be

[47] S. Both, F. Chemat, J. Strube, *Ultrason. Sonochem.*, **2014**, *21*, 1030–1034.
[48] A. M. M. Jalil, A. Ismail, *Molecules*, **2008**, *13*, 2190–2219.

found in the purified polyphenol fraction. Whereas the theobromine concentration in cocoa is much higher than any individual polyphenol, the theobromine peak can repress polyphenols peak on the DAD and MS chromatograms.

The CBS exhausted of lipids and methylxantines was subjected to the UAE, performed at the optimal conditions determined by the Peleg's model. Extraction yield was 19.41% and the extract had TPC of 69.65 mg/g, which corresponds to 13.52 mg/g CBS. The obtained extract was purified on a macroporous resin with a high affinity for polyphenolic compounds. The extract was loaded onto a resin and the compounds with low affinity, like carbohydrates and ash, were eluted using water. This fraction yielded in 63.61%, g/g. The adsorbed polyphenols were eluted using 75% aqueous EtOH. The yield of the polyphenols enriched fraction was 13.95%, g/g. The TPC in this fraction was 474.18 mg/g, providing 85.31% more concentrated sample. Moreover, this result indicates only 5.03% loss of TPC.

Finally, the polyphenols rich fraction was analysed by HPLC-DAD and LC-MS to identify and quantify individual polyphenols. The quantification was performed by an external standard. The identification was done by the UV-Vis spectrum or mass fragmentation comparison with standard compounds or from the literature. The identified and quantified compounds are reported in Table 4.

Table 4. Polyphenols and methylxantines identified and quantified in optimal UAE polyphenol enriched fraction.

Compound	Yield (%, g/g purified fraction)
Theobromine[a]	/
Caffeine[a]	2.20
Epicatechin[a]	0.33
Protocatechiuic acid[a]	0.49
Vanilin[b]	/
Chlorogenic acid[a]	0.23
Quercetin-3-*O*-glucoside[a]	0.07
Proanthocyanidins[b]	/

[a] identification by a comparison with a standard compound;
[b] identification by a comparison with literature data.[49,50]

The prevailing polyphenols in the purified fraction were protocatechuic acid and epicatechin. Generally, the most common polyphenols in CBS are flavanols, in particular epicatechin, catechin, and procyanidins.[37]

Microwave assisted subcritical water extraction (MASWE)

MASWE conditions screening

MASWE was likewise tested for CBS polyphenols extraction. It was performed in a multimode MW reactor where inert gas, in particular N_2, could be introduced to avoid polyphenols oxidation.[51] Whereas the MW cavity was constructed to endure high pressures, subcritical water could be tested for CBS polyphenols extraction. Subcritical water extraction

[49] Y. Shen, C. Han, B. Liu, Z. Lin, X. Zhou, C. Wang, Z. Zhu, *J. Dairy Sci.*, **2014**, *97*, 679–686.
[50] G. Grillo, L. Boffa, A. Binello, S. Mantegna, G. Cravotto, F. Chemat, T. Dizhbite, L. Lauberte, G. Telysheva, *Data Brief*, **2019**, *22*, 56–64.
[51] M. Panić, V. Gunjević, G. Cravotto, I. Radojčić Redovniković, *Food Chem.*, **2019**, *300*, 125185.

has already shown advantages in field of plant bioactive compounds recovery as described in *Chapter 1*. In this case, MW offers a rapid and uniformed heating. However, MASWE was just recently first mentioned in the literature.[52] Therefore, this part of the study aims to present aforesaid novel hybrid technology.

For CBS polyphenols extraction three temperatures were tested: 130, 150 and 170 °C. The extraction time was set at 30 min aiming to achieve a complete extraction. The extracts were evaluated in terms of TPC and the results are reported in Figure 4.

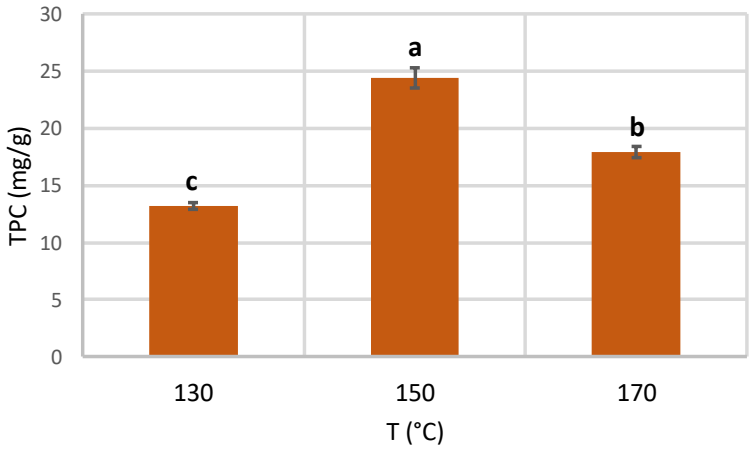


Figure 4. TPC expressed as mg polyphenols/g CBS obtained from MASWE temperature screening. The presented values followed by different lower-case letters (a–c) are significantly different from each other ($p < 0.05$).

Extraction performed at 150 °C gave the highest TPC. Lower yields at other temperatures could be ascribed to water properties change owning to the temperature and pressures applied. Namely, changes in dielectric constant, hydrogen-bond network and hydration enthalpy are observed when modulating subcritical water temperature.[53] Moreover, at 170 °C polyphenols degradation could occur.[54] Therefore, temperature of 150 °C was chosen as the optimal one and the extraction kinetics was evaluated to determine the optimal extraction time. The overall extraction yield at 150 °C was 42.93%, g/g.

Polyphenols MASWE kinetics

The polyphenols MASWE kinetics was, like in the case of UAE determined by Peleg's model. The extraction kinetics was monitored over 30 min. Whereas the equipment did not permit periodical sampling due to the necessary cool down of the microwave cavity, dedicated extractions at 2, 5, 10, 15 and 30 min were performed.

Finally, the extraction kinetics was described as follows:

$$TPC = \frac{t}{0.0123+0.0405\times t} \quad (5)$$

B_0 at $t = t_0$ was 81.30 mg/g min and $c_{t\to\infty}$ 24.69 mg/g.

[52] Z. Yang, B. Uhler, T. Lipkie, *Nat. Prod. Commun.*, **2019**, *June 2019*, 1–4.
[53] N. Galamba, A. Paiva, S. Barreiros, P. Simões, *J. Chem. Theory Comput.*, **2019**, *15*, 6277−6293.
[54] P. P. Singh, M. D. A. Saldaña, *Food Res. Int.*, **2011**, *44*, 2452–2458.

The defined theoretical Peleg curve and the experimental data are shown on Figure 5.

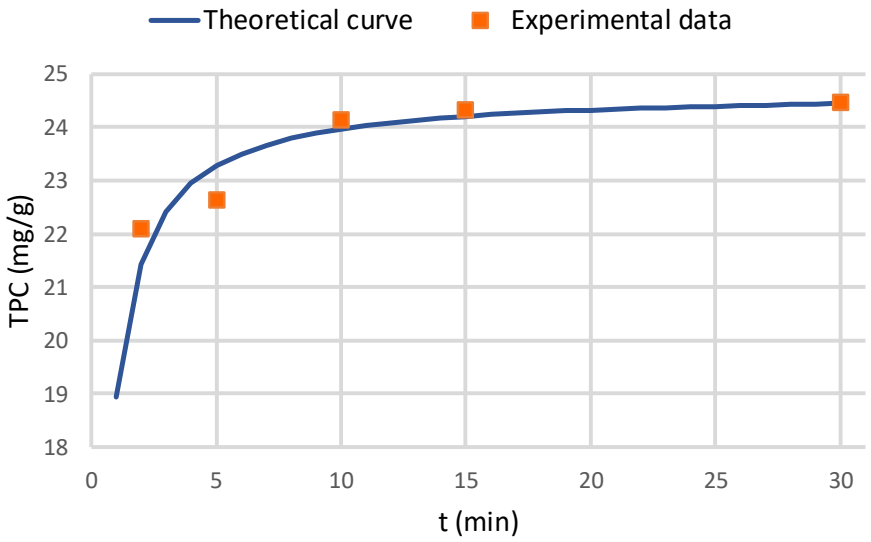


Figure 5. The experimental data on CBS polyphenols extraction and the data elaborated by Peleg's model. TPC is expressed as mg polyphenols/g CBS.

As depicted, CBS MASWE at any extraction time offered a significantly increased TPC extraction yield in respect to the UAE. Namely, in only 2 min TPC of 22.09 mg/g CBS was obtained, which is approx. 37% higher than the optimal UAE. The optimal MASWE time was found to be 10 min where experimentally determined TAC was 24.13 mg/g CBS, while the theoretical one was 23.96 mg/g CBS. This low optimal extraction time is ascribed to the extremely high extraction rate of impressive 81.30 mg/g.

When compared to the conventional exhaustive extraction, MASWE enabled recovery of 99.06% of polyphenols present in CBS. This is a ground-breaking result that demonstrates high efficiency of the tested innovative hybrid technology. Subcritical water combined with MW can eliminate the use of flammable organic solvents and significantly reduce the extraction time, and consequently energy consumption.

Conventional subcritical water extraction

To evaluate the intensification that MW offers in MASWE process, a conventional subcritical water extraction of CBS polyphenols was done. Extraction conditions were transposed to mimic the optimal MASWE.

The obtained extraction yield was 38.59%. The TPC in the extract was 58.45 mg/g and overall 22.56 mg/g CBS of TPC was extracted. Therefore, TPC yield and the extracts composition in terms of TPC is quite similar to the extract obtained in MASWE. Subcritical water extraction is already considered a process intensification technology and it is already well established and thoroughly investigated in the polyphenols extraction field.[55] However, the conventional heating is quite inefficient and time consuming, generating higher energy consumption.[56] In particular, in the conventional extraction set-up, 35 min were necessary to

[55] J. Zhang, C. Wen, H. Zhang, Y. Duan, H. Ma, *Trends Food Sci. Technol.*, **2020**, *95*, 183–195.
[56] M. S. Jesus, Z. Genisheva, A. Romaní, R. N. Pereira, J. A. Teixeira, L. Domingues, *Ind. Crop Prod.*, **2019**, *132*, 99–110.

reach 150 °C, while in the MASWE only 4 min were required to reach the desired temperature. Moreover, the cool-down lasted for 1 h in the conventional reactor, while in the case of MASWE only 15 min were necessary. It can be concluded that MW in this case do not provide a significantly higher extraction yield. However, selective MW heating enables fast water heating diminishing energy consumption and the time needed to complete the extraction process.

Detailed optimal MASWE extract characterisation

The optimal CBS MASWE extract was in detail analysed to determine four major classes of compounds, and water that is absorbed due to the extracts hygroscopicity. The evaluation was performed as for UAE extract and the results are reported in Table 5.

Table 5. TPC, TCC, lipidic, water and ash content in optimal MASWE extract.

	%, g/g extract	**mg/g extract**	**mg/g CBS**
TPC	5.62	56.21	24.13
TCC	36.24	362.44	81.53
Lipids	0.96	9.56	2.15
Water content	21.63	/	/
Ash	17.33	173.32	74.41

This analysis enabled determination of 81.78% of extracts overall chemical composition. The predominant compounds are carbohydrates, while lipids exhibited the lowest content. Therefore, this extract is quite similar in composition to the UAE extract.

The polyphenols in the extract were than thoroughly analysed. For this purpose, raw CBS was extracted firstly with CH_2Cl_2 to eliminate the most part of methylxanthines and like this enable the identification and quantification of the individual polyphenols. Afterwards, the exhausted CBS was subjected to a MASWE performed at above determined optimal conditions. The extracted polyphenols were then purified from the raw extract on a macroporous resin that has a high affinity for phenolic compound. The non-polyphenol fraction yield was 67.10% g/g and the fraction enriched in polyphenols yielded in 15.87% g/g. The polyphenol rich fraction had a TPC of 504.50 mg/g, indicating an approximately 21'fold concentration in these metabolites. In addition, only 7.10% of polyphenols were lost in this purification process.

The obtained fraction was analysed for individual polyphenols on HPLC-DAD and LC-MS. The identified and quantified compounds are reported in Table 6.

Table 6. Polyphenols and methylxantines identified and quantified in optimal MASWE polyphenol enriched fraction.

Compound	Yield (%, g/g purified fraction)
Theobromine[a]	/
Caffeine[a]	0.72
Catechin[b]	/
Epicatechin[a]	0.12
Protocatechiuic acid[a]	0.66
Vanilin[b]	/
Chlorogenic acid[a]	0.06
Quercetin-3-*O*-glucoside[a]	0.07
Proanthocyanidins[b]	/

[a] identification by a comparison with a standard compound;
[b] identification by a comparison with literature data.[49,50]

The analysed sample had a polyphenols profile quite comparable with the one obtained in the optimal UAE.

2.1.2. Conclusions

Unsustainable production is an ever-growing problem in cocoa industry. The cocoa fruit processing generates large amounts of by-products whose improper disposal contributes to the environmental pollution and can propagate various plant diseases. Nevertheless, these by-products are still rich in valuable bioactive compounds. Therefore, this study had an aim to valorise CBS to obtain polyphenols. Moreover, to enable the polyphenols extraction process, two environmentally benign and cost-effective technologies were applied: UAE and MASWE.

Firstly, a benchmark exhaustive CBS polyphenols extraction was performed to determine maximum polyphenols yield that can be obtained. This benchmark extraction yielded in 24.36 mg/g CBS. UAE and MASWE were optimized by extraction kinetics evaluation by Peleg's model to pursue higher process efficiency. In 30 min using only water as the solvent, UAE allowed the extraction of 52.30% polyphenols present in CBS. The optimal extraction conditions were transposed to a conventional system that gave a yield of 6.38 mg/g CBS, emphasising the process intensification that US offers. MASWE, a novel technology that combines two already well-established process intensification techniques, MW and subcritical water extraction, was likewise optimised. At 150 °C in only 10 min 99.06% of polyphenols originally present in CBS were recovered. This is a ground-breaking result that could lead to this novel technique adoption in industry. A subcritical water extraction of CBS was performed in a conventional pressurized system at the MASWE optimal conditions, yielding in TPC of 22.56 mg/g CBS. Therefore, the process intensification in this case could directly be ascribed to subcritical water. Nevertheless, MW offers a much faster water heating to the desired temperature, thus diminishing the necessary time to complete the extraction process.

The optimal extracts were also thoroughly characterised in terms of carbohydrates, ash, lipids and water content. UAE and MASWE extracts had a quite similar composition. Moreover, the polyphenols profile determined by HPLC-DAD and LC-MS showed to be analogues.

These extracts obtained from a food source using water could ensure the absence of toxic compounds. The presented study could facilitate CBS valorisation and pave the way for sustainable cocoa industry with zero-waste generation.

2.1.3. Experimental part

Plant material

The plant material used was dry grinded cocoa bean shell.

Exhaustive extraction

Exhaustive CBS polyphenols extraction was performed as described in Guyot.[42] Briefly, the proanthocyanindins were extracted using 70% aqueous acetone at room temperature and 200 rpm for 8 h. The extract was filtered, and the CBS was subjected to another extraction in 70% acetone. The extract was filtered and both extracts were united. Acetone was evaporated on a rotary vacuum evaporator and the water was freeze dried on lyophilizer (LyoQuest–85, Telstar, Spain). Subsequently, the matrix was subjected to low molecular flavanols extraction in 80% aqueous ethanol for 8 h at room temperature, while the stirring was set at 200 rpm. The extract was separated and the extraction in 80% ethanol was repeated. Finally, solvents from the extracts were evaporated on rotary vacuum evaporator and the water was freeze dried. In both fractions TPC was determined.

Ultrasound assisted extraction (UAE)

Conditions screening and extraction kinetics

CBS polyphenols UAE was done using two titanium immersion horns: first operating at 250 W (Danacamerini sas, Turin, Italy) and the second at 500 W (HNG-20500-SP, Hainertec, Suzhou, China). Both are working at frequency of 20 kHz. Two solvents were tested in each instrumental set-up, in particular hydroalcoholic solution (70% EtOH in water) and water to determine the optimal power and solvent. Extraction time was 15 min and CBS to solvent ratio was 1 to 20. The extraction temperature was kept around 40 °C. For determining UAE kinetics, the optimal power and solvent were used and periodical sampling up to 2 h was performed. The obtained extracts were filtered, ethanol was (if necessary) evaporated in vacuum rotary evaporator, and the water was freeze dried on lyophilizer. The extracts TPC was finally evaluated. The kinetics was described by Peleg's model.

UAE conventional comparison

Aiming to evaluate the US effect during CBS UAE, a silent extraction in the determined optimal conditions was performed: extraction time 30 min and temperature of 40 °C. Water was used as the solvent and the extraction was done in a conventional stirring and heating equipment. CBS to water ratio was 1 to 20. The extract was filtered, and water was freeze dried. In the dried extract, TPC was determined.

Microwave assisted subcritical water extraction (MASWE)

Conditions screening and extraction kinetics

CBS polyphenols MASWE was performed in Synthwave (Milestone, Italy), a pressure-resistant multimode MW reactor, where an inert atmosphere of N_2 was applied. CBS to water ratio was 1 to 20. Firstly, the extraction temperature was tested at 130, 150 and 170 °C. The pressure applied in all the extractions was 10 bar. At the optimal temperature, extraction kinetics was monitored over 30 min. For every extraction time monitored, a dedicated extraction was performed due to the system set-up that does not permit sampling until the microwave cavity is not cooled down. Once the extraction was completed, extracts were filtered, freeze dried and TPC was determined. The extraction kinetics was described using Peleg's model.

Conventional subcritical water extraction

The conventional subcritical water CBS extraction was performed in a Parr Instrument (Span, USA) reproducing the optimal MASWE conditions in order to evaluate the intensification that MW offers in MASWE. Extraction time was 10 min and temperature was set at 150 °C. CBS to water ratio was 1 to 20. Before the extraction, N_2 was introduced to the vessel to imitate the conditions during MASWE. Once the extraction was completed the extract was filtered, water was freeze dried and the TPC was evaluated.

Peleg's model

As mentioned above, UAE and MAE kinetics were described using Peleg's model,[43] which is defined by the following equation:

$$TPC(t) = \frac{t}{K_1 + K_2 \times t} \quad (6)$$

where t is extraction time, K_1 is Peleg's rate constant and K_2 is Peleg's capacity constant. Both the kinetic constants were extrapolated from experimental data *via* the linearisation of Equation 6 ($r^2 \geq 0.99$). From K_1, the value of the extraction rate at $t = t_0$ (B_0) can be obtained:

$$B_0 = \frac{1}{K_1} \quad (7)$$

Using K_2, the maximum TPC yield, when $t \to \infty$ ($C_{t\to\infty}$), can be calculated:

$$C_{t\to\infty} = \frac{1}{K_2} \quad (8)$$

The optimal solvent for UAE and the optimal extraction time for UAE and MAE can be determined using the B_0 and $C_{t\to\infty}$values.

Total phenolic content (TPC) determination

TPC in the CBS extracts was determined according to the method described in Hillis and Swain.[57] 250 µL of the extract solution (1 mg/mL in 50% EtOH) was placed into the test tube and diluted with 4 mL of deionized water. A sodium carbonate solution (10%, w/v) and the Folin–Ciocalteu reagent (diluted 1:1 with deionized water) were added sequentially. The resulting solution was mixed thoroughly. After 25 min, the absorption of the blue complex

[57] W. E. Hillis, T. Swain, *J. Sci. Food Agric.*, **1959**, *10*, 135–144.

was measured at 725 nm, in a 1 cm quartz cuvette, using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, USA), against a blank. Gallic acid was used as the standard. TPC was expressed as gallic acid equivalents (GAE, mg/g) over the dried extract and gallic acid equivalents (GAE, mg/g) over the dried matrix. All analyses were performed in triplicate.

Conventional lipids and methylxantines extraction

Methylxantines and lipids extraction was performed for analytical purposes in heating and stirring system under reflux using dichloromethane (CH_2Cl_2) as solvent. The extracts or CBS to CH_2Cl_2 was 1 to 20. The extraction time was 2 h and the extract was filtered and the extraction was repeated one again. The obtained extracts were united and evaporated on rotary vacuum evaporator.

Phenol-sulphuric method for TCC determination

Total carbohydrates in the extracts were determined by phenol-sulphuric acid method described in work by Nielsen.[58] One millilitre of diluted extracts and 1 mL of deionized water are placed in the test tube followed by 0.05 mL of 80% (w/w) phenol solution in water. Subsequently, 5 mL of H_2SO_4 are rapidly added in the test tube. The resulting solution is mixed thoroughly and left for 10 minutes at 25 °C to cool down. The absorption was measured at 490 nm, in a 1 cm quartz cuvette, using a Cary 60 UV-Vis spectrophotometer, against a blank. Glucose was used as the standard. TCC was expressed as glucose equivalents (GLC, mg/g) over the dried extract and glucose equivalents (GLC, mg/g) over the dried matrix. All analyses were performed in triplicate.

Water content determination

The water content in the extracts was determined by a gravimetric method. Extracts were dried in a furnace muffler (Gelman Instrument Company, Ann Arbor, MI, USA) at 100 °C for 24 h. The analysis was performed in triplicate.

Ash content determination

Ash content was evaluated by a gravimetric method. Firstly, the sample was dried in muffler furnace at 100 °C. Once a constant weight of the sample was achieved, the muffler furnace was set at 600 °C. Dry ashing lasted for 4 h and afterwards, the sample was weighted and the ash content was calculated. The analysis was performed in triplicate.

Extract purification – polyphenols enrichment

Polyphenols isolation from the CBS extract was performed over Sepabeads SP 825L macroporous resins (Mitsubishi Chemical Corporation, Resindion SRL, Italy). After resin preparation using ethanol and water, extract was loaded. The fraction 1 is eluted with 3 bead volumes (BV) of deionized water. The polyphenols (fraction 2) are desorbed with 3 BV of 75% ethanol. Ethanol/water from the fraction 2 is then removed in a rotary vacuum evaporator followed by freeze drying. Finally, dry polyphenols enriched extract is obtained. The polyphenols recovery efficiency was evaluated by measuring TPC in the purified phenolic fraction.

[58] S. S. Nielsen, In: *Food Analysis Laboratory Manual* (ed. S. S. Nielsen), Food Science Texts Series, Springer, Boston, MA, USA **2010**, pp. 47–53.

HPLC-DAD polyphenols analysis

Polyphenols were identified and quantified using a HPLC system (Waters Corp., USA) coupled with a diode array detector (UV/DAD, Waters Corp., USA) and an automatic sampler (Waters Corp., USA). Separation was achieved on a Synergi Hydro RP C18 column (250 mm, 4.6 mm, 5 µm; Phenomenex, USA) using H_2O with 2% AcOH (A) and ACN (B) as the mobile phases. Gradient elution was modified as follows: 0–6.5 min 0% B, 6.5–30 min from 0% to 50% B, 30–36 min from 50% to 100% B, which was maintained for 6 min. The post-running time was 10 min. The flow rate was 1 mL/min. The sample injection volume was 20 µL and the samples were always filtered through 0.22 µm PTFE (polytetrafluoroethylene) filters prior to injection. UV-DAD acquisitions were carried out in 200–600 nm range, while chromatograms were acquired at 280 and 340 nm. Three injections were performed for each sample. The retention time, absorption maximum, λ used for detection, calibration line, linearity range, linear equation fit, LOD and LOQ are reported for every analysed polyphenol in the *Supporting materials* section.

LC-MS polyphenols analysis

Polyphenols were identified using a LC system (Waters Corp., USA) coupled with a MS mass spectrometer (Waters Corp., USA) and an automatic sampler (Waters Corp., USA). Analysis parameters were set using both positive and negative ion mode with spectra acquired over a mass range from m/z 80 to 800. The compound separation was achieved on Synergi Hydro RP C18 column as described in HPLC-DAD analysis.

Statistical analysis

Statistical analyses were performed using the software Statistica (Statsoft Inc., Tulsa, OK, USA), version 10. Where required, the measurements were processed using Tukey's HSD test and statistical difference ($p < 0.05$) were designated by lower-case letters.

2.2. Multi-kilo microwave assisted processes for cascade *Cannabis* terpenes, polyphenols and cannabinoids extraction[59]

Cannabis sativa L. (*Cannabaceae* family), known as hemp, is a widespread plant species cultivated for a broad range of industrial products. These products are fibres, seed oils and biomasses that are used in various fields, including in the pharmaceutical, cosmetic, paper, textile and construction industries, as food and animal-feed additives, phytoremediation agents, biofuel, varnishes and inks.[25,60,61] Hemp has a highly complex chemical composition that includes carbohydrates, terpenoids, alkaloids, stilbenoids, quinones, flavonoids, fatty acids, phenols and cannabinoids.[20,28,62]

In forthcoming years, the popularity of medical *Cannabis* extracts has grown rapidly due to extensive reviews of the pharmacological activity of this plant material, which is mainly attributed to the presence of phytocannabinoids.[27] Nevertheless, recent studies have suggested the application of *Cannabis* extracts as phytocomplexes for various diseases treatment and prevention. Phytocomplexes, a mixture of phytocannabinoids and terpenoids, could have pronounced health beneficial effects due to the so-called entourage effect.[23,30]

The recovery of the aforementioned biologically active compounds from hemp is a crucial step for their further applications in the pharmaceutical and food industries.[25] Hemp volatile fraction consists of monoterpenes, such as α-pinene, myrcene and terpinolene, and bitter-tasting sesquiterpenes, such as E-caryophyllene, α-humulene and caryophyllene oxide.[61] These compounds are traditionally recovered via hydro- or steam distillation using Clevenger apparatus. Supercritical CO_2 can also be exploited.[20,63] Steam and hydro-distillation have numerous drawbacks regarding the extraction efficiency and, moreover, their harsh conditions can affect essential-oil's quality.[63,64,65,66] The main disadvantage of SCO_2E is the fact that processing fresh plant materials is impossible due to the formation of carbonic acid from CO_2 and water.[63] The required desiccation of the matrix dramatically affects the whole volatile-composition fingerprint. Furthermore, it is important to emphasize that extracts obtained using SCO_2E cannot be considered for the recovery of essential oils because the product contains a wide range of different compounds besides cannabinoids (e.g., fatty acids, sterols, hydrocarbons and triterpenes).[61]

Therefore, there is a need for more efficient solution for *Cannabis* volatiles extraction, such as MAHD. During MAHD, MW heats the water in the plant material and in the system, thus steam is released. Heat and steam pressure carry terpenes to the distillation head where

[59] V. Gunjević, G. Grillo, D. Carnaroglio, A. Binello, A. Barge, G. Cravotto, *Ind. Crop. Prod.*, **2021**, *162*, 113247.
[60] Y. Yang, M. M. Lewis, A. M. Bello, E. Wasilewski, H. A. Clarke, L. P. Kotra, *Cannabis Cannabinoid. Res.*, **2017**, *2*, 274–281.
[61] D. Fiorini, A. Molle, M. Nabissi, G. Santini, G. Benelli, F. Maggi, *Ind. Crops Prod.*, **2019**, *128*, 581–589.
[62] Z. Drinić, J. Vladić, A. Koren, T. Zeremski, N. Stojanov, B. Kiprovski, S. Vidović, *J. Chem. Technol. Biotechnol.*, **2020**, *95*, 831–839.
[63] S. Markle, *Cannabis Sci. Technol.*, **2019**, *2*, 50–76.
[64] M. E. Lucchesi, F. Chemat, J. Smadja, *J. Chromatogr. A*, **2004**, *1043*, 323–327.
[65] M. Ferhat, B. Y. Meklati, F. Chemat, *Flavour Fragr. J.*, **2007**, *22*, 494–504.
[66] M. Iriti, G. Colnaghi, F. Chemat, J. Smadja, F. Faoro, F. A. Visinoni, *Flavour Fragr. J.*, **2006**, *21*, 704–712.

they can be recovered. This process is much more efficient than traditional hydro- and steam distillation as the irradiation heats the plant material evenly.[63,67,68] Abovementioned MW technology advantages opened the way to its application in the extraction of phytocannabinoids, to date comprehensively reviewed. MAE enables phytocannabinoids decarboxylation unlike several other extraction methodologies, where the occurrence of this phenomenon is quite negligible. This feature is of great importance as it leads to high quality products with measurable pharmacological activity in patients.[27,28,62]

Therefore, in this work, MW technologies in two setups were used for *Cannabis* secondary metabolites recovery. MAHD was applied for volatiles extraction and was compared to conventional hydrodistillation (CHD). The essential oils, obtained under CHD and various MAHD conditions were qualitatively analysed to compare the oils quality. Moreover, water in which *Cannabis* matrix was immerged during MAHD was analysed in terms of polyphenols. The matrix that was exhausted of terpenes and a part of polyphenols, was subsequently submitted to MAHG extraction to recover phytocannabinoids.

2.2.1. Results and discussion

Plant material growth stage

The *Cannabis sativa* L. (cv. Monoica) was in a 8.7 phenological growth stage as determined by BBCH (Biologische Bundesanstalt, Bundessortenamt and CHemical industry) scale system. BBCH scale is usually used for mono- and dicotyledonous plant species, as *Cannabis*. In 8.7 growth stage, 60% of the seeds are mature. In stage 9 senescence and dormancy begin.[69,70] The phenological growth stage of *Cannabis* plant is an important factor when extracting essential oils. Terpenes and cannabinoids are located in trichomes that appear at the surface of the cotyledons, and therefore their development is crucial for obtaining these metabolites.[71]

Water and inflorescence content in plant material

The collected *Cannabis* consisted of 73.7% ± 3.22% g/g of inflorescence and 36.3% ± 2.98% g/g of stalks and leaves.

The average water content in *Cannabis*, determined by thermogravimetric analysis, amounted in 69.97 ± 2.63%, g/g. In particular, 71.15 ± 0.98% was in the inflorescences, while 59.72 ± 0.89% g/g in separated stalks and leaves.

[67] R. Ciriminna, A. Fidalgo, R. Delisi, D. Carnaroglio, G. Grillo, G. Cravotto, A. Tamburino, L. M. Ilharco, M. Pagliaro, *ACS Sustain. Chem. Eng.*, **2017**, *5*, 5578–5587.
[68] A. Rezvankhah, Z. Emam-Djomeh, M. Safari, G. Askari, M. Salami, *J. Food Sci. Technol.*, **2019**, *56*, 4198–4210.
[69] U. Meier, *Growth Stages of Mono- and Dicotyledonous Plants: BBCH-Monograph*, Federal Biological Research Centre for Agriculture and Forestry, Berlin, Germany, **2001**.
[70] S. Mishchenko, J. Mokher, I. Laiko, N. Burbulis, H. Kyrychenko, S. Dudukova, *Emės Ūkio Mokslai*, **2017**, *24(2)*, 31–36.
[71] S. J. Livingston, T. D. Quilichini, J. K. Booth, D. C. J. Wong, K. H. Rensing, J. Laflamme-Yonkman, S. D. Castellarin, J. Bohlmann, J. E. Page, A. L. Samuels, *Plant J.*, **2020**, *101*, 37–56.

Volatiles extraction

Cannabis volatile fraction was extracted in MAHD. For the sake of comparison, CHD was firstly performed.

Conventional hydrodistillation (CHD)

CHD was carried out to compare the volatile fraction yield, and its terpene profile, with the one derived from a non-conventional extraction procedure, MAHD. It was performed for 4 h, at water boiling point. The recovered hydrodistillate yields obtained in this process were 0.08 ± 0.01% and 0.12 ± 0.01%, g/g, as calculated in relation to the complete dry matrix and the only dry inflorescence, respectively. Terpenoid yields usually vary from 0.01 to 1.5% of the inflorescence dry weight.[22] Low volatiles yield in this extraction could be ascribed either to the *Cannabis* variety or the ineffectiveness of the applied extraction process. Still, CHD is performed using conventional conductive heating, which is inefficient and moreover, has high energy consumption due to thermal dispersion and material calorimetric restrictions. Therefore, low yield could be ascribed to unsuitability of the applied technology.

CHD volatiles analysis

A qualitative analysis of the terpenes was performed by comparing identified compounds retention times and mass fragmentation with the external standards, and to the GC-MS system's libraries for the compounds missing a standard (mass spectra libraries quality ≥95%). The non-assigned compounds show low individual percentages and low quality matching with the libraries, and were hence assumed to be barely significant. The profile of terpenes in volatile fraction obtained by means of CHD is reported in Table 7.

Table 7. CHD volatile fraction profile. Values expressed as percent peak area composition obtained from GC-MS analysis.

Terpene fraction profile			
Compound	Area %	Compound	Area %
Z-caryophyllene[b]	0.19	α-gurjunene[b]	2.26
α-*trans*-bergamotene[b]	1.49	selina-3,7(11)-diene[b]	2.09
α-santalene[b]	0.17	nerolidol[a]	2.53
E-caryophyllene[a]	8.94	germacrene b[b]	2.82
α-guaiene[b]	0.24	γ-muurolene[b]	0.62
β-farnesene[b]	1.94	caryophyllene oxide[b]	8.15
Aromadendrene[b]	2.22	valencene[b]	2.40
α-humulene[a]	4.15	caryophylla-4(12),8(13)-diene-5-β-ol[b]	2.98
β-gurjunene[b]	0.87	α-bisabolol[a]	4.01
γ-selinene[b]	0.89	eudesm-7(11)-en-4-ol[b]	1.43
β-selinene[b]	6.12	heptacosane[b]	0.17
α-selinene[b]	3.22	nonacosane[b]	0.45
β-guaiene[b]	1.82		

[a] identified according to the standard compound;

[b] identified according to Wiley275 and NIST05 GC libraries (matching quality ≥ 95%).

Predominant compounds found in the gas-chromatographic profile include: E-caryophyllene, caryophyllene oxide, α-humulene, β-selinene and α-bisabolol. In addition, this

volatile fraction is quite poor in terpenes variety. A much higher contribution of sesquiterpenes is observed. GC-MS analyses also shown presence of CBD. The percent area of CBD in the resulting essential oil was 23.83%. This value indicates a high contribution of phytocannabinoids in the essential oil, thus may limit the applicability of the CHD product. The analysed sample showed traces of THC as well, proving the harshness of the protocol.

Gulluni et al.[72] analysed *Cannabis* essential oil belonging to the same variety studied in this work (*Cannabis sativa* L. cv. Monoica), prepared in CHD. The essential oil's prevalent compounds, in particular myrcene, terpinolene, caryophyllene, β-humulene, β-ocimene, and limonene, indicate a slightly different composition.

Microwave assisted hydrodistillation (MAHD)

By considering the growing demand for *Cannabis*-derived terpenes from today's hemp market, the aim of this work is to present a novel pilot-scale extraction procedure for their recovery. Extractions were performed in a multimodal MW reactor, Ethos X. Several tests with different extraction conditions and reactor set-ups were investigated (Table 8). All the performed tests are explained when discussing the results.

Table 8. *Cannabis* MAHD tests: screening of parameters.

Test	Plant material (kg)	Water feed	Plant material to water ratio (kg/L)	Additional process alterations
1	2.60	Deionized	1/1	-
2	2.60	Tap water	1/1	-
3	2.64	Deionized	1/1	Hot water added
4	2.70	Deionized	1/1	Matrix moved during MAHD
5	2.72	20% NaCl	1/1	-
6	2.73	Deionized	1/1	PEEK net above the matrix
7	2.84	Deionized	1/1	Matrix in a cotton bag
8	2.63	Deionized	1/1	Matrix in a cotton bag, hot water
9	2.80	Deionized	1/1.5	Matrix in a cotton bag
10	2.50	Deionized	1/1.5	-
11	2.61	Deionized	1/0.5	-
12	2.74	Deionized	1/1	Rectification with Vigreux column
13	2.69	Deionized	1/1	-

The terpene-fraction mass was monitored for each test. Moreover, CBD trend was registered, as a control parameter to describe pyhtocannabinoids extraction behaviour. This metabolite was conveniently chosen being the most abundant in the matrix. The hydrodistillate mass and yield, the time of distillation onset and CBD trend are reported for every MAHD test in Table 9.

[72] N. Gulluni, T. Re, I. Loiacono, G. Lanzo, L. Gori, C. MacChi, F. Epifani, N. Bragazzi, F. Firenzuoli, *Altern. Med.*, **2018**, 1709182.

Table 9. Recovered volatile fraction mass and yield, calculated in relation to the complete dry matrix and based on the only dry inflorescence, distillation onset time and CBD percent area in the GC-MS chromatogram.

Test	Hydrodistillate mass (g)	Yield		Distillation onset time (min)	CBD in hydrodistillate (Area, %)
		over complete matrix (%, g/g) [a]	over only inflorescence (%, g/g) [a]		
1	1.84 ± 0.10	0.24 ± 0.02	0.33 ± 0.02	16	2.49
2	1.91 ± 0.09	0.24 ± 0.02	0.35 ± 0.02	16	1.75
3	1.74 ± 0.12	0.22 ± 0.02	0.31 ± 0.02	12	2.40
4	1.28 ± 0.12	0.16 ± 0.01	0.22 ± 0.02	16	2.34
5	0.66 ± 0.09	0.08 ± 0.01	0.11 ± 0.02	14	10.51
6	1.46 ± 0.08	0.18 ± 0.01	0.25 ± 0.01	16	3.20
7	1.36 ± 0.11	0.16 ± 0.01	0.22 ± 0.02	19	0.30
8	1.26 ± 0.09	0.16 ± 0.01	0.22 ± 0.02	14	0.55
9	1.49 ± 0.13	0.18 ± 0.01	0.25 ± 0.02	19	0.62
10	1.37 ± 0.12	0.18 ± 0.01	0.26 ± 0.02	16	4.10
11	1.54 ± 0.09	0.20 ± 0.02	0.28 ± 0.02	16	2.77
12	1.58 ± 0.09	0.19 ± 0.01	0.27 ± 0.02	15	2.21

[a] calculated on dry plant material

First, the quantity of water added to the system to enhance the stripping power of steam was screened, and its influence on the process was determined (Figure 6). Water addition can increase terpene yield but, more importantly, it prevents the extracted material from burning and thus the metabolites degradation. Material combustion during distillation can also lead to the release of undesired compounds into the terpenes fraction, affecting quality and use of the matrix, such as selective phytocannabinoids recovery, after MAHD.[63]

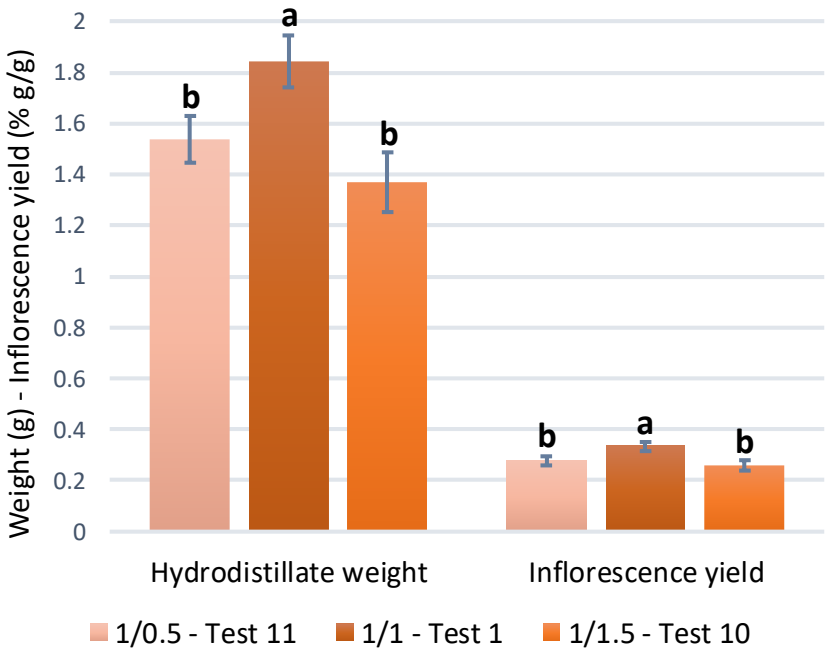


Figure 6. MAHD trend with different water feeds (plant/liquid ratio w/w:1/0.5, 1/1, 1/1,5). Data expressed on hydrodistillate weight (g) and yield on dry inflorescence (%, g/g). Presented values followed by different lower-case letters (a–b) are significantly different from each other ($p < 0.05$).

As reported, the intermediate plant material/water feed ratio of 1:1 proved to be the most efficient, as it kept the matrix hydrated until the end of the extraction. For this reason, the remaining MAHD-screening tests were carried out using this water amount. Fiorini et al.[73] performed MAHD in a similar reactor set-up, and likewise studied the water addition effect. The authors reported the highest essential oil's yield when 30% of water is added. Higher water content caused yield decrease. These results differ much from the results reported in Figure 6. Namely, when expressing the water addition in percentage, the highest volatile fraction's yield was provided when water content was 50%, while lower yields were noted for both 25% and 75% water contents.

Moreover, the effect of having a deionized water (Test 1) or feed with different quantities of solutes, namely tap water and a 20% $NaCl_{aq}$ solution (Test 2 and Test 5) was studied. The greatest yield was observed in Test 2, followed by Test 1 and Test 5, as shown by comparison reported in Figure 7. Test 2 and Test 1 yields were not statistically different. However, since tap water doesn't require additional treatments as the deionized water, tap water use is preferable in industrial scale processes.

[73] D. Fiorini, S. Scortichini, G. Bonacucina, N. G. Greco, E. Mazzara, R. Petrelli, J. Torresi, F. Maggi, M. Cespi, *Ind. Crops Prod.*, **2020**, *154*, 112688.

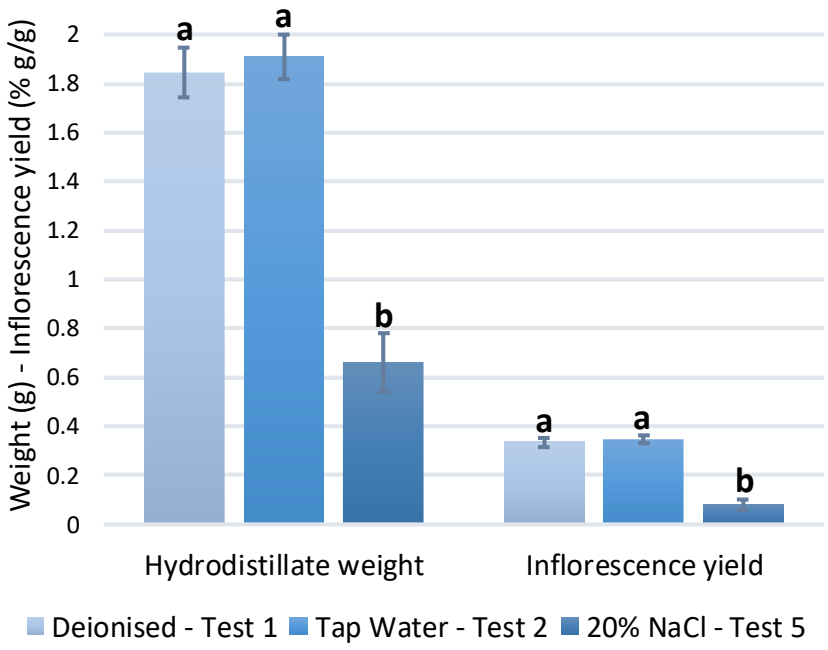


Figure 7. MAHD trend for different water feeds (deionized, tap water, 20% NaCl). Data expressed on hydrodistillation weight (g) and yield on dry inflorescence (%, g/g). Presented values followed by different lower-case letters (a–b) are significantly different from each other ($p < 0.05$).

A high amount of salts is usually exploited to enhance MW absorption, hence leading to higher temperatures and a faster heating ramp. In fact, the onset of distillation was reduced by 2 min for Test 5. However, the rapid temperature increase led to the lowest extraction yield observed, instead of increasing hydrodistillate recovery as assumed. Compound degradation, likely due to the increased boiling point of the system[74,75] and difficult temperature control, is assumed to be the reason. Nevertheless, this peculiar episode requires further study.

The use of hot water (50 °C) as the liquid feed was considered to accelerate the distillation onset, while investigating how this approach could affect the extraction of volatiles. This approach could allow to speed up the distillation onset, reducing the required MW irradiation on the plant material. Thus, the matrix can be preserved from degradation phenomena. As expected, MAHD onset was accelerated from 16 to 12 min, saving a quarter of the total heating step (see Table 9, Test 2 *vs.* Test 3). As depicted in Figure 8, the volatile fraction yield was slightly affected by the hot-water protocol. It can be assumed that the products leaked during hot water addition in the extraction vessel because of the high volatility of the terpenic compounds. Since no statistical difference was noted, remaining tests were performed with room temperature (RT) water addition for being a more simplified protocol.

To prevent any loss during matrix moisturizing and positioning, the same screening was studied using a cotton bag (CB), and both RT and a 50 °C water feed were evaluated. At the same time, the use of a CB had the role of protecting the hemp from overheating, maintaining high wetness and avoiding burning phenomena. Generally, as reported in Figure 8, the use of a CB clearly reduced the average yield of the process showing that the cotton fibres had a

[74] G. W. Mcgraw, R. W. Hemingway, L. L. Ingram, C. S. Canady, W. B. Mcgraw, *Environ. Sci. Technol.*, **1999**, *33*, 4029–4033.
[75] D. Namdar, M. Mazuz, A. Ion, H. Koltai, *Ind. Crops Prod.*, **2018**, *113*, 376–382.

quenching effect. Furthermore, the onset of MAHD was significantly delayed, from 16 to 19 min.

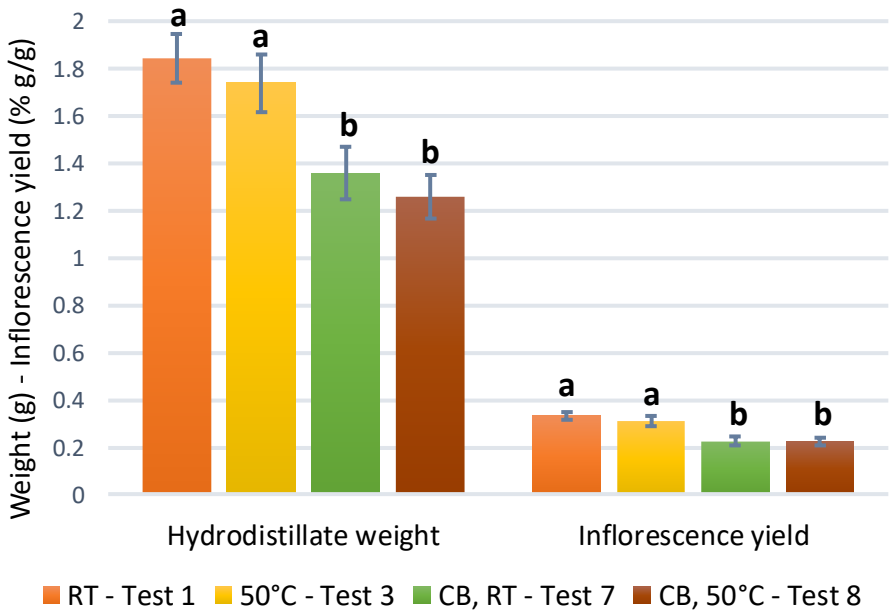


Figure 8. MAHD trend in function of room temperature (RT) and hot-water (50 °C) feed extraction, both for the standard set-up and CB applications. Data expressed on hydrodistillate weight (g) and yield on dry inflorescence (%, g/g). Presented values followed by different lower-case letters (a–b) are significantly different from each other ($p < 0.05$).

A similar approach was tested with a PEEK net (Test 6), aiming to evenly distribute the recycled water on the matrix, during the distillation process. However, also this system led to a decrease in the volatile fraction and inflorescence yield. Test 4 was performed with the matrix being moved every 30 min during extraction. The initial hypothesis was that this should increase the volatile fraction yield by releasing terpenes contained in every spot of the matrix. In contrary, the extraction yield was much lower. The explanation of this result can be related to the necessary equipment extraction and dissembling in order to carry out the matrix movement, that lead to a volatile compounds loss.

Close attention was paid on the state of the vegetal matrix after the extraction treatment, to evaluate any biomass overheating or burning effect. This never happened, even when the plant material was placed in a CB for MAHD. In this case, the matrix appeared to be driest between the screened conditions. Generally, it is possible to state that the hemp that resulted from the MAHD was preserved from combustion and degradation phenomena, thus it may be suitable for further cascade extraction. For this reason, the phytocannabinoid decarboxylation after MW irradiation was investigated. Ethanol extraction under reflux is considered to be the benchmark cannabinoid extraction procedure. Hence, every sample was extracted according to this approach in duplicate, either with a prior heating step of the sample at 120 °C, or directly. The 120 °C heating protocol was applied to promote acidic cannabinoid decarboxylation. Both fresh *Cannabis* inflorescence and the spent matrix after MAHD were analysed. The benchmark phytocannabinoid extraction of fresh plant material enabled CBD quantification by means of UPLC-MS/MS. Similarly, THC was monitored and quantified due to

normative restrictions. Namely, according to the EU regulations, THC content in *Cannabis sativa* crop should not exceed 0.2%.[76] Results are summarized in Table 10.

Table 10. THC and CBD UPLC-MS/MS quantification. Raw inflorescence: percentage yields for acidic and decarboxylated cannabinoids. Test 2 hydrodistillate and depleted inflorescence: decarboxylated and acid forms reported as total amount; expressed as ratio between the phytocannabinoids in hydrodistillate or depleted inflorescence and fresh inflorescence.

	Cannabinoids in inflorescence (%, g/g)	Cannabinoids in hydrodistillate (%)[a]	MAHD inflorescence depletion (%)[a]
THC	0.02	0.04	0.07
THCA	0.05		
CBD	0.34	0.42	0.05
CBDA	0.66		

[a] Test 2 analysis; expressed as total amount of decarboxylated and acid forms.

The results confirmed negligible plant material depletion of phytocannabinoids during MAHD. Less than 1% of both THC and CBD was extracted, indicating the suitability of the terpenes depleted matrix for further phytocannabinoids extraction. The final analysis of the matrix after MAHD confirmed that MW irradiation gave phytocannabinoid decarboxylation of about 70% of the total (69.01% and 74.32% for THC and CBD, respectively). As already mentioned, this feature partially provides more active forms of cannabinoids,[27] hence it can be considered for further investigations.

CBD percent area in the volatile fraction was carefully monitored as a control parameter for phytocannabinoids state in the hydrodistillate, due to their biological activity. Figure 9 compares the CBD trend to hydrodistillate yields, as calculated based on dry inflorescence.

[76] M. Dei Cas, E. Casagni, A. Saccardo, S. Arnoldi, C. Young, S. Scotti, E. Vieira de Manicor, V. Gambaro, G. Roda, *Forensic Sci. Int.*, **2020**, *307*, 110113.

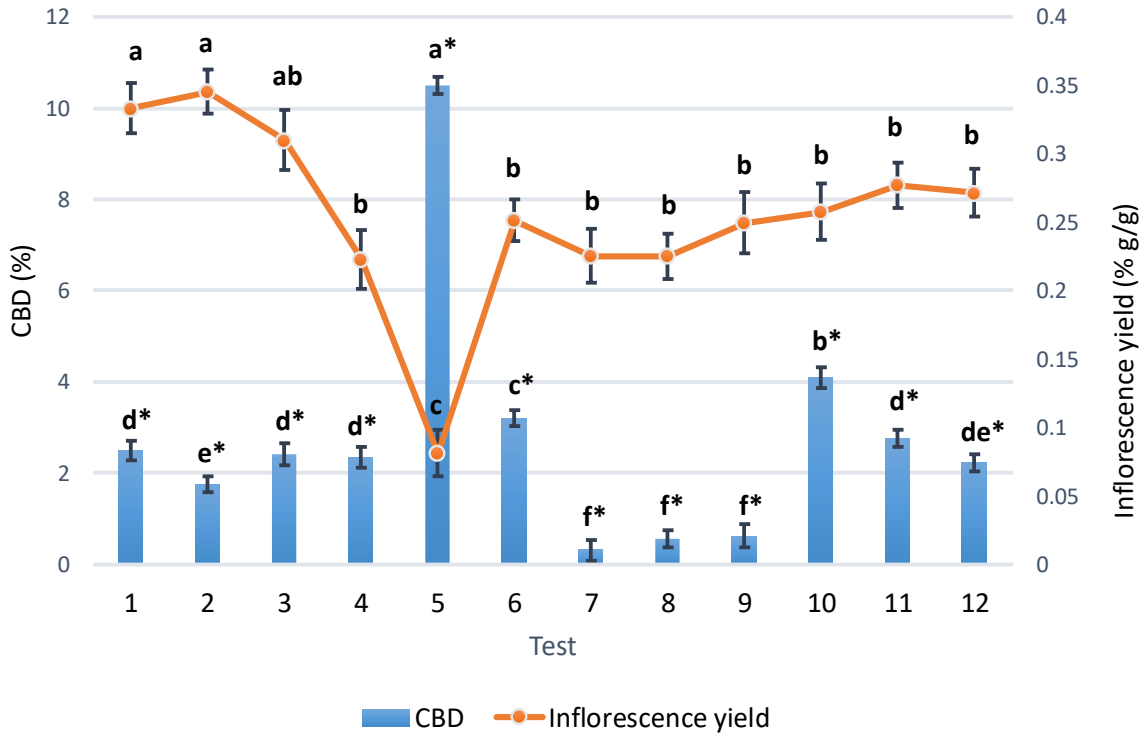


Figure 9. CBD trend and hydrodistillate yield expressed on dry inflorescence in MAHD: general outlook. Data of CBD in all performed tests are reported as percent area of GC-MS chromatograms. Values that are statistically different from each other ($p < 0.05$) are indicated with lower-case letters (a–c and a*–f* for yield and CBD, respectively).

MAHD provides efficient hydrodistillate recovery and good phytocannabinoid decarboxylation before residual matrix extraction with ethanol. Hence, the optimized protocol should maximize terpenoids yield and preserve CBD for the next step. The screening of different plant/water ratios allowed achieving the lowest CBD percent area at a 1/1 ratio (Test 1), while this significantly increased with liquid content (Test 10, Figure 10A). Liquid content reduction to 1/0.5 ratio led to a limited change in CBD area if compared to the 1/1 ratio. Fiorini et al.[73] observed a different trend. In particular, an inverse correlation between CBD percent area and water content was noted.

An even more pronounced increase in area of this cannabinoid was detected using 20% $NaCl_{aq}$ (Test 5), which gave the highest CBD percent area on the GC-MS chromatograms, with 10.51% vs. 2.49% and 1.75% using deionized and tap water, respectively (Figure 10B). This trend can be explained by the increase in the water boiling point, thus permitting the distillation of compounds with lower volatility. Changing the water-feed temperature did not noticeably alter the CBD area in the hydrodistillate, although there was a very slight decrease at 50 °C (Test 1 and Test 3, Figure 10C). On the other hand, the cannabinoid relative area was dramatically lower, namely 0.3, 0.55 and 0.62% for Tests 7, 8 and 9, when the hemp was placed in a CB. The other physical barrier used, a PEEK net placed above the matrix (Test 6), gave higher CBD percent area in the hydrodistillate, more precisely 3.20%. Plant material movement during the extraction (Test 4) did not affect the CBD area (Test 1). In Test 12, a fractionating Vigreux column was assembled to connect the extraction vessel with the distillation head. The Vigreux column permits volatile compounds to be separated by allowing the vapours to cool, condense and vaporize again. Every condensation-vaporization cycle enriches vapours in a certain component, and the larger surface area of the Vigreux column

allows more cycles to be performed.[77] Therefore, this set-up has the objective of distilling the low boiling point terpenes and separating them from the high boiling point cannabinoids. However, CBD percent area on the GC-MS chromatogram of the obtained volatile fraction was 2.21%, which is analogous with the result obtained in Test 1, which used a regular straight column.

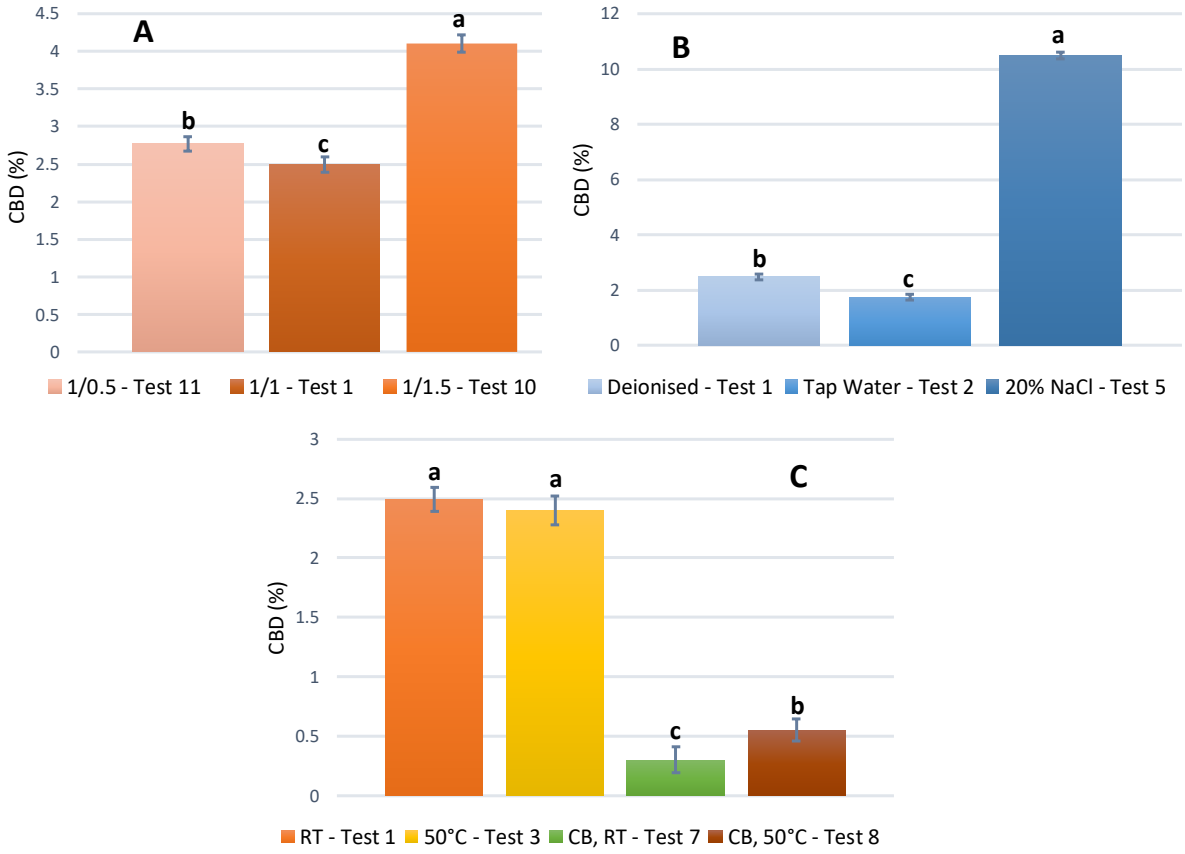


Figure 10. CBD trend for MAHD. A: water/matrix ratio. B: Type of water feed. C: RT/hot water (50 °C), CB application. CBD expressed as GC-MS percent area. Presented values followed by different lower-case letters (a–c) are significantly different from each other ($p < 0.05$).

The analytical data indicate that Test 2 gave the best results, allowing to the highest terpenes yield when performed with tap water, which is preferable on pilot and industrial scales. The volatiles yield expressed on the whole dry matrix was 0.24 ± 0.02% (g/g), which corresponded to 0.35 ± 0.02% (g/g) calculated in relation to the dry inflorescence. The effective cannabinoid content of the hydrodistillate, finally, was evaluated by means of UPLC-MS/MS. Results reported in Table 9. The cannabinoid content in inflorescence and hydrodistillate suggest a negligible depletion of the plant material from these metabolites.

When compared to CHD, MAHD Test 2 offered 3 times higher yield in significantly lower time. In addition, MAHD enabled faster distillation onset. In the case of CHD the terpenes extraction onset was heavily delayed due to the conventional conductive heating. These results confirm that process intensification occurred when MW was applied. As showed by GC-MS analyses, the percent area of CBD in the CHD volatile fraction was 23.83%, ergo about

[77] F. J. Zuiderweg, A. Harmens, *Chem. Eng. Sci.*, **1958**, *9*, 89–103.

14 times higher than in the volatile fraction derived from the optimal MAHD test. Fiorini et al.[73] likewise noted higher CBD yield in CHD volatile fraction, when compared to MAHD. The analysed CHD sample showed traces of THC as well, proving the harshness of the protocol.

MAHD volatiles analysis

The compounds contained in the sample that was obtained from the optimized MAHD Test 2 are listed in Table 11. They were identified by a GC-MS analysis using external standards or by comparison with the GC-MS libraries, finally matching 93.6% of the overall composition. Only compounds with libraries quality matching over 95% are reported. Moreover, the compounds mass fragmentation was revised in literature.[78] The quantity of each component in the sample is expressed as a percent composition of peak areas.

Table 11. Terpene fraction profile obtained in MAHD Test 2. GC-MS peak areas comparison.

Volatile fraction profile			
Compound	Area %	Compound	Area %
α-thujene[b]	0.32	α-Copaene[b]	0.19
α-pinene[a]	10.78	Z-caryophyllene[b]	0.66
camphene[a]	1.65	α-*trans*-bergamotene[b]	0.40
sabinene[b]	0.12	E-caryophyllene[a]	8.91
β-pinene[a]	4.09	β-farnesene[b]	1.82
β-myrcene[b]	6.74	α-humulene[a]	4.32
δ-3-carene[a]	3.55	β-patchoulene[b]	0.95
α-terpinene[a]	0.19	β-selinene[b]	4.22
o-cymene[b]	0.08	α-selinene[b]	2.88
limonene[a]	1.82	δ-cadinene[b]	1.78
1,8-cineole[b]	1.16	α-gurjunene[b]	2.46
β-ocimene[b]	7.02	aromadendrene[b]	2.65
γ-terpinene[a]	0.28	selina-3,7(11)-diene[b]	3.32
trans-sabinene hydrate[b]	0.14	nerolidol[a]	1.95
α-terpinolene[a]	2.55	germacrene B[b]	2.69
p-cymene[a]	0.04	caryophyllene oxide[b]	4.93
dehydro-linalool[a]	0.13	allo-aromadendrene[b]	0.54
cis-sabinene hydrate[b]	0.07	7-epi-α-selinene[b]	1.41
fenchol[a]	0.08	caryophylla-4(12),8(13)-diene-5-β-ol[b]	1.84
pinocarvone[b]	0.04	α-bisabolol[a]	0.94
borneol[a]	0.05	eudesm-7(11)-en-4-ol	0.29
terpinen-4-ol[b]	0.15	hexahydrofarnesyl acetone[b]	0.11
α-terpineol[a]	0.11	heptacosane[b]	0.02
n-tridecane[b]	0.06	nonacosane[b]	0.07
α-ylangene [b]	0.14		

[a] identified according to the standard compound;
[b] identified according to Wiley275 and NIST05 GC libraries (matching quality ≥ 95%).

[78] R. P. Adams, *Identification of essential oil components by gas chromatography/mass spectrometry*, 4 ed., Allured Publishing Corporation, Carol Stream, IL, USA, **2007**.

The main terpenoids are as follows: monoterpenes: α-pinene, β-myrcene, β-ocimene; and sesquiterpenes: E-caryophyllene, α-humulene, caryophyllene oxide and β-selinene. These are the compounds typically present in the volatile hydrodistillate of European *Cannabis sativa* L.[20] α-Pinene has a characteristic pine fragrance and exhibits antiseptic properties. β-Myrcene is characterized by a musky fragrance as well as antioxidant and chemo-protective effects. Caryophyllene has a peppery fragrance, and gastro-protective and anti-inflammatory biological activities.[21] Moreover, it is a Food and Drug Administration (FDA) approved food additive. Caryophyllene oxide is used as the marker compound for marijuana detection by trained dogs.[61]

Fiorini et al.[73] performed MAHD of *Cannabis* volatiles, obtaining a CBD enriched essential oil. The main components present in this oil were caryophyllene, CBD, α-humulene, α-pinene, caryophyllene oxide and myrcene. Therefore, the extracted terpenes composition is similar to Test 2 extract.

When compared to CHD, volatile fraction obtained in MAHD possess a great diversity of different terpenes, while CHD extract has a quite reduced variety. A much higher contribution of sesquiterpenes in CHD extract is observed. However, this highlights how for a vegetable matrix like *Cannabis*, which possesses a little essential oil content, better extractive yields in volatile compounds, such as terpenes and sesquiterpenes, can be obtained thanks to the action of unconventional techniques such as MW. MAHD allows to a process intensification shortening extraction time, thus avoiding the loss of more volatile compounds and secondary metabolite degradation. Therefore, terpene profile does not only depend on *Cannabis sativa* variety, growth stage and cultivation position, but also on the extraction method.

As already mentioned, the sample with a higher hydrodistillate yield (Test 2) was repeated in order to observe how the composition profile evolves during extraction (Test 13). The terpenic fraction was sampled six times after the distillation onset. After sampling, the florentine vase was thoroughly washed with acetone and water to avoid the remaining compounds interfering with the following sample. No significant changes in general terpene composition in relation to extraction time were noted. Nevertheless, the trend of the relative areas of the main monoterpenes (α-pinene, β-myrcene, β-ocimene) and sesquiterpenes (E-caryophyllene, α-humulene and caryophyllene oxide) was investigated (see Figure 11) at each sampling time. For the sake of comparison, percentage compositions were normalized exclusively in relation to the abovementioned compounds.

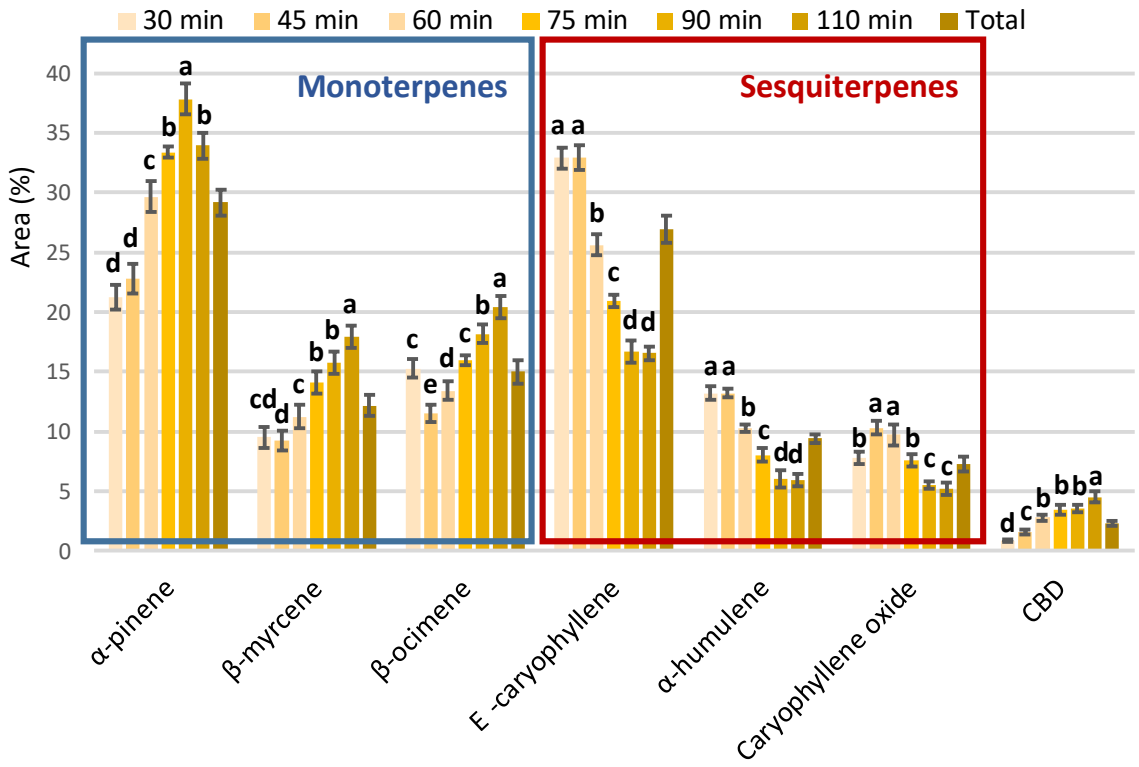


Figure 11. Test 13 MAHD sampling: main terpenes composition distribution. CBD relative area variation in time. Both main terpenes and CBD are expressed as relative peak areas obtained by GC-MS quantification. The main terpenes values are normalized on the reported compounds. Presented values followed by different lower-case letters (a–d) are significantly different from each other ($p < 0.05$). Statistical analysis of the total united samples was not depicted on this Figure, since herein comparison of specific fractions was performed.

On average the monoterpenes relative area constantly increased as extraction progressed, while the E-caryophyllene, α-humulene and caryophyllene oxide area decrease; during extraction, monoterpene area overtakes the decreasing sesquiterpene percent area. Nevertheless, lighter terpenes were the most abundant compounds in all of the analysed samples. Subsequently, all of the tests were united and analysed by GC-MS to verify the overall composition and efficiency of the sampling. The prevalent terpenes were found to be quite comparable with the terpenes from the volatile fraction that was obtained in the optimal MAHD test. The decreasing trend of sesquiterpene amount during extraction may be related to the progressive depletion of the matrix, as the lower quantity of these compounds in hemp inflorescences is well known.[79,80] Monoterpenes, which are usually predominant, have an even more pronounced presence in the extracted matrix, due to the post-harvesting strategies. CBD, whose percent area is shown for every sample in Figure 11, was found to be present across all the sampling times, with correlated percent area changes during extraction. The reported plot shows a gradual increase in CBD area over extraction time. When the

[79] O. Aizpurua-Olaizola, U. Soydaner, E. Öztürk, D. Schibano, Y. Simsir, P. Navarro, N. Etxebarria, A. Usobiaga, *J. Nat. Prod.*, **2016**, *79*, 324–331.
[80] J. K. Booth, J. E. Page, J. Bohlmann, *PLoS One*, **2017**, *12*, 1–20.

sampled fractions were combined, the CBD area was 2.29%, which is comparable to the optimal MAHD test.

MAHD polyphenols rich water

The water added to the *Cannabis* plant material before the extraction was, in the most cases, around 2.5 L in quantity. As is already known, MW assisted solid/liquid extraction is widely used in the field of green extraction.[46] As mentioned in this chapter, polyphenols are some of today's most interesting phytochemicals.[81] The MAE of polyphenols has been described in many papers. Teh et al.[82] have investigated the use of MW as a prior step to US-assisted polyphenol extraction from defatted hemp seed cake. The results have shown that the irradiation of this residue can enhance the metabolites extraction, maximizing the polyphenols yield. Drinić et al.[62] unified the aforementioned approaches, optimizing hemp phenols, flavonoids, and phytocannabinoids MAE in ethanol. In this case, MAE was found to be a simple, fast, and efficient extractive method for the cited classes of metabolites, preserving at the same time a high antioxidant activity.

Therefore, in present work, the liquid fraction after the optimized MAHD Test 2 was analysed to evaluate its TPC. This value estimated using the Folin–Ciocalteu test, was found to be 1.49 mg polyphenols/g matrix and 53.54 mg polyphenols/g extract. Hence, the obtained extract contained 5.35%, g/g of polyphenols.

The solid-phase extraction (SPE) was used to purify polyphenolic water fraction obtained from optimized MAHD for the sake of analysis. The concentrated polyphenol fraction yield reached 10.31%, g/g, calculated in relation to the dry raw extract. The process led to an overall TPC content of 51.71%, g/g, calculated on dry purified sample, achieving a nearly 10-fold metabolite concentration, in respect to the raw dry extract. Literature suggests that the prevailing polyphenols in *Cannabis sativa* L. are flavonoids.[83,84] Nevertheless, phenolic acids have also been detected in *Cannabis* plant.[85] Therefore, the polyphenols belonging to the aforesaid classes in the enriched sample was analysed by HPLC-DAD. The polyphenols enriched sample was analysed by HPLC-DAD, and the two main peaks were identified belonging to flavones: luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, with an amount of 2.84%, g/g and 2.58%, g/g, respectively, when calculated in relation to the dry purified water fraction. Moreover, the absorption spectrum of each compound detected was thoroughly revised. In particular, six signals, besides luteolin and apigenin glucosides, were detected at 340 nm and featured a spectrum that is characteristic for flavones. Unfortunately, the lack of specific standards means that identification and quantification were impossible. Other flavonoids and phenolic acids were not detected. Nevertheless, flavones were found as main polyphenols in low-THC *Cannabis* cultivars.[20]

Thanks to their additive nutritional value, flavones have received increased attention in recent years. Their main activity is their ability to scavenge oxygen species that contain free radicals that cause oxidative stress.[86] Moreover, their beneficial effects on the prevention of

[81] G. Cravotto, F. Mariatti, V. Gunjevic, M. Secondo, M. Villa, J. Parolin, G. Cavaglià, *Foods*, **2018**, *7*, 1–14.
[82] S. S. Teh, B. E. Niven, A. E. D. A. Bekhit, A. Carne, E.J. Birch, *Food Bioprocess Technol.*, **2014**, *7*, 3064–3076.
[83] H. Koltai, D. Namdar, *Trends Plant Sci.*, **2020**, *25*, 976–984.
[84] D. U. Nagy, K. Cianfaglione, F. Maggi, S. Sut, S. Dall'Acqua, *Chem. Biodivers.*, **2019**, 16, e1800562.
[85] L. Izzo, L. Castaldo, A. Narváez, G. Graziani, A. Gaspari, A., Y. Rodríguez-Carrasco, A. Ritieni, *Molecules*, **2020**, *25*, 631.
[86] N. Jiang, A. I. Doseff, E. Grotewold, *Plants*, **2016**, *5*, 1–1256.

cardiovascular, cerebrovascular and some other chronic diseases, such as asthma, cataracts, diabetes and rheumatoid arthritis, have been reported.[87] This by-product of MAHD can be considered as a cheap source for the isolation of some of these natural compounds, bringing additional value to the overall process.

Cannabinoids extraction in MAHG

Reflux is the conventional extraction method to recover cannabinoids from *Cannabis* inflorescence.[23,88] It entails a long extraction time and ineffective conductive heating. Moreover, it requires ethanol, a widely used but potentially flammable solvent. A preliminary MAHG test was performed to evaluate the possibility of overcoming the disadvantages of the classical extraction approach. Unfortunately, the fresh inflorescence MAHG gave a dry extract of 4.65% g/g on dry inflorescence, with CBD and THC content of 0.0085% and 0.0017%, that corresponds to an extraction yield of 0.01% and 0.04%, respectively. This result is therefore quite incomparable with the conventional extraction yield, meaning that MAHG could not be considered as a suitable alternative for cannabinoids extraction.

2.2.2. Conclusions

More than 2.5 kg of *Cannabis* plant material was efficiently processed by MAHD in a 12 L vessel, allowing to hydrodistillate yield of 0.35% g/g, expressed in relation to dry inflorescence mass. The extract was extremely rich in the characteristic *Cannabis* terpenes: α-pinene, β-myrcene, β-ocimene, E-caryophyllene, α-humulene, caryophyllene oxide and β-selinene. Furthermore, the absence of solvents strengthens the sustainability of the whole process, as benign by design. Sampling performed during the distillation highlighted a progressive enrichment in monoterpenes and a decrease in sesquiterpene extraction efficiency during the process. The terpene yield and profile from MAHD were compared to those obtained from CHD, for which the oil amount was only 0.12%, g/g in relation to inflorescence, also having a significantly different volatiles fingerprint.

The residual *Cannabis* biomass was still rich in phytocannabinoids, which mainly results decarboxylated after the irradiation (69.01% for THC and 74.32% for CBD). Hence, residual hemp, unaltered from MAHD protocol, is suitable for subsequent cannabinoid recovery.

Furthermore, water resulting from MAHD provided a TPC in the dry water extract of 5.35%, g/g. The two main metabolites, namely luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, were identified and quantified by means of HPLC-DAD.

In conclusion, *Cannabis* terpenes were extracted from the inflorescence by means of MW irradiation in water-only media. Polyphenolic rich by-product has also been valorised, and an active enriched fraction was obtained. Moreover, the phytocannabinoids CBD and THC in the optimised hydrodistillate have been thoroughly evaluated and the decarboxylation effects of the explored technique have been defined. Further investigations should be performed to more completely describe the features of MAHG in hemp treatment, which has so far revealed limited cannabinoids recovery. Taken together, the results reported in this study draw attention to the use of MAE in extraction from *Cannabis*, and have proven the versatility and

[87] B. A. Graf, P. E. Milbury, J. B. Blumberg, *J. Med. Food*, **2005**, *8*, 281–290.
[88] J. Baranauskaite, M. Marksa, L. Ivanauskas, K. Vitkevicius, M. Liaudanskas, V. Skyrius, A. Baranauskas, *Phytochem. Anal.*, **2020**, *31*, 516–521.

efficiency of this technique. All tests were performed in a scaled-up MW reactor, thus encouraging the further industrial development of the proposed cascade extraction process.

2.2.3. Experimental part

Plant material

The plant material studied was *Cannabis sativa* L. cv. Monoica, and was kindly provided by the company Egeria s.r.l. (Milano, Italy). The matrix was collected in the middle of September 2019 at the fields of Azienda Agricola Prina Pietro (Pavia, Italy, N 45°13'10.3", E 9°11'22.1", 2.7 ha) and was in a 8.7 phenological growth stage (60% ripe fruit). After collection, the fresh plant material was vacuum packed (see Figure 12) and stored at -18 °C. In all experiments, the plant material was used without defrosting.

Figure 12. Plant material storage under vacuum.

Inflorescence content in plant material

The plant material collected contained inflorescences, leaves and stalks. 1 Kg of Cannabis was thoroughly selected and weighed in order to obtain the ratio between the inflorescence and the other components of the matrix.

Water content in plant material

The water content in frozen *Cannabis* was determined using the gravimetric method. Plant material was sampled in triplicate from 1 kg frozen bag and dried in a furnace muffler (Gelman Instrument Company, USA) at 100 °C for 24 h.

Volatiles extraction

CHD

CHD was performed with the aim to compare the efficiency of MAHD terpene extraction to a conventionally applied procedure. It was carried out according to the essential oils

extraction methods described in European Pharmacopoeia[89] with few modifications due to the equipment limitations. The *Cannabis* was placed in a 2 L round bottom flask and deionized water was added at a solid/liquid ratio of 1 to 5. The round bottom flask was placed inside a heating mantle, whilst a Clevenger-type apparatus and a refrigerant were assembled. The extraction time was 4 h. The recovered hydrodistillate yield and composition was compared to the ones obtained in MAHD tests.

MAHD

The terpene fraction from the *Cannabis* was recovered using MAHD. It was performed in an ETHOS X (Milestone s.r.l., Italy), a multimode MW reactor, at a maximum delivered power of 1800 W (Figure 13). All extractions were performed in a 12 L vessel. The temperature was monitored using an infrared sensor. The MW power during the extractions was set as follows: 500 W for 3 min, 1100 W for 3 min, 1600 W for 14 min and finally 1500 W for 90 min. The overall time, necessary to complete volatile compound extraction, was then 1 h and 50 min.

Figure 13. ETHOS X, MAHD set-up.

Twelve tests were performed under different conditions. 2.5 to 2.8 kg of matrix were extracted in all tests. The plant was always placed evenly in the extraction vessel directly from the freezer. Even though the *Cannabis* was fresh and still hydrated, supplementary water was placed in the extraction vessel together with the material prior the extraction to enhance the steam formation. All sources of water (added and contained in the plant) were heated during the extraction, generating steam that releases the terpenes from the *Cannabis* inflorescence and carries them to the distillation head. The extractions were performed with matrix-to-liquid ratios of 1/0.5 to 1/1.5 (kg/L). Moreover, the use of tap water, deionized water and a NaCl solution (20%) was tested. Once the vessel was filled with plant material and water, it was placed into the MW cavity of the reactor. The distillation head was assembled with a florentine vase and the extraction process was started. As the terpenes are distilled together

[89] Council of Europe, *European Pharmacopeia* 7.0, 8th ed., Strasbourg, Germany, **2013**.

with a large amount of liquid, the water was able to recirculate from the florentine vase back into the vessel.

Table 8 reports the mass of extracted *Cannabis*, the plant-material-to-water-ratio and water feed used in every test, as well as the equipment and method alterations made to the processes. As reported in Table 8, additional alterations were made for some tests. In Tests 7, 8 and 9, the plant material was placed in a CB during extraction. A PEEK net was placed above the matrix in Test 6. Both the CB and net were used to homogenize the re-circulated water distribution and to enhance overall wetness during extraction, thus helping to prevent the browning effect and potential degradation. In Test 4, the plant material was moved every 30 min during extraction. Hot water (50 °C) was added at the beginning of Test 3 to hasten the onset of terpene distillation. In Test 12, a fractionating Vigreux column (20 cm length) was assembled to connect the MW cavity and the distillation head, instead of the regular straight column, in order to investigate the variation in the volatiles fingerprint. Finally, in Test 13, the sampling of the recovered terpenes was performed every 15 min to follow changes in terpene profile with extraction time.

Once the run was completed, the terpenes fraction was recovered from the florentine vase of the MW system. The extracted terpenes are not miscible with water and hence can be found as the lighter oily phase above the water column. Avery run was performed in triplicate and the mass of the obtained volatile fraction was noted for every extraction and expressed as average ± S.D. Yield of the volatile fraction was calculated both in relation to dry matrix and on dry inflorescences. The volatile fraction was then analysed using GC-MS. The CBD and THC quantitative analysis of the extract that was obtained in the optimal MAHD test was performed using UPLC-MS/MS.

Hydrodistillate analysis

The GC-MS qualitative analyses of the volatile fractions obtained with MAHD and CHD were performed in an Agilent Technologies 6850 Network GC System fitted with a 5973 Network Mass Selective Detector, 7683B Automatic Sampler and a capillary column Mega 5MS (length 30 m; i.d. 0.25 mm; film thickness 0.25 µm, Mega S.r.l., Italy). The injector and detector temperature were set at 250 and 280 °C, respectively. The temperature program started at 50 °C for 5 min, increasing to 100 °C at 10 °C/min, afterwards increasing to 230 °C at 20 °C/min and, finally, to 300 °C at 20 °C/min, which was held for 10 min. The carrier gas was helium at an initial flow rate of 1.2 mL/min and a constant pressure of 9.78 psi. For the analysis, 1 µL of essential oil was diluted in hexane (1:10, v/v) and injected in split mode (1:20). The identification of the individual compounds was performed with two approaches: 1) by comparing the retention time e mass spectrum with standard compounds form Cannabis Terpene Mix A and Cannabis Terpene Mix B, 2) by using GC-MS Wiley275 and NIST05 GC libraries from the acquired chromatograms, considering only matching qualities over 95%. The summed molar areas of the relevant peaks were normalized to 100%. Relative peak areas, calculated as percentages, were used to evaluate extract composition. The retention times and mass fragmentation spectrum for every compound detected are reported in *Supporting materials* section.

MAHD polyphenols rich water

Due to the addition of an abundant amount of water to the plant material before MAHD, there is a significant amount of liquid remaining in the extraction vessel after the process. The

aqueous fraction was filtered, freeze dried (LyoQuest – 85 lyophilizer, Azbil Telstar Technologies, Spain) and analysed in terms of dry extract yield and TPC.

Total polyphenolic content determination

TPC in the water fraction after MAHD was determined by Folin–Ciocalteu test as described in *2.1. Green extraction technologies for cocoa been shell polyphenols recovery* paragraph. TPC was expressed as gallic acid equivalents (GAE, mg/g) over the dried extract and gallic acid equivalents (GAE, mg/g) over the dried matrix. All analyses were performed in triplicate.

Polyphenols enrichment

Polyphenols from the water fraction after the optimal MAHD protocol were enriched using SPE on a C18 Sep-Pak cartridge (Waters, USA). 50 mg of sample were dissolved in 1 mL 0.5% AcOHaq. After bonded phase activation using MeOH and 0.5% AcOHaq, sequentially, the sample solution was loaded onto the cartridge. Fraction 1, which mostly contains low activity metabolites (likely sugars and carbohydrates), was eluted using 0.5% AcOH. The polyphenol-enriched fraction (fraction 2) was desorbed using 80% $MeOH_{aq}$. Finally, fraction 3 was eluted using pure MeOH. The amount of solvents used for elution in all cases was 3 bead volumes. Fraction 2 was analysed using HPLC-DAD for polyphenols.

HPLC polyphenols analysis

Polyphenols, in particular luetolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, catechin, epicatecthin, chlorogenic acid, caffeic acid, and quercetin-3-*O*-glucoside, were identified and quantified using a HPLC system (Waters Corp., USA) coupled with a diode array detector (UV/DAD, Waters Corp., USA) and an automatic sampler (Waters Corp., USA). Separation was achieved on a Synergi Hydro RP C18 column as reported in *2.1. Green extraction technologies for cocoa been shell polyphenols recovery* part. UV-DAD acquisitions were carried out in 200–600 nm range, while chromatograms were acquired at 340 nm. Three injections were performed for each sample. The retention time, absorption maximum, λ used for detection, calibration line, linearity range, linear equation fit, LOD and LOQ are reported for every analysed polyphenol in the *Supporting materials* section.

Phytocannabinoids extraction

Phytocannabinoids were extracted both from the fresh matrix and from the depleted biomass in the optimal MAHD process. Cannabinoid extraction from fresh plant material was performed for two purposes. The first objective was the fresh plant determination of CBD and THC content. The second target was to provide a control parameter for cannabinoid decarboxylation after MAHD. Together with the conventional benchmark, a MW-assisted protocol, MAHG, was tested on the fresh plant to investigate the technique's phytocannabinoid-recovery efficiency. The *Cannabis* inflorescence was separated from the rest of the plant in every extraction.

Conventional extraction under reflux

For analytical purposes, conventional reflux cannabinoid extractions were performed using ethanol (99%) as the solvent. The extraction time was 2.5 h and the solid-to-liquid ratio was 1 to 10. The obtained extract was filtered and the ethanol was evaporated. Moreover, two extractions were performed for every sample to evaluate the decarboxylation efficiency

of the MAHD extraction method. In one of these extractions, the *Cannabis* inflorescence was placed in a furnace muffler for 30 min at 120 °C before extraction to promote cannabinoid decarboxylation, while this step was skipped in the other extraction. For every obtained extract, the yield was noted and the CBD and THC contents were evaluated.

MAHG

MAHG was also performed in the ETHOS X MW reactor, but using a different system configuration in which the extract was recovered in the flask placed under the reactor (see Figure 14). The frozen Cannabis (200 g) was placed evenly in the 5 L extraction vessel, which was housed in the MW cavity of the reactor. The condensation system and the collection flask were assembled from the bottom of the device. Thanks to the opening on the top of the MW cavity, steam can be introduced into the system and thus enhance the extraction efficiency thanks to the continuous stripping and water supply to the plant. The extraction method provided a continuous irradiation of 200 W for 60 min. Steam was fed into the vessel for 30 s every 5 min. The temperature was monitored with an infrared sensor and never exceeded 100 °C. During the extraction, the extract was continuously collected in the receiving flask. Once the process was completed, the collected extract was freeze-dried and analysed for its extraction yield, and CBD and THC content.

Figure 14. ETHOS X, MAHG set-up.

UPLC-MS/MS phytocannabinoids analysis

Quantitative determination of phytocannabinoids CBD and THC was carried out on a Waters Acquity TQD UPLC system, Using a Waters BEH C18 (2.1x50, 1.7µ) column, 0.1% water solution of formic acid and 0.1% acetonitrile solution of formic acid ad eluents (A and B respectively). The elution was conducted with the following gradient: (min, B%) 0,50; 0.44,50; 3.04, 100; 4.77, 100. The flow rate was set at 0.4 mL/min. The injected sample was tempered at 15 °C and injection volume was 5 µL. The column temperature was set at 40 °C. Ionization type was APCi+ (ESCI source) with following conditions: corona 2.6uA, cone 30 V, source temp. 120 °C, solvation temp. 220 °C, solvation flow 500 L/h, con flow 50 L/h, collision gas flow 0.20 mL/min, collision voltage 28 V. The samples were injected in pure ethanol (≥ 99.9%). The detection was carried out in MS/MS mode on the transition 315->193 for quantification and using the transition 315->135 as qualitative control. The retention time, linear equation fit,

LOD and LOQ are reported for THC and CBD in the *Supporting materials* section. Each sample was divided in two specimens: the first was directly analysed whilst the second was firstly decarboxylated in a furnace. THCA and CBDA were quantified as difference between cannabinoids detected in the two specimens.

Statistical analysis

Statistical analysis was performed as described in *2.1. Green extraction technologies for cocoa been shell polyphenols recovery* part.

2.3. *Salacia impressifolia* (Miers) A. C. Sm. roots anti-parasitic compounds recovered by supercritical CO_2 extraction

Numerous ancient civilisations used plants for biological, pharmacological and medical purposes. In addition, a notable portion of drugs used nowadays originate from medical plants. Together with the increasing interest of general public in phytochemistry, ethnopharmacology is gaining growing attention in Europe. Ethnopharmacology is a relatively new filed of research, although it has strong bases in traditional medicine history. Great contribution to this scientific filed comes from China, India, Southeast Asia and several South and Central American countries.[90,91]

Salacia impressifolia (Miers) A. C. Sm., also known as Panu or Chuchuasi, is used in traditional medicine of Tacana community based in Bolivia, to treat bones and joints pain, inflammation, respiratory issues and male impotence in older adults.[92,93] In the last decade, *S. impressifolia* was investigated for several biological activities, such as anti-tumour (human leukemia and melanoma), anti-fungal, anti-parasitic (nematode and malaria) and anti-diabetic effects.[94,95,96,97,98]

Conventional extraction methodologies, in particular maceration and Soxhlet extraction are usually used for medicinal plants bioactive compounds extraction. These technologies possess already known limitations, such as long extraction time and use of VOCs hazardous for environment and humans.[99] As an alternative for non-polar lipophilic compounds recovery SCO_2E can be an ideal procedure due to low dielectric constant of supercritical CO_2. Thanks to low extraction temperature and the absence of light and oxygen, biological activities of the extracted compounds can be preserved. In addition, SCO_2E yields in a safe extract free of organic solvents and microorganisms.[100] Therefore, in this work, SCO_2E was used for *S. impressifolia* roots extraction. Periodical sampling during the extraction was performed to

[90] A. W. K. Yeung, M. Heinrich, A. G. Atansaov, *Front. Pharmacol.*, **2018**, *9*, 215.
[91] A. H. Gilani, Atta-ur-Rahman, *J. Ethnopharmacol.*, **2005**, *100*, 43–49.
[92] A. Giménez, E. Udaeta, N. Nina, J.C. Ticona, E. Salamanca, I. Limachi, N. Flores, C. Paredes, A. Serato, N. Marupa, B. Chao, *Saberes tradicionales tacana y actividad biológica*, Universidad mayor de San Andres, La Paz, Bolivia, **2020**.
[93] D. Arévalo López, *Evaluacion in vitro de biomoleculas naturales y sinteticas sobre cultivos de kinetoplastidos-Leishmania y Trypanosoma*, Universidad mayor de San Andres, La Paz, Bolivia, **2017**.
[94] E. S. P. Aranha, *Efeito antitumoral de 22ϐ-hidroxitingenona obtida de Salacia impressifolia (Miers) A.C. (Celastraceae) contra células de melanoma humano*, Universidade Federal do Amazonas, Manaus, Brasil, **2020**.
[95] M. M. Laikowski, *Avaliação dos principais metabólicos secundários por espectrometria de massas e atividade hipoglicêmica de Salacia impressifolia Miers A. C. Smith*, Universidade de Caxias do Sul, Caxias do Sul, Brasil, **2015**.
[96] L. D. R. Acho, *Avaliação da atividade antidiabética de extratos das cascas de Salacia impressifolia*, Universidade Federal do Amazonas, Manaus, Brasil, **2018**.
[97] A. C. B. da C. Rodrigues, F. P. de Oliveira, R. B. Dias, C. B.S. Sales, C. A. G. Rocha, M. B. P. Soares, E. V. Costa, F. M. A. da Silva, W. C. Rocha, H. H. F. Koolen, D. P. Bezerr, *J. Ethnopharmacol.*, **2019**, *231*, 516–524.
[98] M. R. P. S. Soares, C. A. Caneschi, M. das G. A. M. Chaves, M. Mota, P. H. F. Stroppa, W. Barbosa, N. R. B. Raposo, *Afr. J. Tradit. Complement. Altern. Med.*, **2018**, *15(4)*, 13–21.
[99] N. N. Azwanida, *Med. Aromat. Plants*, **2015**, *4(3)*, 1000196.
[100] B. Diaz-Reinoso, A. S. Moure, H. Dominguez, J. C. Parajo, *J. Agric. Food Chem.*, **2006**, *54*, 2441−2469.

monitor extracts composition. The extracts yield and composition was compared to the conventional extract obtained in Soxhlet procedure with CH_2Cl_2 as solvent. The obtained extracts were additionally purified by liquid-liquid extraction, size exclusion chromatography and silica gel chromatography.

S. impressifolia secondary metabolites consist in triterpenoids, sesquiterpenes, flavonoids and phenolic acids.[101] However, the roots of this medicinal plant are rich in triterpenoids.[102] Triterpenes are a part of widespread trepenoid family. These compounds have up to date been reviewed for their anti-cancer, anti-inflammatory, antioxidant, anti-viral, anti-bacterial and anti-fungal properties.[103] Nevertheless, they are unique for possessing strong anti-parasitic activity. Parasitic infections are still one of the leading health problems causing high mortality and economic issues, especially in developing countries.[104] Hence, this study focuses on evaluating the obtained extracts activity against two parasites: *Leishmania amazonensis* and *Trypanosma cruzi*. Up to date, the number of drugs for leishmaniasis treating is quite scarce, suggesting the neglectance of this tropical disease. Treatments currently available, besides being scarce, exhibit several limitations such as toxicity, cost, access, difficulties in obtaining oral formulations, and/or growing drug resistance and treatment failure.[105,106] Therefore, there is a need for new drugs that could meet current requirements. Chagas disease, caused by parasite *Trypanosoma cruzi*, is a cause of most hearth failure cases in Latin America. This disease is, as leishmaniasis, considered a neglected tropical disease. There is still a need for effective and safe treatment.[107]

2.3.1. Results and discussion

Soxhlet extraction

Conventional exhaustive *S. impressifolia* root extraction, lasting for 12 h, yielded in 1.12% (g/g) of extract.

Supercritical CO_2 extraction

SCO_2E of *S. impressifolia* roots gave a yield of 1.09% (g/g). It was performed for 6 h and every hour sampling was performed. The samples and the conventional extracts composition is evaluated on TLC (Figure 15).

[101] F. M. A. da Silva, W. H. P. Paz, L. S. F. Vasconcelos, A. L. B. da Silva, F. A. da Silva-Filho, R. A. de Almeida, A. D. L. de Souza, M. L. B. Pinheiro, H. H. F. Koolen, *Biochem. Syst. Ecol.*, **2016**, *68*, 77–80.
[102] W. H. P. Paz, F. M. A. da Silva, F. A. da Silva-Filho, R. A. de Almeida, M. L. B. Pinheiro, H. H. F. Koolen, *Chem. Nat. Compd.*, **2018**, *54*, 200-201.
[103] M. Chudzik, I. Korzonek-Szlacheta, W. Król, *Molecules*, **2015**, *20*, 1610-1625.
[104] M. B. Isah, M. A. Ibrahim, A. Mohammed, A. B. Aliyu, B. Masola, T. H. T. Coetzer, *Parasitology*, **2016**, *143*, 1219–1231.
[105] A. Hefnawy, M. Berg, J. C. Dujardin, G. De Muylder, *Trends Parasitol.*, **2017**, *33*, 162–174.
[106] A. Ponte-Sucre, F. Gamarro, J. C. Dujardin, M. P. Barrett, R. López-Vélez, R. García-Hernández, A. W. Pountain, R. Mwenechanya, B. Papadopoulou, *PLoS Negl. Trop. Dis.*, **2017**, *11*, 1–24.
[107] L. E. Echeverria, C. A. Morillo, *Infect. Dis. Clin. North Am.*, **2019**, *33*, 119–134.

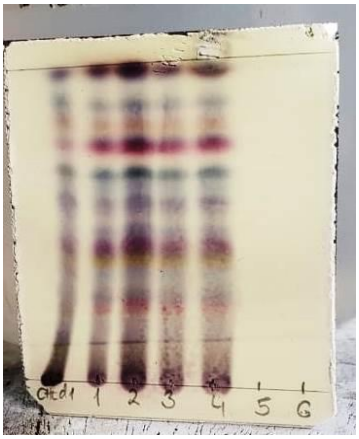


Figure 15. TLC of conventional extract and samples obtained in SCO_2E by periodical sampling. First line represents conventional extract and the following lines show SCO_2E samples.

The performed TLC indicates that there are no major differences in composition of different SCO_2E samples. Therefore, all the obtained SCO_2E samples were unified. In addition, SCO_2E samples shown a similar composition to the conventional Soxhlet extract. Herein is demonstrated how this green extraction methodology can intensify the extraction process by offering a 2' fold reduction in extraction time. Moreover, SCO_2E did not affect the extracts composition, and it provided an extract with unmodified quality. SCO_2E, being performed at relatively low temperature, prevents thermo-labile compounds degradation.[108,109] Nowadays, SCO_2E is considered as innovative green alternative for medicinal plants extraction since it yields in extract free of harmful organic solvents at a relatively low investment cost.[99] Castola et al.[110] compared *Quercus suber* L. conventional CH_2Cl_2 and SCO_2E triterpene rich extracts composition. The extraction yields were comparable, however, SCO_2E, besides triterpenes, enabled sterols extraction.

Both obtained extracts were fractionated. The obtained extracts and fractions were evaluated for anti-parasitic activity.

Extracts fractionation

Since complex extracts sometimes do not show a specific biological activity, extracts purification was performed aiming to obtain fractions that could yield in greater anti-parasitic activity. Since the conventional and non-conventional extract have a comparable composition, the attempts on anti-parasitic fractions purification were performed for both extracts. Four fractionation methods were performed: liquid-liquid extraction with different organic solvents, size exclusion chromatography and liquid-liquid extraction with NaOH for the conventional extract, and silica gel chromatography for the SCO_2E extract. The efficiency of the extract purification was monitored by TLC.

<u>Liquid-liquid extraction with organic solvents</u>

Liquid-liquid extraction using various organic solvents was performed aiming to separate the extracts lipophilic compounds based on their polarity. The extract dissolved in 80% aqueous MeOH was extracted with ether, obtaining ether phase (EtOP) that should contain

[108] K. Y. Khaw, M. O. Parat, P. N. Shaw, J. R. Falconer, *Molecules*, **2017**, *22*, 1186.
[109] N. Polikhronidi, R. Batyrova, A. Aliev, I. Abdulagatov, *J. Therm. Sci.*, **2019**, *28*, 394-430.
[110] V. Castola, B. Marongiu, A. Bighelli, C. Floris, A. Lai, J. Casanova, *Ind. Crop. Prod.*, **2005**, *21*, 65–69.

the most lipophilic compounds. MeOH was then evaporated and the remaining aqueous phase was extracted with CH_2Cl_2, obtaining CH_2Cl_2 phase (DiOP). The aqueous phase was once again extracted with EtOAc yielding in EtOAc phase (EAOP) with the least lipophilic compounds. The fractionation efficiency was evaluated by TLC (Figure 16). On Figure 17 are depicted yields for every fraction.

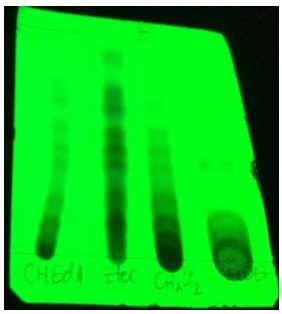


Figure 16. TLC analysis of fractions obtained from liquid-liquid extraction at 254 nm (samples are shown in following order: raw extract, EtOP, DiOP, EAOP).

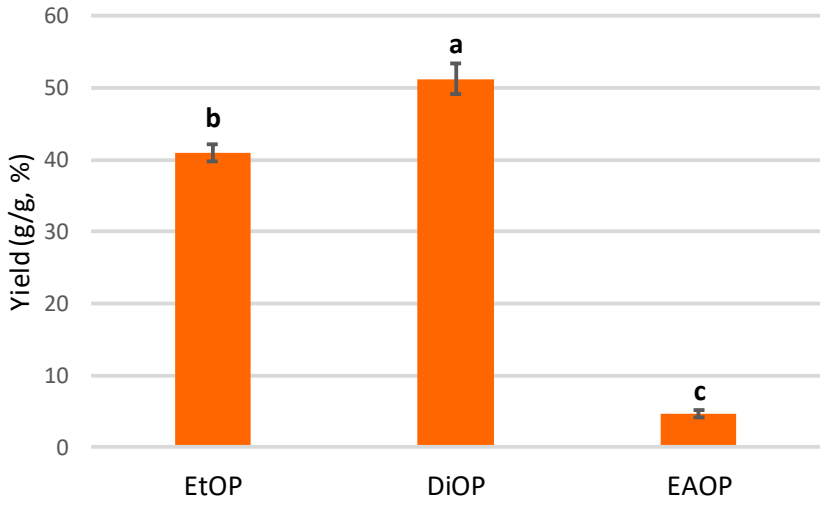


Figure 17. Liquid-liquid extraction fraction yields. Presented values followed by different lower-case letters (a–c) are significantly different from each other ($p < 0.05$).

TLC analysis shown a difference in the fractions composition. DiOP has the most similar composition to the raw extract. Nevertheless, this result was expected since the raw extract was prepared in CH_2Cl_2. EtOP and DiOP fractions had the highest yield. Anti-parasitic activity of the obtained extract was evaluated.

Size exclusion chromatography (SEC)

To achieve extracts compounds separation based on their molecular weight, SEC was performed. The fractions were collected in test tubes. The samples were analysed on TLC and unified based on their composition. Finally, 5 fractions were obtained (Figure 18). The fractions yields are reported in Figure 19.

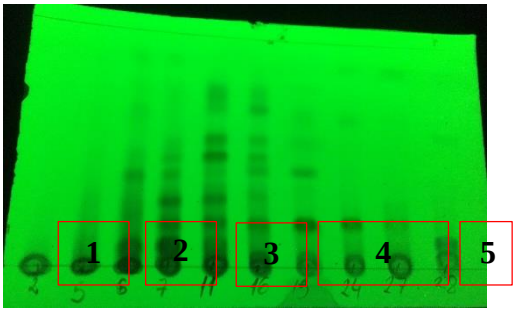


Figure 18. TLC analysis of fractions obtained from SEC (the specific fraction is indicated with the numbers 1-5).

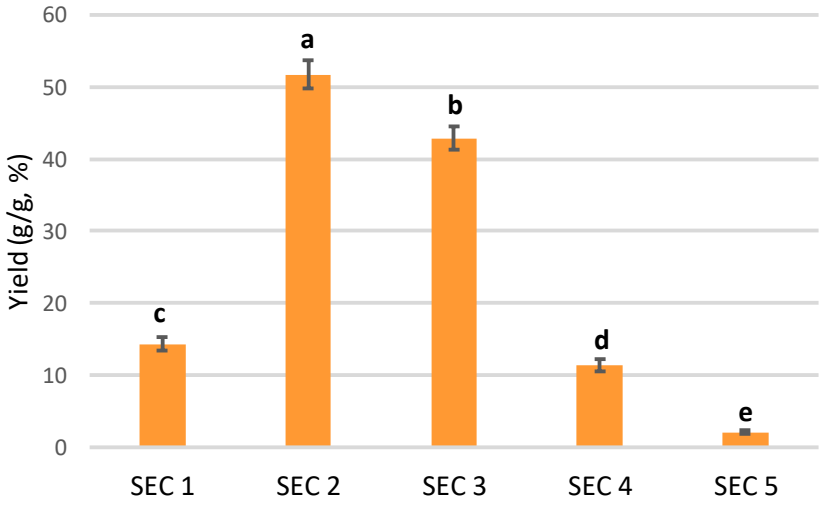


Figure 19. SEC fractions yield. Presented values followed by different lower-case letters (a–e) are significantly different from each other ($p < 0.05$).

The fractionation was quite successful. Fraction 3 has the widest range of the lipophilic compounds. Fractions SEC2 and SEC3 had the highest yield. Anti-parasitic activity for fractions 2 to 5 was evaluated.

Silica gel column chromatography

To fractionate different SCO_2E extracts compounds based on their polarity, chromatography over silica gel was performed. Six different eluents were used, with gradually increasing polarity. The fractions were analysed on TLC (Figure 20) and the yields for every fraction are shown on Figure 21.

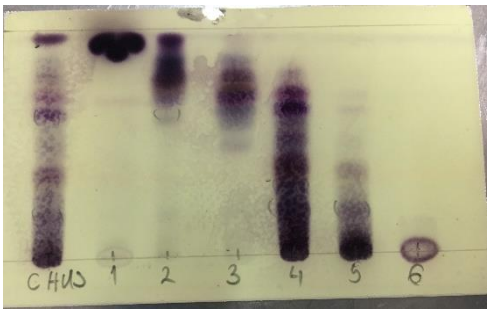


Figure 20. TLC analysis of SGC fractions (samples are shown in following order: raw extract, SGC fractions).

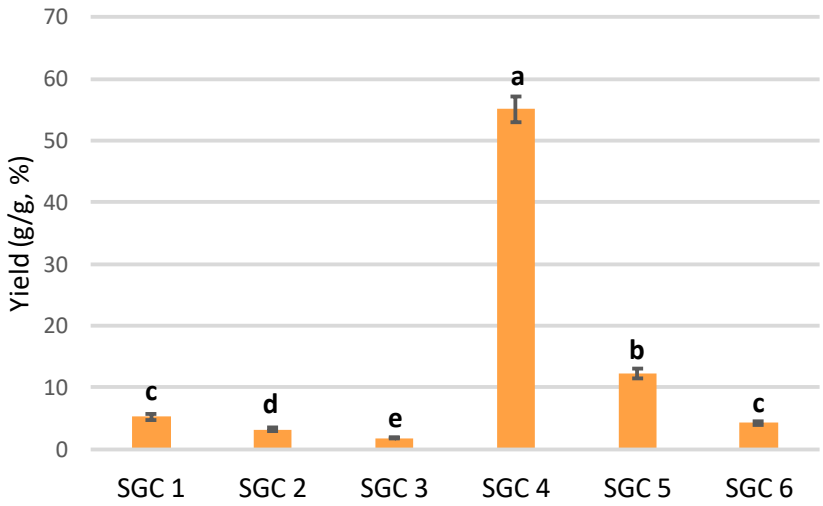


Figure 21. SGC fractions yields. Presented values followed by different lower-case letters (a–e) are significantly different from each other ($p < 0.05$).

The performed SGC provided 6 fractions with a quite different composition. SGC4 containing widest range of the compounds also was the most abundant in term of yield. Since the separation was successful, fractions anti-parasitic activity was evaluated.

Liquid-liquid extraction with NaOH

Liquid-liquid extraction with NaOH was performed in 3 steps. Firstly, the extract in EtOAc was extracted in NaOH and the obtained organic phase was once again extracted with stronger NaOH. The obtained organic phase (OP1) should be free from acidic compounds. The aqueous phase from this step was then brought to pH 3 and once again extracted with EtOAc, obtaining OP2, that should contain strongly acidic compounds. The aqueous phase form first step was also acidified to pH 3 and extracted with EtOAc, resulting in OP3. OP3 should have contained weak acidic compounds. TLC analysis of the fractions is shown on Figure 22.

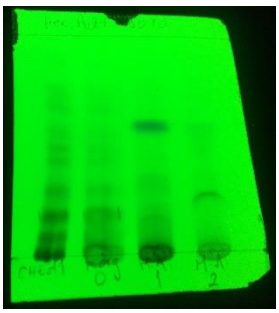


Figure 22. Fractions form liquid-liquid NaOH extraction under 254 nm (samples are shown in following order: raw extract, OP1, OP2, OP3).

The obtained fractions have a quite similar composition. Especially OP1 is extremely similar to the raw extract. Therefore, this fractionation methodology was considered unsuccessful and the fractions anti-parasitic activity was not evaluated.

Extracts and fractions anti-parasitic activity

Leishmanisasis, caused by parasites of *Leishmania* type, and Chagas disease, caused by parasite *Trypanosoma cruzi*, are considered two neglected tropical diseases by World Health

Organisation which indicated the need for research in this particular area.[111] Hence, in this work, *S. impressifolia* root extracts and their purified fractions have been evaluated for their anti-parasitic activity against the aforesaid parasites. Extracts cytotoxicity was evaluated on adherent murine macrophage-like RAW cells, whereas it is already well established cell line in this field of research. In addition, these cells are found in every organ and tissue in the body so by performing this test a general overview on extracts cytotoxicity can be provided.[112]

IC_{50} and LD_{50}, respectively, was calculated for *L. amazonensins*, *T. cruzi* and RAW treated with extracts and fractions. Tested samples anti-parasitic activity was estimated based on Selective Index (SI), a ratio between LD_{50} and IC_{50}, that is the ratio between the control cells and parasitic cells viability. The results are presented on Table 12.

Table 12. Extracts anti-parasitic activity on *Leishmania amazonensis* and *Trypanosma cruzi*.

Sample	LD_{50}	IC_{50}	**$SI_{L.\ amazonensis}$**	IC_{50}	**$SI_{T.\ cruzi}$**
	Control (RAW)	*L. amazonensis*		*T. cruzi*	
Soxhlet raw extract	4.2±1.3	3.5±1.3	1.2	3.8±1.2	1.1
SCO_2E raw extract	1.4±0.4	3.4±0.8	0.4	1.7	0.8
SEC2	24.2±2.7	>50.0	/	>50.0	/
SEC3	18.3±1.6	15.5±0.4	1.2	9.2±2.7	**2**
SEC4	1.5±0.5	1±0.1	1.5	1.1	1.4
SEC5	0.5±0.1	0.25±0.05	**2**	0.3	1.7
EtOP	16±2	26±4	0.6	29.7	0.5
DiOP	9±1.5	4.2±0.2	**2.1**	5.4	1.7
EAOP	3.4±0.5	2±0.2	1.7	2.9	1.2
SGC1	44.4±1.2	75±5	0.6	35.4	1.2
SGC2	53±4.1	20±0.6	**2.7**	41.3	1.3
SGC3	57±1.1	29±4	**2**	32.6	1.7
SGC4	1±0.4	2±0.6	0.5	4	0.2
SGC5	1.1±0.2	0.85±0.05	1.3	0.8	1.4
SGC6	1.3±0.1	1±0.1	1.3	0.7	1.9
CAT	27.3±6	21.05±1	1.3	11±1.1	**2.5**
Miltefosin	16.0±4.2	3.0±0.6	**5.3**	/	/
Amphotericin B	>50.0	0.4±0.1	**176.3**	/	/
Nifurtimox	8.7±2.2	/	/	0.65±0.15	**13.4**
Benznidazol	>50.0	/	/	7.3±0.8	>**9.5**

The extracts having SI greater than 2 were considered to have anti-parasitic activity, because such extracts or fractions are not cytotoxic, but possess an ability to inhibit propagation of a specific parasite. The SI lower than 1, suggests that the extract is cytotoxic.

[111] WHO, *Investing to overcome global impact of neglected tropical diseases*, WHO Library Cataloguing-in-Publication Data, Geneva, Sweden, **2015**.

[112] B. Taciak, M. Białasek, A. Braniewska, Z. Sas, P. Sawicka, Ł. Kiraga, T. Rygiel, M. Król, *PLoS One*, **2018**, *13*, 1–13.

Both conventional and SCO_2E raw extracts did not display atiparasitic activity. Moreover, SCO_2E extract shown to be cytotoxic. Therefore, both extracts were fractionated. For *T. cruzi*, only one fraction resulting from SEC, in particular fraction SEC3, has displayed activity. This fraction had a particularly high yield in respect to other portions obtained in SEC. TLC on Figure 18 indicated presence of wide range of compounds. Four fractions shown leishmanicidal activity: SEC5, DiOP, SGC2 and SGC3. Figure 18 suggests presence of more polar compounds in SEC5 fraction. DiOP on the other hand has a composition quite similar to the raw extract (Figure 16). Both SGC2 and SCG3 are rich in quite lipophilic compounds (Figure 20), and both fractions had a low yield. SGC2 exhibited the highest activity of all the tested samples. Therefore, this fraction was analysed by HPTLC-MS.

These extracts and fractions were also compared to commercial anti-parasitic drugs, in particular miltefosine and amphotericin B for leishmaniasis and nifurtimox and benzniadol for Chagas disease. Many *Leishmania* and *Trypanosoma* types show drug resistance to these well-established synthetic drugs. In addition, the treatment can be extremely expensive. For example, to treat Chagas disease 10 mg per kg of body weight of nifurtimox is required for 60 to 120 days.[113,114,115,116] This implies the necessity of more efficient and cost-effective drugs. In Bolivia novel phytotherapeutic used to treat several parasitoses, Alcaloides Totales de Corteza (CAT), emerged. It is a standardized *G. longiflora* extract, and was tested as well in this study.[117] Purified fractions of *S. impressifolia* root extract showed a superior leishmanicidal activity to the CAT (SI of 2.7 for SGC2 instead of 1.3 for CAT). However, the *S. impressifolia* fractions did not have an activity as strong as commercial pharmaceuticals (miltefosine and amphotericin B). Regarding the activity against *T. cruzi*, both CAT and commercial drugs were superior to *S. impressifolia* extracts and their fractions.

The SGC2 fraction could be considered promising alternative for commercial drugs, avoiding the possibility of drug resistance development. Animal and human trials should be performed to evaluate the feasibility of these extracts implementation as treatment against leishmaniasis.

HPTLC-MS analysis

Whereas it showed a promising leishmaniadical activity, SCG2 fraction was further analysed by HPTLC-MS. Mass spectra of the detected compounds was compared to those in literature.[118,119] The compounds identified in leishmaniadically active SGC2 fraction were triterpenes α-amyrin, β-amyrin, lupeol and friedelin. To date, several triterpenoids have been identified in *S. impressifolia* extracts, namely quinovic acid, 3-oxo-quinovic acid, 3-O-β-D-quinovopyranosyl quinovic acid, 3-O-β-D-fucopyranosyl quinovic acid, chincholic acid, friedelin, α-amyrin, β-amyrin, lupeol, salicinin B and pristrimerin.[101,102,120] α-amyrin, β-amyrin

[113] S. R. Wilkinson, M. C. Taylor, D. Horn, J. M. Kelly, I. Cheeseman, *PNAS*, **2008**, *105(13)*, 5022-5027.
[114] J. B. T. Carnielli, R. Monti-Rocha, D. L. Costa, A. M. Sesana, L. N. N. Pansini, M. Segatto, J. C. Mottram, C. H. N. Costa, S. F. G. Carvalho, R. Dietze, *Am. J. Trop. Med. Hyg.*, **2019**, *101(4)*, 789–794.
[115] C. T. Trinconi, J. Q. Reimao, A. C. Coelho, S. R. B. Uliana, *J. Antimicrob. Chemother.*, **2016**, *71*, 1314 –1322.
[116] B. Purkait, A. Kumar, N. Nandi, A. H. Sardar, S. Das, S. Kumar, K. Pandey, V. Ravidas, M. Kumar, T. De, D. Singh, P. Das, *Antimicrob. Agents Chemother*., **2012**, *56*, 1031–1041.
[117] G. Vechi, A. Tenfen, E. S. Capusiri, A. Gimenez, V. Cechinel-Filho, *Nat. Prod. Res.*, **2020**, *0*, 1–5.
[118] C. Mathe, G. Culioli, P. Archier, C. Vieillescazes, *J. Chromatogr. A*, **2004**, *1023*, 277–285.
[119] D. I. Falev, D. S. Kosyakov, N. V. Ul'yanovskii, D. V. Ovchinnikov, *J. Chromatogr. A*, **2020**, *1609*, 460458.
[120] H. S. Ripardo Filho, M. L. S. Costa, L. C. Pacheco, E. S. Andrade, R. N. M. Araújo, R. H. V. Mourão, G. M. S. P. Guilhon, L. S. Santos, *Ew. J. Nat. Prod. Res.*, **2015**, *1(1)*, 1–4.

and friedelin have already been reported in literature for having anti-trypanosomal activity, and β-amyrin and lupene have been reported for lieishmaniadical activity.[104]

2.3.2. Conclusions

Leishmaniasis and Chagas disease are ever growing problem in rural areas of South America and Asia contributing to mortality and economic issues. Several synthetic drug are present in the market for this disease treatment. However, due to their price and drug resistance occurrence, the scientific community is focused on finding novel more effective pharmaceuticals, and has for this purpose moved to the ethnopharmacology filed. *S. impressifolia* is medicinal plant commonly used by native Bolivian Tacana community. Its roots are rich in triterpenoids which are terpenoids that have emerged as effective anti-parasitic agents. These compounds extraction requires extraction with hazardous volatile organic compound and high time and energy consuming extraction methodologies, yielding often in unsafe extracts with questionable quality. SCO_2E could be a key innovation for these compound extraction.

In presented work, *S. impressifolia* roots were extracted in SCO_2E. This process yielded in 1.09% of extract. For the sake of the comparison, conventional Soxhlet extraction in CH_2Cl_2 was performed. The extraction yield and the extracts composition were comparable with the extract obtained in SCO_2E. However, SCO_2E enabled 2'fold decrease in extraction time. The extract was further fractionated by several procedures, in particular liquid-liquid extraction, SEC and SGC. The extracts and obtained fractions activity against *L. amazonensis* and *T. cruzi* was evaluated against RAW control cells. Moreover, traditional leismaniadical drugs, miltefosine and amphotericin B, and Chagas disease drugs, nifurtimox and benzniadol, were joined in the test together with new Bolivian anti-parasitic drug CAT, to evaluate the extracts efficiency. For Chagas disease, SCE3 fraction showed activity, but weaker than the traditional drugs and CAT. On the other hand, SGC2 fraction showed a strong leishmaniadical activity, superb to the CAT's activity. SGC2 was analysed on HPTLC-MS. Triterpenes α-amyrin, β-amyrin, lupeol and friedelin were detected, some of them previously reported for having anti-leishmaniadical activity. These promising result suggest that *S. impressifolia* root SGC2 fraction may be effective agent against leishmaniasis. However, tests on more *Leishmania* type parasite and *in vivo* trials should be performed.

2.3.3. Experimental part

Plant material

Plant material was dried and milled roots of *Salacia impressifolia* (Miers) A. C. Sm. (Figure 23). It was collected in 2014 in Buena Vista (14°22'410"S; 67°34'203"W; 229 meters above sea level).

Figure 23. *S. impressifolia* tree.

Soxhlet extraction

Conventional exhaustive *S. impressifolia* roots triterpenes extraction was performed in Soxhlet type apparatus according to Sporstøl et al..[121] 30 g of dry plant material was extracted with 400 mL of CH_2Cl_2. Extraction was performed over 12 h at CH_2Cl_2 boiling point. After the extraction, the extract was filtered and the solvent was evaporated. The extracts composition was evaluated using TLC.

SCO_2 extraction

Supercritical CO_2 extraction was performed in the reactor depicted in Figure 24.

Figure 24. SCO_2E reactor.

250 g of dry *S. impressifolia* roots were placed in the extractor. The temperature in the extractor was 38 °C and in the separators it was 49–60 °C. Pressure in the extractor was 138

[121] Sporstøl, S., Gjøs, N. & Carlberg, G. E. Extraction efficiencies for organic compounds found in aquatic sediments. *Anal. Chim. Acta* **151**, 231–235 (1983).

bar and the pressure in the separators and accumulator was 41 bar. The CO_2 flux was constant and the extraction was performed for 6 h. Every hour, sampling was performed. The samples were evaluated using TLC.

Extracts fractionation

Whereas the conventional and SCO_2 extract possess a quite similar composition, the results of conventional extracts purification could be applied also for the SCO_2 extract and *vice versa*. The conventional extract was purified *via* liquid-liquid extraction with aqueous NaOH, liquid-liquid extraction with organic solvent and size exclusion chromatography (gel chromatography). The SCO_2 extract was purified by silica gel column chromatography.

Liquid-liquid extraction with organic solvents

Liquid-liquid extraction using different organic solvents was performed to fractionate *S. impressifolia* extract. The schematic representation of the process is depicted in Figure 25. Firstly, 1.5 g of the extract was dissolved in 150 mL of 80% MeOH (aqueous) and extracted in a separation funnel with EtOAc. The organic phase (EtOP) was dried with Na_2SO_4 and ether was evaporated. MeOH was evaporated form the aqueous phase and the remaining water was extracted with CH_2Cl_2. The obtained organic phase (DiOP) was dried. The remaining aqueous phase was extracted with EtOAc and the resulting organic phase (EAOP) was died. The purification efficiency was evaluated on TLC.

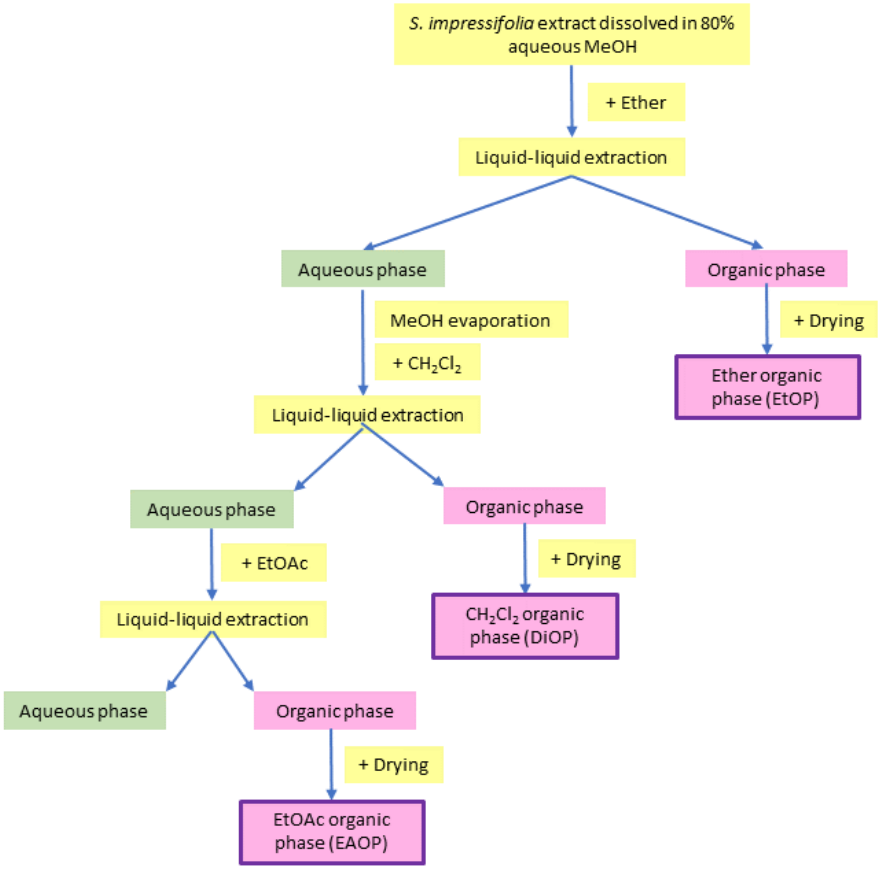


Figure 25. Schematic representation of liquid-liquid extraction for *S. impressifolia* extract purification.

Size exclusion chromatography (SEC)

For extract fractionation, SEC was also evaluated. It was performed over Sephadex LH-20, with MeOH:$CHCl_3$ (1:1, v/v) as the mobile phase. 400 mg of extract were dissolved in a small amount of mobile phase and subjected to the separation. 100 mL of solvent was used for compounds separation. The obtained fractions were evaluated by TLC. Finally, similar fractions were unified and the solvent was evaporated.

Silica gel column chromatography

1.6 g of sample was prepared with silica gel. The prepared sample was subjected to the chromatography on silica gel column. The column was 3.6 cm wide and silica was 19 cm high, the mass of silica was 65 g. A gradient elution was performed. Solvent used were following: ether, ether:CH_2Cl_2 (1:1), CH_2Cl_2, CH_2Cl_2:EtOAc (1:1), EtOAc, EtOAc:MeOH (8:2), and 200 mL of every phase were used. The solvents were evaporated from the fractions on a rotary vacuum evaporator. The separation efficiency was evaluated by TLC.

Liquid-liquid extraction with NaOH

S. impressifolia roots extract fractionation was also performed *via* liquid-liquid extraction with NaOH, brefely described on Figure 26. 100 mg of extract was dissolved in 50 mL od EtOAc and mixed with 50 mL 0.1 N NaOH (in water). Liquid-liquid extraction was performed in separating funnel. The organic phase was once again extracted with 50 mL 1 N NaOH. The obtained organic phase (OP 1) was dried with Na_2SO_4. The aqueous phase was brought to pH 3 (using 1 N HCl) and once again extracted with EtOAc (50 mL). The resulting organic phase 2 (OP 2) was dried. Aqueous phase from the first liquid-liquid extraction was brought to pH 3 and extracted with EtOAc (50 mL). The obtained organic phase 3 (OP 3) was dried with Na_2SO_4. The OPs composition was evaluated using TLC.

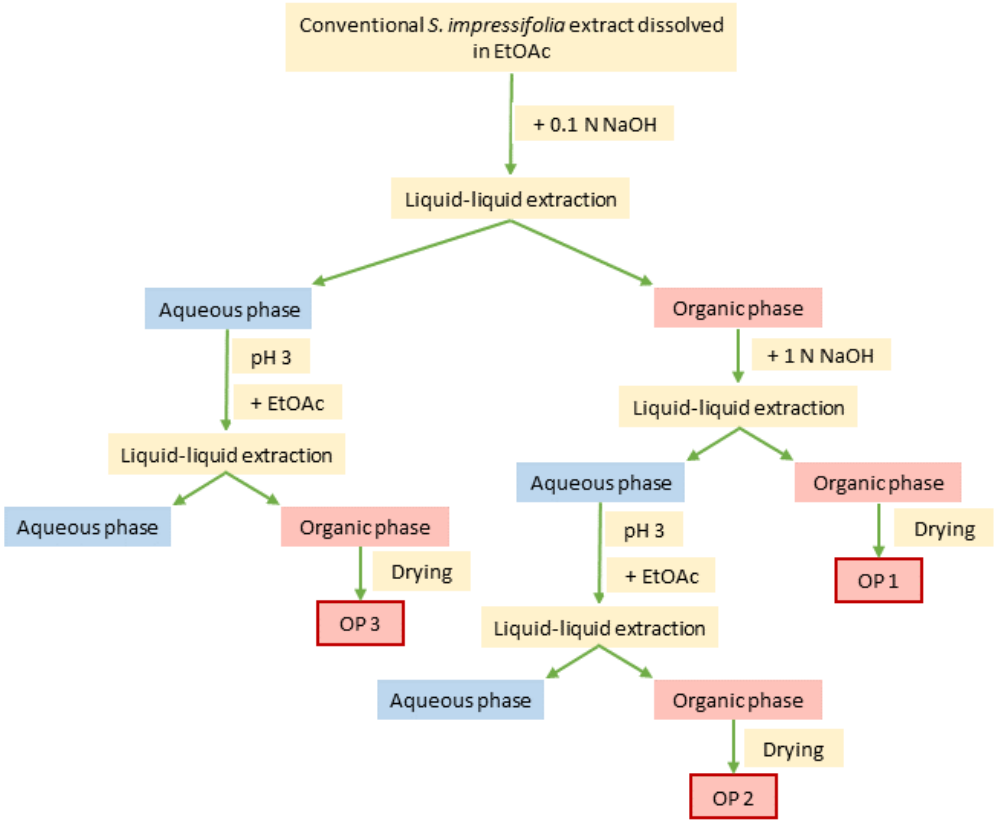


Figure 26. Schematic representation of liquid-liquid extraction for *S. impressifolia* root extract purification.

TLC conditions

Thin layer chromatography (TLC) analyses were performed on alumina plates coated with silica gel, using hexane and ethyl acetate (EtOAc) 7:3 or 9:1 v/v as eluent. Spots were visualized under UV light (254 and 365 nm) and/or by spraying/heating with vanillin/H_2SO_4 (2% vanillin in concentrated H_2SO_4).

Anti-parasitic activity evaluation

In vitro leishmanicidal activity (IC_{50})

L. amazonensis, Clone 1, MHOM-BR-76-LTB-012 (Lma, donated by the Paul Sabatier Université, France) strain was cultured in Schneider's insect medium, (pH 6.2) supplemented with 10% FBS and incubated at 26 °C. Medium changes were made every 72 h to maintain a viable parasitic population. Leishmanicidal activity was determined according to Williams et al.[122] with some modifications. Samples were dissolved in DMSO (maximum final concentration 1%) at 10 mg/mL. Promastigotes in logarithmic phase of growth, at a concentration of 3×10^6 parasites/mL, were distributed (100 µL/well) in 96-well flat bottom microtiter plates. Samples at different concentrations (0.1–100 µg/mL) were added (100 µL). Miltefosine and anfotericina B (0.1–100 µg/mL) were used as control drug.[123] Moreover, a standardized *G. longiflora* extract (CAT) (0.1–100 µg/mL) was also tested. Assays were

[122] C. Williams, O. A. Espinosa, H. Montenegro, L. Cubilla, T. L. Capson, E. Ortega-Barría, L. I. Romero *J. Microbiol. Methods*, **2003**, *55*, 813–816.
[123] F. Campos-Buzzi, M. Fracasso, B. K. Clasen, J. C. Ticona, A. Gimenez, V. Cechinel-Filho, *Methods Find. Exp. Clin. Pharmacol.*, **2010**, *32*, 707—711.

performed in triplicate. The microwell plates were incubated for 72 h at 26 °C. After incubation, a solution of XTT (1 mg/mL) in PBS (pH 7.0 at 37 °C) with PMS (0.06 mg/mL) was added (50 µL/well) and incubated for 3 h at 26 °C. Optical density of each well was obtained using a Synergy HT microplate reader (Biotek, Winooski, VT, USA) at 450 nm test wavelength and 650 nm as reference filter. The IC_{50} values were calculated using the GEN5 program (Biotek) and expressed in µg/mL.

In vitro activity against *Trypanosoma cruzi* (IC_{50})

Cultures of *Trypanosoma cruzi* (epimastigotes, donated by the Parasitology Department of INLASA, Tc-INLASA), were maintained in medium LIT (pH 7.2), supplemented with 10% FBS and incubated at 26 °C. Medium changes were made every 72 h to maintain a viable parasitic population. Trypanocidal activity was determined according to Muelas-Serrano et al.[124] with some modifications. Samples were dissolved in DMSO (maximum final concentration 1%) at 10 mg/mL. Epimastigotes in logarithmic phase of growth, at a concentration of $3x10^6$ parasites/mL, were distributed (100 µL/well) in 96-well flat bottom microtiter plates. Samples at different concentrations (0.1–100 µg/mL) were added (100 µL). Benznidazol and nifurtimox (0.1–100 µg/mL) were used as the control drug. Moreover, a standardized G. longiflora extract (CAT) (0.1–100 µg/mL) was also tested. Assays were performed in triplicates. The microwell plates were incubated for 72 h at 26 °C. After incubation, a solution of XTT (1 mg/mL) in PBS (pH 7.0 at 37 °C) with PMS (0.06 mg/mL) was added (50 µL/well) and incubated for 4 h at 26 °C. The optical density of each well was measured and the IC_{50} values were calculated.

Cytotoxicity activity (LD_{50})

The RAW 264.7 murine macrophage cell line was purchased from the American Type Culture Collection (ATCC-TIB71). The cytotoxicity of the samples was determined using the resazurin assay,[125] with some modifications. The cells were maintained in DMEM-HG medium supplemented with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin, and sodium bicarbonate (2.2 g/L) in humidified atmosphere at 37 °C with 5% CO_2. Samples were prepared as described above and added (in 100 µL DMSO) at different concentrations (0.7–100 µg/mL). Medium blank, control drugs and cell growth controls were included to evaluate cell viability. The plates were incubated for 72 h at 37 °C with 5% CO_2. After incubation for the indicated time, the cells were washed, after which 10 µL of Resazurin reagent (2.0 mM) was added. They were further incubated at 37 °C for 3 h in a humidified incubator. The LD_{50} values were assessed using a fluorometric reader (540 nm excitation, 590 nm emission) and the Gen5 software. All assays were performed in triplicate.

Selective Index (SI)

The SI values were calculated as the ratio between control cell LD_{50} and parasites IC_{50} values:

$$SI = \frac{LD_{50}}{IC_{50}} \quad (9)$$

HPTLC-MS analysis

The analysed fraction was eluted on alumina plates coated with silica gel, using hexane and ethyl acetate (EtOAc) 9:1 v/v as eluent. The spots were visualised by UV light and marked.

[124] S. Muelas-Serrano, J. J. Nogal-Ruiz, A. Gómez-Barrio, *Parasitol. Res.*, **2000**, *86*, 999–1002.
[125] R. C. Borra, M. A. Lotufo, S. M. Gagioti, F. de M. Barros, P. M. Andrade, *Braz. Oral Res.*, **2009**, *23*, 255–262.

The plate was directly placed in Plate Express HPTLC-MS Interface (Advion, NY, USA) coupled with ESI (Electrospray Ionization) mass detector (Advion, NY, USA). Mobile phase used was methanol. The MS system was operated in the full scan mode (total ion current) between m/z 100 and 1000 in both positive and negative ESI mode. Nitrogen was used as curtain and collision gas.

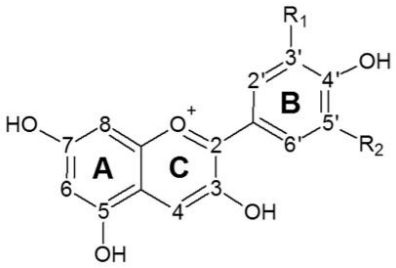
Statistical analysis

Statistical analysis was performed as described in *2.1. Green extraction technologies for cocoa been shell polyphenols recovery* part.

Chapter 3: Enabling technologies and natural deep eutectic solvents for anthocyanins extraction

The enabling technologies closely described in *Chapter 2* have been highly efficient for phytochemicals extraction in respect to their conventional analogues. Therefore, these methodologies were combined with natural deep eutectic solvent (NADES). As described in *Chapter 1*, these solvents are formed by complexation between hydrogen bound acceptor and donor, both of natural origin, making these solvents bio-compatible, bio-degradable and non-toxic for humans and environment. These traits imply that NADES could be directly incorporated in food, pharmaceutical and cosmetic application. There is a wide range of compounds that could be used for NADES formation, and consequently a specific solvent with desired properties could be synthetized, improving the extraction yield and selectivity.[1,2,3]

In these thesis, NADES were applied for anthocyanins extraction. Anthocyanins are red, purple or blue polyphenolic pigments. The most common anthocyanins and their structures are reported in Figure 1.[4]



Anthocyanin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OH	OCH_3
Malvidin	OCH_3	OCH_3

Figure 1. The most common anthocyanins structures.[4]

Like other secondary metabolites, their presence does not affect plant growth or reproduction. However, these compounds have several hypothesized functions in plant tissues such as protection of chloroplasts from the adverse effects of excess light, attenuation

[1] M. H. Zainal-Abidin, M. Hayyan, A. Hayyan, N. S. Jayakumar, *Anal. Chim. Acta*, **2017**, *979*, 1–23.
[2] H. Vanda, Y. Dai, E. G. Wilson, R. Verpoorte, Y. Hae Choi, *C. R. Chimie*, **2018**, *21*, 628–638.
[3] K. Radošević, M. Cvjetko Bubalo, V. Gaurina Srček, D. Grgas, T. Landeka Dragičević, I. Radojčić Redovniković, *Ecotoxicol. Environ. Saf.*, **2015**, *112*, 46–53.
[4] J. Fang, *Nutrition*, **2015**, *31*, 1301–1306.

of UV-B radiation, antioxidant activity and repellence of herbivores and parasites.[5] Plant-derived anthocyanins are used as dyes and natural food colorants. Anthocyanins possess strong antioxidant activity and are therefore able to prevent cellular damage and the formation of degenerative diseases. Their numerous beneficial effects on human health have been thoroughly studied and reported in the literature, and include antidiabetic, anticancer, anti-inflammatory, antimicrobial, anti-aging, neuroprotective and cardio-protective effect.[6,7,8,9] Their popularity is currently increasing due to the aforementioned beneficial effects and the global anthocyanin market experiences constant growth. In 2019, the size of the anthocyanin market was USD 318 million and that is expected to reach USD 388 million by 2024.[10]

Anthocyanins are conventionally extracted with mixtures of water and ethanol, methanol and acetone with associated flammability and toxicity risks.[11] Moreover, anthocyanins are stable in acidic medium[12] and therefore addition of acids, most commonly HCl, is required imposing the application of dedicated equipment. NADES, as already indicated, could be synthetized with various compounds, and therefore could be prepared with organic acids resulting in acidic NADES.[13] Consequently, HCl use could be avoided.

[5] K. Gould, K. Davies, C. Winefield, *Anthocyanins: Biosynthesis, Functions, and Applications*, Springer, New York, NY, USA, **2009**.
[6] H. E. Khoo, A. Azlan, S. T. Tang, S. M. Lim, *Food Nutr. Res.*, **2017**, *61(1)*, 1361779.
[7] B. Yousuf, K. Gul, A. A. Wani, P. Singh, *Crit. Rev. Food Sci. Nutr.*, **2016**, *56*, 2223–2230.
[8] A. Smeriglio, D. Barreca, E. Bellocco, D. Trombetta, *Phytother. Res.*, **2016**, *30*, 1265–1286.
[9] D. Li, P. Wang, Y. Luo, M. Zhao, F. Chen, *Crit. Rev. Food Sci. Nutr.*, **2017**, *57(8)*, 1729–1741.
[10] Market Data Forecast, **2020**, https://www.marketdataforecast.com/market-reports/anthocyanins-market.
[11] Y. Dai, E. Rozema, R. Verpoorte, Y. H. Choi, *J. Chromatogr. A* **2016**, *1434*, 50–56.
[12] A. Castañeda-Ovando, M. L. Pacheco-Hernández, M. E. Páez-Hernández, J. A. Rodríguez, C. A. Galán-Vidal, *Food Chem.*, **2009**, *113*, 859–871.
[13] T. Bosiljkov, F. Dujmić, M. Cvjetko Bubalo, J. Hribar, R. Vidrih, M. Brnčić, E. Zlatić, I. Radojčić Redovniković, S. Jokić, *Food Bioprod. Process.*, **2017**, *102*, 195–203.

3.1. Combined ultrasound and microwave irradiation as an efficient technology for grape-stalk anthocyanin recovery[14]

The winery industry generates a substantial amount of wastes and by-products. Grape stalks (GS) represent 1.4% and 7.0% of the total raw matter processed in wine making. This by-product has a quite low commercial value and up to date, has mostly been used as fertilizers or animal feed.[15] However, it is rich in polyphenols, and this valorisation option could bring added value to the overall wine making process.[16,17]

In this work, firstly different NADES solutions were tested for GS anthocyanins isolation by a conventional methodology. Subsequently, UAE, MAE and combined US and MW assisted extraction (UMAE) were tested for process intensification. US and MW separately offer a great number of advantages already described in *Chapters 1* and *2*. Nevertheless, combining these two emerging technologies can increase extraction efficiency to an even greater extent. To date, UMAE is one of the most promising hybrid extraction technologies.[18,19] Since experiments proven the UMAE's efficiency, this extraction was further optimized. The aim of the process optimization was to, of course, maximize the anthocyanins yield and moreover, to minimize energy consumption. For this purpose, response surface methodology (RSM) approach was used. RSM is a collection of statistical and mathematical techniques that could be used for process development, improvement or optimization. This approach is widely used in industry, mainly for processes optimization, where several parameters influence particular performance or quality characteristic of the product or process.[20]

In addition, the NADES from this extraction was recycled. Whereas NADES have a negligible vapour pressure, four recycling techniques can be considered: liquid-liquid extraction using another solvent, supercritical CO_2 extraction, precipitation via antisolvents addition and adsorption chromatography.[21,22] The former was applied in this work.

[14] V. Gunjević, I. Radojčić Redovniković, L. Stevanato, G. Cravotto, *Int. J. Green Tech.*, **2020**, *6*, 51–63.
[15] S. Maicas, J. J. Mateo, *Sustainability*, **2020**, *12*, 559.
[16] G. Spigno, D. M. De Faveri, *J. Food Eng.* **2007**, *78*, 793–801.
[17] G. Grillo, L. Boffa, S. Talarico, R. Solarino, A, Binello, G. Cavaglià, S. Bensaid, G. Telysheva, G. Cravotto, *Antioxidants*, **2020**, *9*, 730.
[18] F. Chemat, M. Rombaut, A. G. Sicaire, A. Meullemiestre, A. S. Fabiano-Tixier, M. Abert-Vian, *Ultrason. Sonochem.*, **2017**, *34*, 540–560.
[19] L. Wen, Z. Zhang, D. S. Sun, S. P. Sivagnanam, K. Brijesh, B. K. Tiwari, *Crit. Rev. Food Sci. Nutr.*, **2020**, *60(11)*, 1826–1841.
[20] R. H. Myers, D. C. Montgomery, C. M. Anderson-Cook, *Response surface methodology: Process and products optimization using designed experiments*, 3 ed., John Wiley & Sons, Inc., Hoboken, NJ, USA, **2009**.
[21] K. M. Jeong, M. S. Lee, M. W. Nam, J. Zhao, Y. Jin, D. K. Lee, S. W. Kwon, J. H. Jeong, J. Lee, *J. Chromatogr. A*, **2015**, *1424*, 10–17.
[22] M. Ruesgas-Ramón, M. C. Figueroa-Espinoza, E. Durand, *J. Agric. Food Chem.*, **2017**, *65*, 3591–3601.

3.1.1. Results and discussion

Conventional GS anthocyanins extraction

A conventional heating and stirring extraction of GS anthocyanins was performed to evaluate the intensification that extraction protocol with NADES and enabling technologies offers. The extraction lasted for 2 h and the solvent used was acidified ethanol (0.8% HCl in 60% hydroalcoholic solution), traditionally used for anthocyanins extraction. The removal of a chlorine-based strong inorganic acid appears to be crucial for environment and furthermore for the process's overall cost since HCl handling requires dedicated equipment. Ethanol, besides being a taxed solvent, requires ATEX (EXplosive ATmospheres) and anti-flammable plants.

The conventional extraction yielded in 1.04 mg/g of total anthocyanins content (TAC). The energy required for this process is 1.14 kWh.

NADES screening

Due to the aforementioned organic solvents and inorganic acids drawbacks, six NADES were tested for the GS anthocyanins extraction. All the NADES had 30% (v/v) of water. The tested solvents are reported in Table 1.

Table 1. NADES tested for GS anthocyanins extraction, their abbreviations and molar ratios.

NADES	Abbreviation	Molar ratio	Reference
Choline chloride: Malic acid	ChCl:MA	1.5:1	[23,24]
Choline chloride: Citric acid	ChCl:CA	2:1	[24,25]
Choline chloride: Oxalic acid	ChCl:OA	1:1	[23]
Choline chloride: Urea	ChCl:U	1:2	[26]
Choline chloride: Glycerol	ChCl:Gly	1:2	[23,27]
Choline chloride: Glucose	ChCl:Glc	1:1	[11,28]

Besides cholinium-based NADES, anthocyanins extraction in acidified ethanol was also carried out, for the sake of comparison. All the extractions were performed as conventional stirring extractions at room T, to avoid the anthocyanins degradation. As acidified ethanol and NADES, with 30% of water, are not viscous, the extraction time was only 30 min. The results in terms of TAC are depicted in Figure 2.

[23] M. Cvjetko Bubalo, N. Ćurko, M. Tomašević, K. Kovačević Ganić, I. Radojčić Redovniković, *Food Chem*., **2016**, *200*, 159–166.
[24] M. Panić, M. Radić Stojković, K. Kraljić, D. Škevin, I. Radojčić Redovniković, V. Gaurina Srček, K. Radošević, *Food Chem.*, **2019**, *283*, 628–636.
[25] H. Vanda, R. Verpoorte, P. G. L. Klinkhamer, Y. H. Choi, In: *Deep Eutectic Solvents: Synthesis, Properties, and Applications* (ed. D. J. Ramón, G. Guillena), John Wiley & Sons, Inc., Hoboken, NJ, USA, **2020**, pp 61–81.
[26] A. P. Abbott, G. Capper, D. L. Davies, R. K. Rasheed, V. Tambyrajah, *Chem. Commun.*, **2003**, 70–71.
[27] K. Radošević, N. Ćurko, V. Gaurina Srček, M. Cvjetko Bubalo, M. Tomašević, K. Kovačević Ganić, I. Radojčić Redovniković, *LWT - Food Sci. Technol.*, **2016**, *73*, 45–51.
[28] R. Craveiro, I. Aroso, V. Flammia, T. Carvalho, M. T. Viciosa, M. Dionísio, S. Barreiros, R. L. Reis, A. R. C. Duarte, A. Paiva, *J. Mol. Liq.*, **2016**, *215*, 534–540.

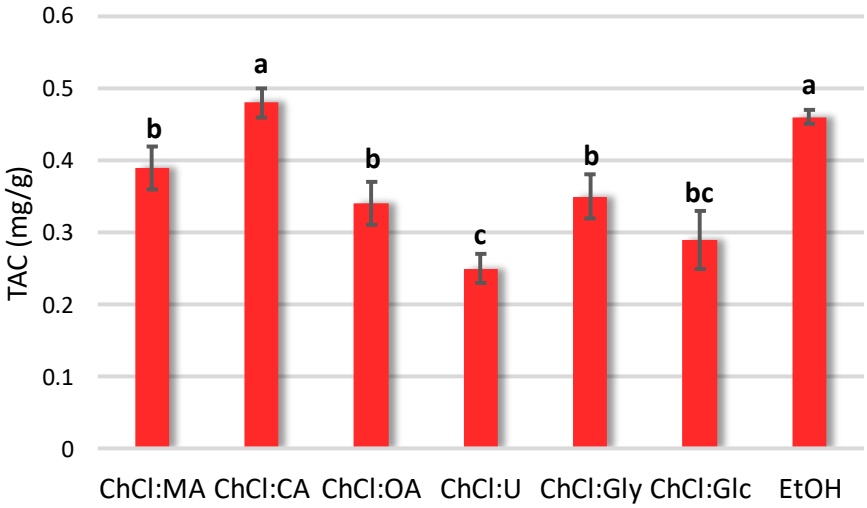


Figure 2. TAC in extracts obtained using different solvents. The presented values followed by different lower-case letters (a–c) are significantly different from each other ($p < 0.05$).

Hydrogen bond donor used for NADES preparation has a considerable impact on anthocyanin extraction. NADES mixtures containing carboxylic acids provided higher TAC yields. As they are composed of acids, it is clear that these solvents have quite an acidic pH. As mentioned in the introduction, acidic pH solvents are preferable for anthocyanin extraction and preservation because they help to prevent their degradation.[29] NADES ChCl:CA gave the highest yields, namely 0.48 mg/g, and was hence selected as the optimal one. Acidified ethanol (EtOH), a conventional solvent for anthocyanin extraction, gave a very similar yield to ChCl:CA, implying that this biocompatible solvent could replace the traditional solvent.

Enabling technologies screening

To find a suitable process intensification technique for GS anthocyanin extraction, UAE, MAE and UMAE were coupled with optimal NADES, ChCl:CA. All the extractions were carried out for 10 min. Whereas these techniques offer significant heat and mass transfer enhancement, longer extraction times are not necessary.[30] The MAE and UMAE were performed at 80 °C as the anthocyanins should not degrade at this temperature during this short period of time.[31,32] The TAC in the obtained extracts is reported in Figure 3.

[29] S. Silva, E. M. Costa, C. Calhau, R. M. Morais, M. E. Pintado, *Crit. Rev. Food Sci. Nutr.*, **2017**, *57(14)*, 3072–3083.
[30] G. Cravotto, P. Cintas, *Chem. Eur. J.*, **2007**, *13*, 1902–1909.
[31] R. Buckow, A. Kastell, N. S. Terefe, C. Versteeg, *J. Agric. Food Chem.*, **2010**, *58*, 10076–10084.
[32] M. Cisse, F. Vaillant, O. Acosta, D. M. Claudie, M. Dornier, *J. Agric. Food Chem.*, **2009**, *57*, 6285–6291.

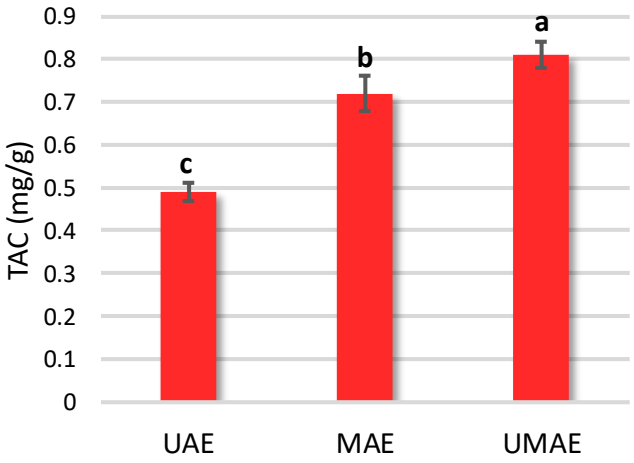


Figure 3. TAC yield in GS extracts obtained using different enabling technologies. The presented values followed by different lower-case letters (a–c) are significantly different from each other ($p < 0.05$).

Compared to the conventional heating and stirring method, UAE did not significantly increase the extraction yield, while MAE and UMAE provided extracts with considerably higher TAC. UMAE yielded 0.81 mg/g TAC in 15 min, whilst the initial test for solvent screening, over 30 min, gave 0.48 mg/g TAC. This considerable process intensification could be ascribed to US and MW working in synergy. While US offers plant-cell breakdown, as well as improving mass transfer and solvent penetration, microwaves provide fast heat transfer.[33] As already mentioned, MW irradiation has a strong impact on dipoles, due to the constantly changing electric field.[34] Whereas anthocyanins, CA and ChCl are all dipoles, it can be easily concluded that MW can have a positive impact on this extraction process.

UMAE was further optimized with the aim of developing a fast and cost-effective anthocyanin extraction process.

UMAE of GS anthocyanins optimization

Even though there is an ever growing trend in environmental friendly processes, industries are still mainly focused on reducing production costs. Thus, this study aims to optimize UMAE, presenting a fast and efficient process for anthocyanin recovery from a cheap source. The impact of extraction parameters on the TAC in the extracts was observed. Moreover, aiming to develop a cost-effective process, energy consumption (EC) during the extraction was monitored.

Experimental plan preparation and application

The extraction parameters that could have a significant impact on GS anthocyanins extraction are time, temperature and water content in ChCl:LA. To determine the optimal conditions RSM was applied. Experimental design employed was Box-Benken design, whereas it offers to optimized the process in three evenly spaced levels.[20] It was prepared in Design Expert 7.0.0 software. The investigated independent variables were extraction time,

[33] T. J. Mason, F. Chemat, M. Vinatoru, *Curr. Org. Chem.*, **2011**, *15*, 237–247.
[34] V. Mandal, Y. Mohan, S. Hemalatha, *Pharmacogn. Rev.*, **2007**, *1(1)*, 7–18.

temperature and water content in NADES. US and MW power were kept constant over time. Responses monitored were TAC in the obtained extracts and EC during extraction.

First of all, to prepare the experimental plan boundary conditions for the independent variables were evaluated. An extraction with periodical sampling was performed to find the range for which the extraction time was to be monitored. Results are depicted in Figure 4.

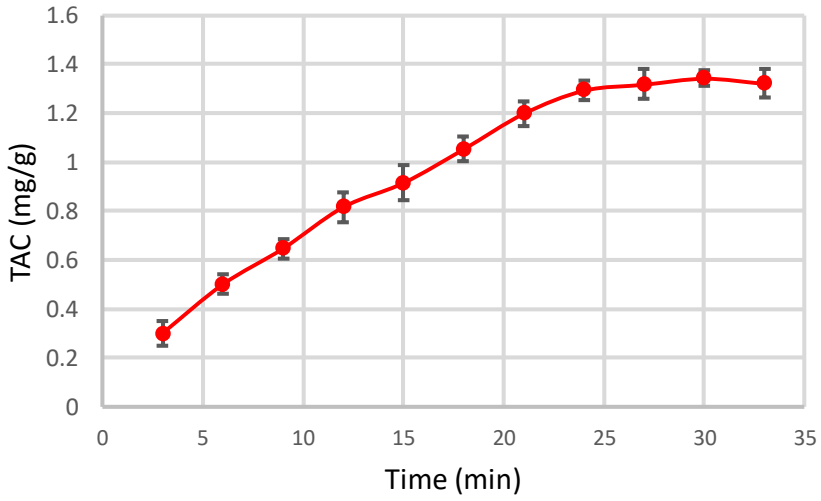


Figure 4. TAC during UMAE as determined by periodical sampling.

A quite linear TAC yield increase was observed until 24 min. Therefore, the area that could be optimized was between 3 and 24 minutes. As 3 minutes is a very short period of time, 5 min was selected as the lower boundary level, and 25 min was chosen as the higher boundary level. Temperature boundary conditions were determined using the information in the literature. Significant anthocyanin degradation should generally not occur over 25 min of extraction in range of 50 to 90 °C.[31,32] In the MicroSYNTH MW reactor, MW power is controlled by the temperature of the solution in the cavity. Low temperature extraction would not permit MW irradiation to be emitted, and thus MW would not have a significant effect on the extraction process. Therefore, the lower and upper boundary conditions were 50 and 90 °C, respectively. For the water content in NADES, boundary conditions were also evaluated using the literature. As NADES mixtures preserve the hydrogen bonds between their components when the water content ranges from 0 to 50%, this range was applied.[35] Finally, the Box-Behnken experimental plan was created (Table 2).

Table 2. Independent variables and experimental testing area in coded levels used for GS anthocyanin UMAE optimization.

Factor	Lower axial level	Central level	Upper level
Independent variables	-1	0	1
Time (X_1, min)	5	15	25
Temperature (X_2, °C)	50	70	90
Water content in NADES (X_3, %)	0	25	50

The planned experiments were conducted and the results are reported in Table 3.

[35] Y. Dai, G. J. Witkamp, R. Verpoorte, Y. H. Choi, *Food Chem.*, **2015**, *187*, 14–19.

Table 3. TAC in extracts and EC during extraction for every test performed according to the Box-Behnken design.

Run	Factor X_1	Factor X_2	Factor X_3	Response 1	Response 2
	Time (min)	Temperature (°C)	Water content in NADES (%)	TAC (mg/g)	EC (kWh)
1	25	70	50	0.58	0.15
2	5	70	50	0.39	0.04
3	15	50	50	0.45	0.08
4	15	70	25	0.83	0.09
5	25	70	0	0.66	0.15
6	15	90	0	0.55	0.09
7	25	90	25	1.45	0.17
8	15	70	25	0.83	0.10
9	25	50	25	0.61	0.13
10	5	70	0	0.16	0.03
11	5	50	25	0.25	0.03
12	15	90	50	0.81	0.13
13	15	70	25	0.83	0.09
14	15	50	0	0.27	0.08
15	5	90	25	0.89	0.05

A thorough statistical analysis of TAC and EC results was performed.

Mathematical model development and fitting

The independent variables (extraction conditions) were correlated with the responses (TAC and EC) using two second-order polynomial equations, one for each response monitored, as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j \quad (1)^{20}$$

where Y is the predicted response, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the cross-product coefficient and $X_{i,j}$ are studied independent variables.

Statistical ANOVA (Analysis of variance) analyses were conducted on the obtained equations in Design Expert software. The statistical analysis for TAC response is reported in Table 4.

Table 4. Statistical analysis of experimentally determined TAC approximation suitability with polynomial model.

Variability source	SS	df	MS	F value	p-value Prob > F
Model	4.5725	9	0.5081	22.4251	0.0016
X_1-time	1.2752	1	1.2752	56.2875	0.0007
X_2-temperature	1.4748	1	1.4748	65.0967	0.0005
X_3-water content	0.3443	1	0.3443	15.1982	0.0114
$X_1 X_2$	0.0408	1	0.0408	1.8002	0.2374
$X_1 X_3$	0.2602	1	0.2602	11.4847	0.0195
$X_2 X_3$	0.0038	1	0.0038	0.1689	0.6981
X_1^2	0.1664	1	0.1664	7.3444	0.0423
X_2^2	0.0002	1	0.0002	0.0099	0.9248
X_3^2	1.0568	1	1.0568	46.6462	0.0010
Residual	0.1133	5	0.0227		
Lack of Fit	0.1133	3	0.0378		
Pure Error	0	2	0		
Cor Total	4.6858	14			

*SS – sum of squares
df – degrees of freedom
MS – mean square

The statistical analysis of the equation that approximated the Ln(TAC) response implies that the model is statistically significant, as the models Prob>F is less than 0.0500. In addition, the model's F-value of 22.43 confirms its significance. Finally, the Ln(TAC) response model was defined:

$$Ln(TAC) = -0.19 + 0.40 \times X_1 + 0.43 \times X_2 + 0.21 \times X_3 - 0.26 \times X_1 \times X_3 - 0.21 \times X_1^2 - 0.53 \times X_3^2 \quad (2)$$

Only significant model terms (Prob>F less than 0.0500) were considered in defining the model.

Analogous statistical analysis was performed for EC response (Table 5).

Table 5. Statistical analysis of experimentally determined EC approximation suitability with polynomial model.

Variability source	SS	df	MS	F value	p-value Prob > F
Model	0.028	9	$3.108*10^{-3}$	81.07	< 0.0001
X_1-time	0.025	1	0.025	660.33	< 0.0001
X_2-temperature	$1.800*10^{-3}$	1	$1.800*10^{-3}$	46.96	0.0010
X_3-water content	$3.125*10^{-4}$	1	$3.125*10^{-4}$	8.15	0.0356
$X_1 X_2$	$1.000*10^{-4}$	1	$1.000*10^{-4}$	2.61	0.1672
$X_1 X_3$	$2.500*10^{-5}$	1	$2.500*10^{-5}$	0.65	0.4560
$X_2 X_3$	$4.000*10^{-4}$	1	$4.000*10^{-4}$	10.43	0.0232
X_1^2	$6.410*10^{-7}$	1	$6.410*10^{-7}$	0.017	0.9021
X_2^2	$1.603*10^{-5}$	1	$1.603*10^{-5}$	0.42	0.5464
X_3^2	$6.410*10^{-7}$	1	$6.410*10^{-7}$	0.017	0.9021
Residual	$1.917*10^{-4}$	5	$3.833*10^{-5}$		
Lack of Fit	$1.250*10^{-4}$	3	$4.167*10^{-5}$	1.25	0.4733
Pure Error	$6.667*10^{-5}$	2	$3.333*10^{-5}$		
Cor Total	0.028	14			

Similarly, the model that approximated the EC response was also shown to be statistically significant. The EC response model was finally defined with significant model terms:

$$EC = 0.093 + 0.056 \times X_1 + 0.015 \times X_2 + 0.00625 \times X_3 + 0.01 \times X_2 \times X_3 \quad (3)$$

These polynomial equations could therefore be applied for the optimization of the GS anthocyanin extraction. Based on the models, the predicted TAC and EC values were calculated for every extraction performed. These predicted values were compared with the actual TAC and EC experimental values (Figure 5).

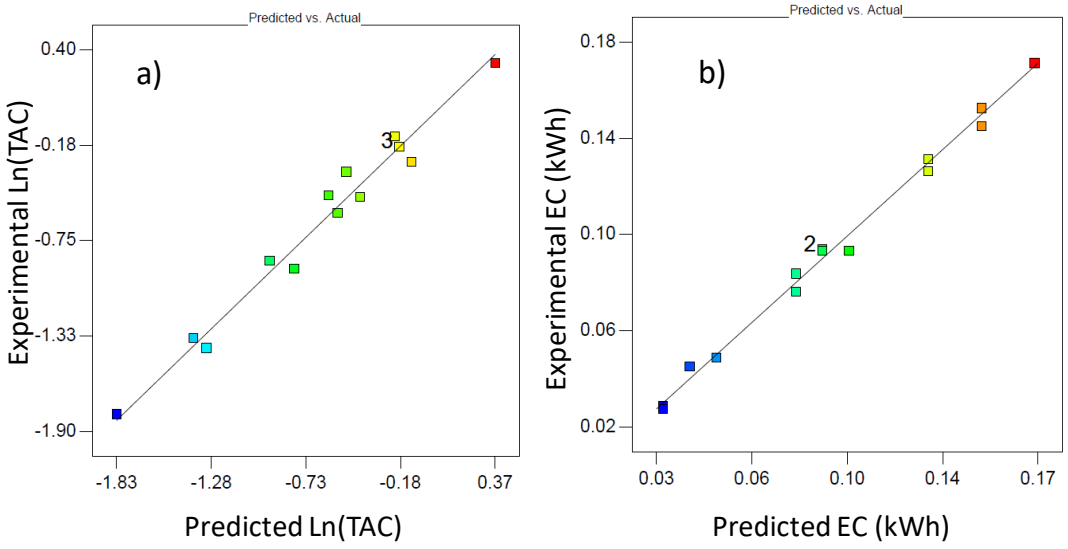


Figure 5. a) Experimental Ln(TAC) values vs. Ln(TAC) values predicted by the model; b) experimental EC values vs. EC values predicted by the model.

Graphs show good correlation between actual experimental values and the values predicted by the models for both responses, suggesting the suitability of these models to describe the studied extraction processes. To have a clearer insight into how the extraction conditions affect TAC yield, 3D diagrams showing the interaction between TAC yield and the independent variables were designed (Figure 6).

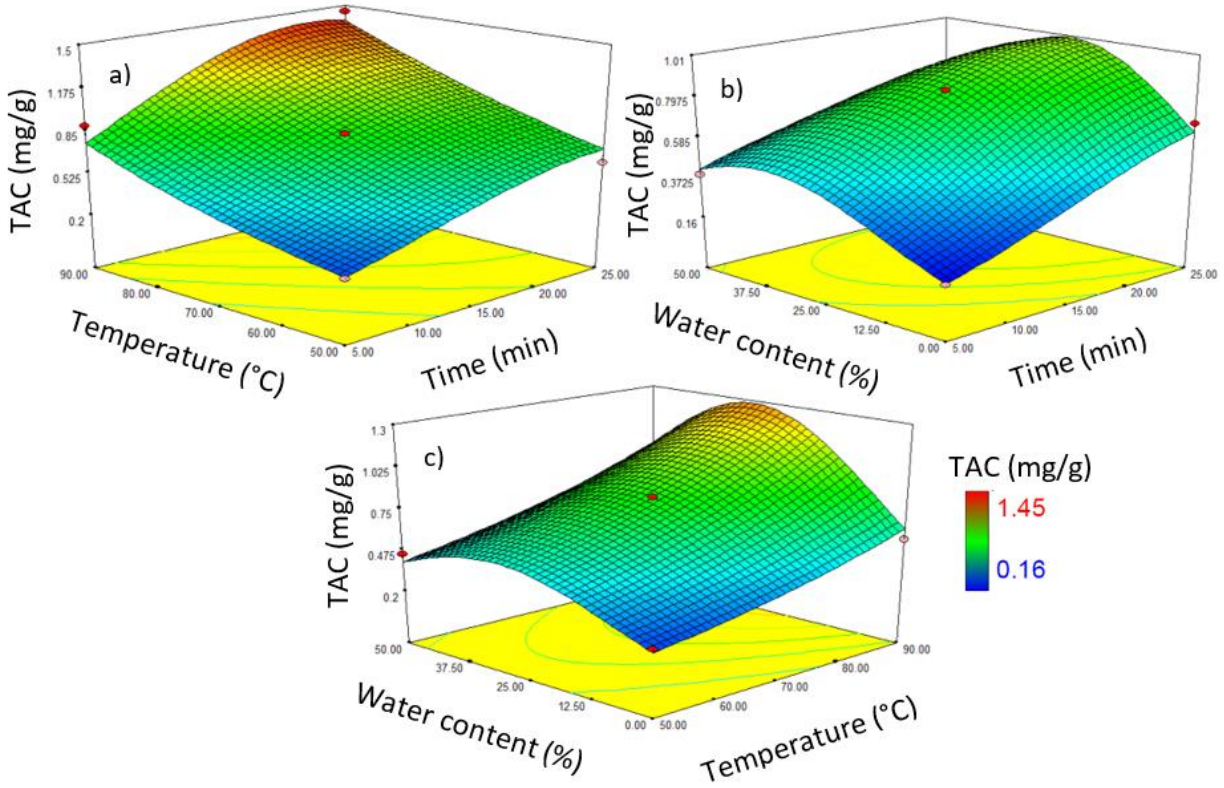


Figure 6. 3D diagrams demonstrating the TAC response surface as a function of: a) extraction time and temperature, b) extraction time and water content in NADES, c) temperature and water content in NADES.

Figure 6 a) shows that longer extraction time and higher temperature lead to higher TAC yield. In many extraction processes this is an expected trend. However, anthocyanins are easily degradable phytochemicals that are highly sensitive to pH, temperature, light, oxygen exposure and the presence of some enzymes.[12,36] Anthocyanin degradation was thus expected at higher boundary conditions of time and temperature variables. However, anthocyanin degradation did not occur. This result can most likely be attributed to ChCl:CA, which stabilizes anthocyanins. Dai et al.[11] have demonstrated the higher stability of anthocyanins in NADES lactic acid:glucose than in conventional acidified ethanol. Moreover, based on its constituents, it is clear that ChCl:CA has a quite acidic pH, which is favourable for anthocyanin extraction and preservation. Figures 6 b) and c) show how the best extraction yield can be obtained when NADES contains around 25% of water. Extremely low yields are obtained when there is no water in NADES, and this trend has also been reported in the literature.[13,37,38] Generally, NADES are viscous solvents (usually 200–500 mm^2/s at 40 °C), which is the main disadvantage

[36] M. M. Giusti, R. E. Wrolstad, *Current Protocols in Food Analytical Chemistry*, **2001**, *00*, F1.2.1-F1.2.13.
[37] Y. Dai, G. J. Witkamp, R. Verpoorte, Y. H. Choi, *Anal. Chem.*, **2013**, *85*, 6272–6278.
[38] M. Panić, V. Gunjević, G. Cravotto, I. Radojčić Redovniković, *Food Chem.*, **2019**, *300*, 125185.

of their application. In the extraction processes, the biggest issue is low mass transfer.[37,39] Higher temperature should reduce NADES viscosity, however, even at 90 °C, a significant increase in TAC yield was not observed (Figure 6 c). Moreover, even the enhancement of mass transfer provided by UMAE did not enable anthocyanin extraction in this case. In addition, it is visible that higher water content causes a decrease in TAC yield. This event could be attributed to the increased polarity of the solvent when water is added.[40] Nevertheless, it could also be ascribed to decreased anthocyanin stability in water-diluted NADES, which has already been reported in the literature.[41]

Numerical optimization and model verification

Numerical optimization was carried out in Design Expert software. The final aim was to maximize TAC yield and minimize EC, in order to develop an economically viable process. Finally, the determined optimal conditions were: extraction time of 5 min, temperature 90 °C and water content of 30%. Under these conditions, 0.81 mg/g TAC can theoretically be extracted, while the EC required for this extraction is only 0.05 kWh. The model was successfully verified by performing an extraction under these conditions. The obtained TAC was 0.79 mg/g and EC was 0.05 kWh. The conventional extraction yielded in 22% higher TAC yield. However, due to the long extraction time, EC was 95% higher. The optimized UMAE anthocyanin extraction using NADES is therefore much more efficient and economically feasible. It offers drastic cuts in extraction time and, subsequently, EC reduction.

In Table 3, the results show that the highest TAC yield (1.45 mg/g) is achieved when the extraction time is 25 min, temperature 90 °C and water content is 30%. However, this extraction requires around 70% higher EC (0.17 kWh). These EC values do not differ much, since this is a lab scale extraction. Nevertheless, these values would be much higher if a pilot- or industrial-scale process was conducted. Moreover, TAC obtained in this extraction is considerably greater than the yield from the conventional extraction, demonstrating how combined US and MW technology can enable the extraction process.

Anthocyanins isolation and NADES recycling from the extract

Reuse and recycle is one of important circular economy principles.[42,43,44] Generally, NADES recycling is feasible due to their remarkable physiochemical properties. For this study, recycling was conducted via adsorption chromatography.

Firstly, the NADES structure in the optimal anthocyanin extract was disrupted by the addition of water, up to a 50% of v/v ratio.[35] In this way, the availability of anthocyanins for adsorption is enhanced. Moreover, extract viscosity is significantly decreased. After dilution, the extract was loaded onto a column where the anthocyanins were adsorbed and NADES was eluted using only water. 88.89% of NADES was recovered, suggesting that this process was quite successful. The recycled NADES could be used for further extraction processes, making this extraction profitable and green.

[39] B. Ozturk, C. Parkinson, M. Gonzalez-Mique, *Sep. Purif. Technol.*, **2018**, *206*, 1–13.
[40] F. Gabriele, M. Chiarini, R. Germani, M. Tiecco, N. Spreti, *J. Mol. Liq.*, **2019**, *291*, 111301.
[41] Y. Dai, R. Verpoorte, Y. H. Choi, *Food Chem.*, **2014**, *159*, 116–121.
[42] P. Ghisellini, C. Cialani, S. Ulgiati, *J. Clean. Prod.*, **2016**, *114*, 11–32.
[43] K. Winans, A. Kendall, H. Deng, *Renew Sustain. Energy Rev.*, **2017**, *68*, 825–833.
[44] S. Sauvé, S. Bernard, P. Sloan, *Environ. Dev.*, **2016**, *17*, 48–56.

Anthocyanins were desorbed using acidified ethanol with a recovery efficiency of 74.76%. The observed anthocyanin loss could be ascribed to their presence in the recycled NADES; the recycled ChCl:CA has a slightly pink colour. However, since this NADES will be used in further GS anthocyanin extraction processes, the presence of anthocyanin is not an issue. Dai et al.[45], have reported the co-elution of polyphenols with NADES ChCl:sucrose in the absorption chromatography process. Panić et al.[38] have noted colour retention in recycled ChCl:CA NADES after anthocyanin recovery from grape-pomace extract.

3.1.2. Conclusion

To address emerging trends, an environmentally friendly and cost-efficient methodology for anthocyanin isolation from a winery industry by-product has been developed. NADES have been tested for GS anthocyanin extraction with the aim of replacing conventionally used acidified ethanol. ChCl:CA gave the highest TAC yield, which was quite comparable to that obtained in the extraction with acidified ethanol. This NADES was coupled with 3 energy sources: US, MW and UMAE, and UMAE was the most effective one. Therefore, UMAE was further optimized by RSM; extraction time, temperature and water content in ChCl:CA were investigated. Besides TAC yield, EC was also monitored in order to reduce the operative costs of the process. After thorough statistical analyses, the optimal extraction conditions were defined as follows: extraction time 5 min, temperature 90 °C and 30% of water in NADES. This extraction yielded 0.79 mg/g of TAC. The EC for this extraction was 0.05 kWh. In addition, the obtained result was compared to a conventional extraction performed for 2 h in a heating and stirring equipment with acidified ethanol. The conventional extraction gave a 22% higher TAC yield. However, the energy consumption was 95% greater. In addition, NADES recycling and anthocyanin isolation from the optimal extract was performed successfully. Solvent recycling provides another economic benefit for this process. In conclusion, the present study has provided new insight into GS anthocyanin UMAE, in view of potential exploitation at pilot- and semi-industrial scales, paving the way for zero-waste winery industry.

3.1.3. Experimental part

Plant material

GS were dry and milled obtained *via* mechanical separation from grapes, provided by Cantine Ascheri Giacomo (Bra-CN, Italy).

Conventional GS anthocyanins extraction

The conventional GS anthocyanin extraction protocol was performed using acidified ethanol (0.8% of HCl in 60% hydroalcoholic solution) according to Dai et al.[11] and Panić et al.[38], with some modifications. The extraction time was 2 h and the GS-to-solvent ratio was 1 to 20. The extraction temperature was 55 °C and the stirring was set at 200 rpm. EC was measured during the extraction. The obtained extract was filtered and TAC was determined.

[45] Y. Dai, J. van Spronsen, G. J. Witkamp, R. Verpoorte, Y. Hae Choi, *Anal. Chim. Acta*, **2013**, *766*, 61–68.

NADES preparation

NADES were synthesised at fixed molar ratios of choline chloride (ChCl) to hydrogen bond donor (HBD). The two components were placed in appropriate ratios in a round-bottomed glass flask with 30% (v/v) of deionized water. The compounds were stirred and heated to 50 °C for 2 h until a homogeneous transparent colourless liquid was formed.

Solvents screening for GS anthocyanins extraction

All NADES, with water contents of 30% and acidified ethanol (60% hydroalcoholic solution with 0.8% of HCl), were tested in conventional condition for GS anthocyanin extraction to find the optimal NADES. Acidified ethanol extraction was performed for the sake of comparison. Extraction process parameters were adapted from the previous application of NADES in anthocyanin extraction,[11] with some modifications. In particular, extractions were performed using the conventional stirring approach for 30 min at room temperature and at 200 rpm. The GS-to-solvent ratio was 1 to 20. The extracts were finally filtered and TAC was determined.

Enabling technologies screening

UAE, MAE and UMAE were combined with the optimal NADES and tested for GS anthocyanins extraction. UAE was performed using a titanium immersion horn (Danacamerini sas, Italy) working at 20.2 kHz. MAE extraction was performed in a SynthWAVE (Milestone, Italy); a pressurized multimode MW reactor, with the possibility to feed an inert gas into the cavity (N_2). UMAE was carried out in a MW reactor MicroSYNTH (Milestone, Italy), combined with a Pyrex® horn as the US source. All the extractions were performed for 10 min and the GS-to-NADES ratio was 1 to 20. The extraction temperature and US and MW powers are listed in Table 6. The obtained extracts were filtered and TAC was determined.

Table 6. UAE, MAE and UMAE parameters.

Extraction technique	Power (W)	Temperature (°C)
UAE	40	~ 40
MAE	300	80
UMAE	US 40, MW 300	80

GS anthocyanins UMAE optimization

Response surface methodology (RSM) was used for the optimization of GS anthocyanin UMAE. Extractions were designed using the Box-Behnken experimental plan with constant MW power (400 W) and US power (40 W, 23 kHz), and variable extraction time (5–25 min), temperature (50–90 °C) and water content in NADES (0–50%). The experimental plan was prepared in Design Expert 7.0.0 software (Stat-Ease Inc., MN, USA). The boundary conditions of each variable, i.e. lower and upper axial level, were either determined experimentally or from the literature. Extraction-time boundary conditions were determined experimentally via periodical sampling during extraction. The GS-to-ChCl:CA ratio was 1 to 20, average temperature was 80 °C, MW power was 300 W and US power was 40 W. The sampling of roughly 1 mL was performed every 3 minutes and TAC in samples was measured. Total extraction time was 33 min. Extraction temperature and water content in NADES were defined according to the literature.

The determined boundary conditions of the variables were transformed into coded values using the following equation:

$$X_i = \frac{x - \frac{(x_{max} + x_{min})}{2}}{\frac{(x_{max} - x_{min})}{2}} \quad (4)$$

where X_i is the coded variable, x is the natural variable, x_{max} is the upper axial level and x_{min} is the lower level of the natural variable.

Fifteen experiments were performed according to the experimental plan with 3 repetitions in the central level of the experimental testing area. All extractions were carried out in a MicroSYNTH multimode MW reactor combined with a Pyrex® horn as the US source (Figure 7).

Figure 7. MicroSYNTH MW reactor combined with a Pyrex® horn.

The ratio between dry GS and NADES was 1 to 20 in all the performed extractions. The obtained extracts were filtered and the TAC was evaluated. EC expressed in kWh, was measured using an electric power meter (Brennenstuhl, Tübingen, Germany). The obtained experimental results were used to create a correlation between the independent variables and responses using a second-order polynomial equation (model). A detailed statistical analysis, ANOVA, was carried out in Design Expert software to validate both TAC and EC models. The statistical significance of the models and independent variables for the observed process were evaluated. After the models was finally defined, an extraction was performed under optimal conditions to verify the data predicted by models. The optimal conditions were defined by numerical optimization where the goal was to maximize TAC and minimize EC.

Anthocyanins isolation and NADES recycling

Anthocyanin isolation from the optimal GS ChCl:CA extract and the NADES recycling was performed over Sepabeads SP 825L macroporous resins (Mitsubishi Chemical Corporation, Resindion SRL, Italy) as described in Panić et al..[38] Before the separation process, the extract was diluted to more than 50% of water in NADES. The resin was prepared using ethanol and water. After the preparation resin bead volume (BV) was calculated by following equation:

$$BV = r^2 \times h \times \pi \quad (5)$$

where r^2 is the column radius and h is the resin height. Subsequently, the extract was loaded. Fraction 1, containing the recycled NADES was eluted with 3 BV of deionized water. The anthocyanins (fraction 2) were desorbed with 3 BV of 75% ethanol with 0.1% of HCl. The water from fraction 1 and ethanol/water from fraction 2 were then removed in a rotary vacuum evaporator followed by freeze drying. Finally, recycled NADES and dry anthocyanins are obtained.

Anthocyanin recovery efficiency was evaluated by measuring the TAC in the obtained anthocyanin fraction. NADES recycling efficiency was estimated by weighting the obtained recycled NADES.

TAC determination

The TAC in the extracts and recovered anthocyanin fraction was determined according to the method described in Ribéreau-Gayon and Stonestreet.[46] 100 µL of extract solution was placed in the test tubes. Sequentially, samples were diluted with a 60% ethanol solution containing 0.1% of HCl and 2 mL of 2% HCl solution. In one parallel, 400 µL of distilled water was added to the sample solution and, to the other, 400 µL of 15% (w/v) sodium bisulfite solution was added. The resulting solution was mixed thoroughly. After 15 min, the absorbance of the solutions was measured at 520 nm, in a 1 cm quartz cuvette, using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), against a blank. The TAC was calculated using the following equation:

$$TAC = 875 \times (D_1 - D_2) \quad (6)$$

where D_1 is the absorbance of the control sample and D_2 is the absorbance of the bisulfite bleached sample. TAC is expressed as mass of anthocyanins over mass of dry GS (mg/g). All analyses were performed in triplicate.

Statistical analysis

Statistical analysis was performed as described in *Chapter 2: 2.1. Green extraction technologies for cocoa been shell polyphenols recovery*.

[46] P. Ribéreau-Gayon, E. Stonestreet, *Chimie Analitique*, **1966**, *48*, 188-196.

3.2. Deep eutectic solvents and non-conventional technologies for blueberry-peel extraction: kinetics, anthocyanin stability and antiproliferative activity[47]

Blueberry peels (BP) are one of the main by-products of blueberry fruit processing. Blueberries have increased in popularity in recent years thanks to their health benefits, nutritional value and sensory properties. In particular, they possess one of highest antioxidant activities of all fruits, and this is mainly due to their exceptional concentration of anthocyanins.[48] The most common anthocyanins found in blueberries are monoarabinosides, monoglucosides and monogalactosides of cyanidin, petunidin, peonidin, delphinidin and malvidin.[49] Nevertheless, it is worth noting that the peels have the highest content of these metabolites, compared to the remaining berry components.[50]

In this work, a novel green extraction methodology for the recovery of high added-value phytochemicals from blueberry-processing waste was designed. Several NADES mixtures have been tested for anthocyanin extraction and the most sustainable was selected according to its process efficiency, cost, viscosity and toxicity. The shelf-life of extracts prepared in different NADES was estimated. Moreover, percentage polymeric colour was determined to study anthocyanin stability in NADES. UAE and MAE were tested to enhance anthocyanin recovery, and process kinetics were described using Peleg's model. The results achieved were compared with those of the conventional anthocyanins extraction methodology. NADES were recycled and the extracted anthocyanins were recovered using a macroporous resin.

As already mentioned, anthocyanins possess strong chemo-protective and anti-tumour effect. Cancer is the second leading cause of mortality, as it led to 9.6 million deaths globally in 2018.[51] As chemotherapy and many anti-cancer drugs that are currently available on the market have potential life-threatening side effects, it is necessary to find new, less dangerous agents.[52] As a result, chemo-protection by naturally occurring compounds has gained increasing attention,[53] and anthocyanins can be considered promising chemo-protective agents, especially for tumorigenesis that occurs at directly accessible targets, such as the gastrointestinal tract and skin.[54]

Therefore, in this study, the antiproliferative activity and cytotoxicity of the extracts were evaluated in *in-vitro* tests on human tumour cells against a human skin cell line. Moreover, the mechanism of cell-growth inhibition and cell death were evaluated using flow cytometry.

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[47] G. Grillo, V. Gunjević, K. Radošević, I. Radojčić Redovniković, G. Cravotto, *Antioxidants* **2020**, *9*, 1069.
[48] S. Skrovankova, D. Sumczynski, J. Mlcek, T. Jurikova, J. Sochor, *Int. J. Mol. Sci.*, **2015**, *16*, 24673–24706.
[49] S. Norberto, S. Silva, M. Meireles, A. Faria, M. Pintado, C. Calhau, *J. Funct. Foods*, **2013**, *5*, 1518–1528.
[50] S. Y. Wang, M. J. Camp, M. K. Ehlenfeldt, *Food Chem.*, **2012**, *132*, 1759–1768.
[51] WHO, Cancer, **2020**, https://www.who.int/health-topics/cancer#tab=tab_1.
[52] T. Wang, J. Jiao, Q. Y. Gai, P. Wang, N. Guo, L. L. Niu, Y. J. Fu, *J. Pharm. Biomed. Anal.*, **2017**, *145*, 339–345.
[53] H. Gali-Muhtasib, R. Hmadi, M. Kareh, R. Tohme, N. Darwiche, *Apoptosis*, **2015**, *20*, 1531–1562.
[54] P. Jing, M. M. Giusti, In: *Berries and Cancer Prevention* (ed. N. Seeram, G. Stoner) Springer, New York, NY, USA, **2010**; pp 3-40.

3.2.1. Results and discussion

Water content in plant material

The water content in the frozen BP, as determined by freeze drying, was 56.70 ± 0.19%, w/w. This value was used to calculate the effective water addition necessary to prepare NADES with 25% H_2O content.

Conventional BP anthocyanins extraction

Conventional BP anthocyanin extraction was performed in a stirring and heating system using acidified ethanol (0.8% of HCl in 60% hydroalcohloc solution) as the solvent. The extraction lasted for 2 h and was performed at 55 °C whereas degradation should not occur at this condition.[55] Finally, 22.70 mg/g of TAC were obtained in extract, and this value was set as the benchmark for all further extractions.

NADES screening

Preliminary NADES screening was conducted on five different choline chloride-based systems. The tested NADES mixtures are reported in Table 7. All prepared NADES had 22% (v/v) of water.

Table 7. NADES tested for BP anthocyanins extraction and molar ratios between the mixed compounds.

NADES	Abbreviation	Molar ratio	References
Choline chloride: Malic acid	ChCl:MA	1.5:1	23,24
Choline chloride: Citric acid	ChCl:CA	2:1	25,38
Choline chloride: Lactic acid	ChCl:LA	1:1	25,45
Choline chloride: Glycerol	ChCl:Gly	1:2	23
Choline chloride: Glucose	ChCl:Glc	1:1	11,56

Extractions were performed in a heating and stirring system under the same conditions applied for the benchmark extraction. In addition, the plant material was cryo-milled to avoid any matrix effect on mass transfer. The TAC for every extraction performed is reported in Figure 8.

[55] C. P. Kechinski, P. V. Ramos Guimaraes, C. P. Zapata Norena, I. C. Tessaro, L. D. Ferreiramarczak, *J. Food Sci.*, **2010**, *75*, 173–176.

[56] R. Craveiro, I. Aroso, V. Flammia, T. Carvalho, M. T. Viciosa, M. Dionísio, S. Barreiros, R. L. Reis, A. R. C. Duarte, A. Paiva, *J. Mol. Liq.*, **2016**, *215*, 534–540.

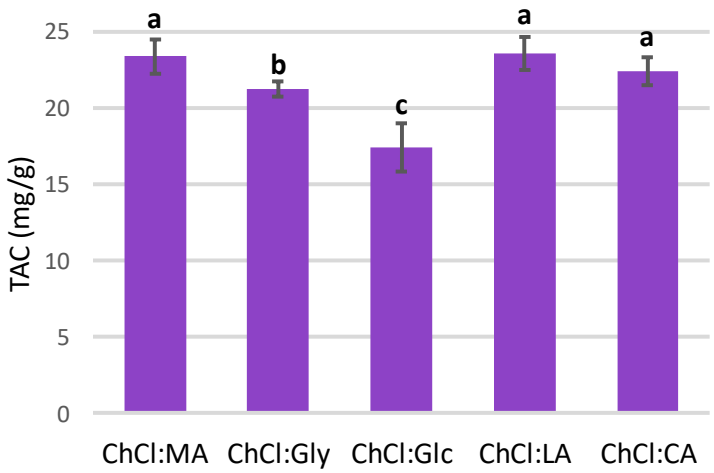

Figure 8. TAC for the extracts obtained from NADES screening. The presented values followed by different lower-case letters (a–c) are significantly different from each other ($p < 0.05$).

ChCl:MA and ChCl:LA gave the highest but very similar TAC values, in particular 23.41 and 23.59 mg/g, respectively. The optimal NADES was selected on the basis of viscosity, price and cytotoxicity. The HBD prices were reviewed on Sigma Aldrich. 1 kg of LA costs €55.14 and the price of the same mass of MA is €49.00. These NADES, therefore, have a comparable preparation costs. Although the viscosity of all of the NADES mixtures can be decreased by the addition of water, ChCl:LA appears to be generally less viscous, and hence, could be more easily incorporated into industrial processes.

Radošević et al.[57] have evaluated the chemical cytotoxicity of numerous NADES and cholinium-based ionic liquids on Channel catfish ovary cells. The study proved that ChCl:MA has a large influence on cell viability, while ChCl:LA showed barely any effect at all. Zhao et al.[58] have investigated the toxicity of different NADES on several bacteria species (*Escherichia coli, Staphylococcus aureus, Salmonella enteritidis, Listeria monocytogenes*). ChCl:MA was shown to be one of the most toxic NADES of those tested. The increasing interest in the sustainable production of lactic acid and the development of suitable biorefinery approaches was an aspect that was evaluated further.[59] Based on the data found in literature, ChCl:LA was therefore selected as the optimal solvent system and was adopted for the subsequent investigations.

NADES systems shelf-life

The shelf-life of the BP extracts prepared using different NADES mixtures was monitored in order to evaluate their stability. The extracts that were prepared during the NADES screening were stored at +4 to +8 °C. After 1 week, 3 weeks and 9 months, the TAC in the stored extracts was evaluated. The results are reported in Figure 9. Data for the ChCl:Glc extract are not shown as the sample separated after 1 week of storage. The initial extracts are labelled as fresh extracts.

[57] K. Radošević, J. Železnjak, M. Cvjetko Bubalo, I. Radojčić Redovniković, I. Slivac, V. Gaurina Srček, *Ecotox. Environ. Safe.*, **2016**, *131*, 30–36.
[58] B. Y. Zhao, P. Xu, F. X. Yang, H. Wu, M. H. Zong, W.Y. Lou, *ACS Sustain. Chem. Eng.*, **2015**, *3*, 2746−2755.
[59] J. P. López-Gómez, M. Alexandri, R. Schneider, J. Venus, J. *Process Biochem.* **2019**, *79*, 1–10.

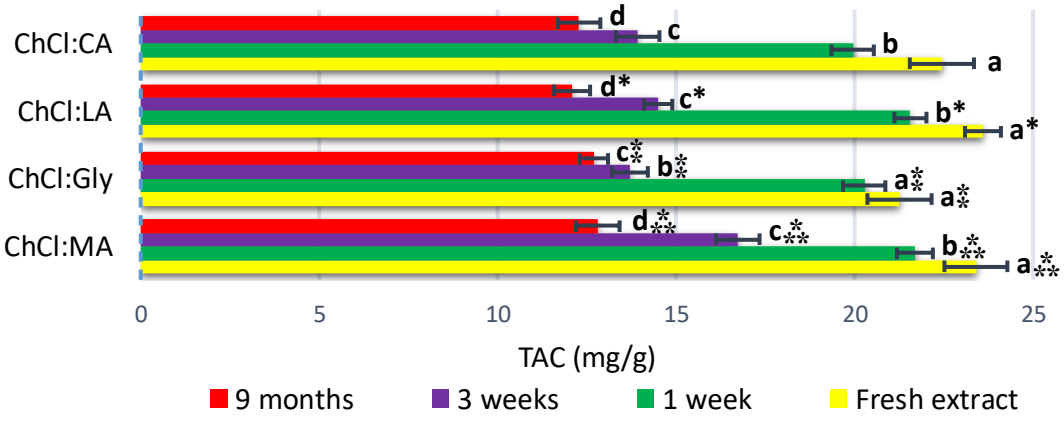

Figure 9. TAC content in BP extracts prepared with different NADES mixtures, stored at +4 to +8 °C over 9 months. The presented values followed by different lower-case letters (a–d) are significantly different from each other ($p < 0.05$) according to the NADES systems.

The stability, in terms of TAC, was very similar for all the monitored systems. Great TAC loss was noted between the 1st and 3rd week of storage. In between the 3rd week and 9 months, there was no significant TAC loss. Similar behaviour has been observed by Srivastava et al..[60] The stability of the anthocyanins and other polyphenols in the blueberry extract that was prepared using water was monitored over 60 days. Significant anthocyanin degradation occurred in the extracts stored at 6 °C from day 15 to day 30. Afterwards, there was no great degradation.

The type of NADES did not have a great effect on anthocyanin stability. After 9 months of storage, the TAC for all the extracts was more or less halved, in respect to the fresh extract. In particular, the average TAC loss for the monitored extracts was 44.92 ± 3.45%. Laleh et al.[61] have prepared blueberry anthocyanin extracts using an acidified ethanol solution and they were stored at 5 °C for 84 days (i.e. 12 weeks). Anthocyanin stability varied greatly with blueberry species. The TAC loss varied from 10.22 to even 57.81%. Moldovan et al.[62] have studied anthocyanin stability in cranberry bush fruit extracts that were prepared in water and ethanol and stored at 2 °C. The solvent acidity effect was also monitored. The highest anthocyanin half-life was observed in water that was acidified at pH 3, and was 48.12 days. The next highest anthocyanin stability was found in ethanol that was acidified at pH 3. However, the TAC was halved to 22.21 days. Conversely, Tao et al.[63] have noted a different trend. They performed the UAE of wine lees using an ethanol/water solution, and stored the extract for 30 days at 4 °C. No degradation was observed. However, this result may not be representative, in terms of the solvent's effect on anthocyanin stability, as the ethanol was evaporated from the extract before storage. Panić et al.[38] have monitored anthocyanin stability in grape pomace extracts at 4 °C for 60 days. The extracts were prepared using acidified ethanol, ChCl:CA and ChCl:proline:MA. ChCl:CA showed the highest stabilising capacity, with 14% degradation being observed after 60 days of storage, whereas this value was 70% for acidified ethanol and ChCl:proline:MA. The literature therefore indicates that the extraction solvent has a significant effect on anthocyanin stability, although information on

[60] A. Srivastava, C.C. Akoh, W. Yi, J. Fischer, G. Krewer, *J. Agric. Food Chem.*, **2007**, *55*, 2705–2713.
[61] G. H. Laleh, H. Frydoonfar, R. Heidary, R. Jameei, S. Zare, *Pakistan J. Nutr.*, **2006**, *5*, 90–92.
[62] B. Moldovan, L. David, C. Chişbora, C. Cimpoiu, *Molecules*, **2012**, *17*, 11655–11666.
[63] Y. Tao, D. Wu, Q. A. Zhang, D.W. Sun, *Ultrason. Sonochem.*, **2012**, *21*, 706–715.

anthocyanin-extract stability over long term storage is currently quite scarce. The results obtained herein on the shelf-life of BP extracts offers a deeper view into anthocyanin stability in different NADES media.

Percent polymeric colour for conventional and NADES systems

Anthocyanin degradation is usually associated with the formation of polymers,[64] and it is therefore possible to evaluate alterations in these metabolites in different solvents using percent polymeric colour (PPC) determination. PPC is a monomeric/polymeric ratio of anthocyanins and anthocyanin-like compounds. Hence, high PPC values can be used as an indicator of anthocyanin degradation. All the extracts were prepared in conventional heating and stirring equipment under the same conditions (2 h, 55 °C, 200 rpm). After filtration, PPC was evaluated. The ChCl:Glc system was not taken into account because of its degradation issues, as reported in Paragraph discussing NADES systems shelf-life. The results are shown in Table 8.

Table 8. PPC in extracts prepared using different solvents.

Extraction Solvent	PPC (%)
Acidified EtOH	16.37
ChCl:CA	19.02
ChCl:LA	19.90
ChCl:MA	19.10
ChCl:Gly	65.71

The lowest PPC was detected for acidified ethanol. ChCl:CA, ChCl:LA and ChCl:MA gave a very similar PPC values, which were quite comparable to that of the acidified ethanol extract. The ChCl:Gly extract gave a dramatically higher PPC value. These results can most likely be attributed to the solvent pH. Based on their constituents, it is clear that lactic-, malic- and citric-based NADES have quite an acidic pH, which is favourable for anthocyanin extraction and preservation, whereas glycerol moves toward a neutral or basic pH, and is therefore unsuitable for these purposes. ChCl:CA, ChCl:LA and ChCl:MA can easily replace traditional acidified ethanol.

Whereas NADES have proven to be a suitable solvent for anthocyanins extraction and degradation, they were implemented in innovative extraction methodologies, aiming to intensify the TAC extraction. UAE and MAE were evaluated for this purpose. In particular, TAC extraction kinetics was studied for both techniques and these extraction methodologies were closely compared to their conventional analogues.

MAE of BP anthocyanis

Extraction kinetics

The MAE were performed in multimode MW reactor (Synthwave, Milestone, Italy), under an inert atmosphere (N_2) to avoid anthocyanin oxidation. In all the test non-milled BP were extracted, whereas MW offers significant mass transfer improvements. Preliminary tests were performed by investigating the role that temperature and S/L (solid/liquid ratio) play in BP MAE. Two BP-to-ChCl:LA ratios, namely 1 to 20 and 1 to 30, were tested at four different

[64] A. Martynenko, Y. Chen, *J. Food Eng.*, **2016**, *171*, 44–51.

temperatures, 40, 60, 80 and 100 °C. The different solvent ratios were evaluated in consideration of the moderate mass transfer provided by the system, due to vials and magnetic stirrer geometries. Lower BP-to-NADES ratios were not considered because of the inefficient matrix stirring. This set of extractions was performed for 15 min. Results, in terms of TAC, are reported in Figure 10.

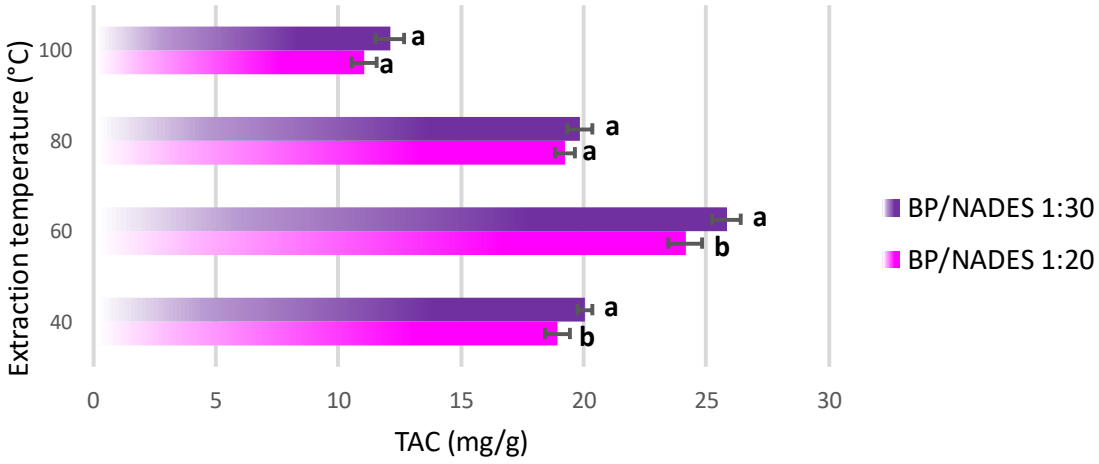

Figure 10. TAC yields of BP anthocyanin MAE under different extraction conditions (S/L ratio and temperature), for 15 min. The presented values followed by different lower-case letters (a–b) are significantly different from each other ($p < 0.05$) according to extraction temperature.

In all cases, the extractions performed with a matrix-to-NADES ratio of 1 to 30 gave the best TAC yields. Regarding temperature, the maximum TAC was achieved at 60 °C, and a decrease was visible at higher temperatures. The optimal MAE parameters were therefore a BP-to-NADES ratio of 1 to 30 and temperature of 60 °C. Under these conditions, a TAC yield of 25.83 mg/g was achieved, which is more than 12% higher than that of the benchmark protocol (55 °C, 2 h, acidified ethanol as solvent). The significantly higher extraction yields and extremely low extraction times can be attributed to the process-intensification effect of MW. In addition, their chemical characteristics indicates that they are well suited to MAE.[38] Extraction efficiencies can be enhanced by this combination of green solvent and MW.

Extraction at 40 °C at a ratio of 1 to 30 yielded 20.03 mg/g TAC, which is quite near to the benchmark yield. The low yields of the MAE performed at 80 and 100 °C suggest the occurrence of anthocyanin degradation at these temperatures. Zheng et al.[65] have investigated anthocyanin MAE from blueberry powder, using acidified ethanol as the solvent. In this study, metabolite degradation was observed at temperatures of approximately above 50 °C, and was influenced by ethanol concentration and process time. Moreover, Pendey and Pendey[66] reported that NADES polarity increases with temperature increase. Therefore, at temp higher than 60 °C, ChCl:LA could be to polar to extract anthocyanins.

The obtained data were used to evaluate anthocyanin MAE kinetics for the best-performing results (60 °C, S/L ratio of 1 to 30). Sampling during the extraction was not feasible due to the reactor security set-up; the irradiation chamber cannot be opened without prior

[65] X. Zheng, X. Xu, C. Liu, Y. Sun, Z. Lin, H. Liu, *Sep. Purif. Technol.*, **2013**, *104*, 17–25.
[66] A. Pandey, S. Pandey, *J. Phys. Chem. B*, **2014**, *118*, 14652–14661.

RT cool-down. A dedicated extraction was therefore carried out for every sample. Optimal MAE parameters were monitored over a time range of 40 min, starting at 2 min and 5 min with subsequent 5 min/step increases. The TAC values for all the performed MAEs are shown in Figure 11.

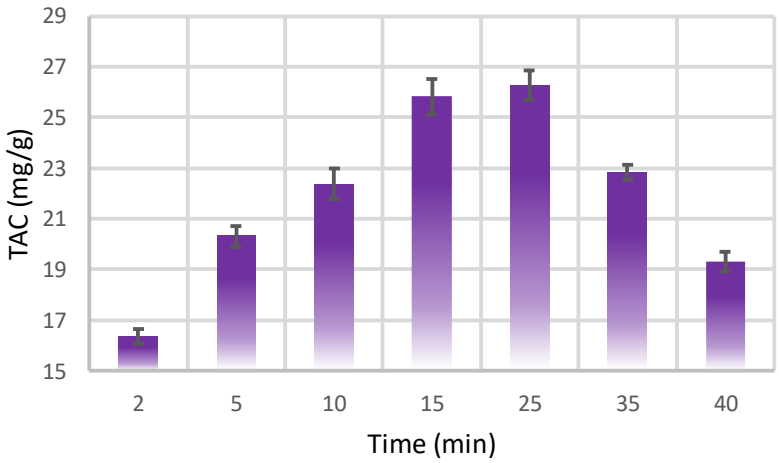

Figure 11. TAC during MAE time, screening with optimised parameters (1 to 30 S/L ratio, 60 °C).

The yield after only 5 min was extremely high at 20.30 mg/g, which is quite close to the result obtained in the benchmark extraction (22.70 mg/g). After 25 min of extraction, the anthocyanin concentration greatly decreased, which suggests that degradation phenomena occurred. As has already been mentioned, anthocyanin are degradable phytochemicals whose stability is strongly dependent on pH, temperature, light, oxygen exposure and the presence of some enzymes.[12,67] Metabolite stability during MAE does not only depend on the extraction temperature, but also involves the energy irradiated towards the sample. Some studies have reported that faster anthocyanin degradation occurs under MW treatment at 700 W than in a conventional thermal bath at 98 °C.[68,69] All tests were performed in a pH 3.5 buffer solution. Their hypothesis was that the MW irradiation induces Baeyer–Villiger oxidation via the nucleophilic attack of hydrogen peroxide, which can cause rapid anthocyanin degradation. However, the degradation mechanism in our case requires further investigation as NADES was used as the solvent.

Finally, the data in Figure 11 were used to describe the extraction kinetics. In particular, Peleg's model was used to describe the kinetics. As Peleg's hyperbolic equation does not take degradation phenomena into consideration, only extraction times up to 25 min were considered for linearization. The obtained model was defined by the following equation:

$$TAC(t) = \frac{t}{0.0652+0.0355\times t} \quad (7)$$

Extraction rate (B_0) at $t = t_0$ had a value of 15.34 mg/g min. The maximum TAC yield, when $t \to \infty$ ($C_{t\to\infty}$) was 28.17 mg/g.

[67] M. Rein, *Copigmentation reactions and color stability of berry anthocyanins*, PhD Thesis, University of Helsinki, Helsinki, Finland, **2005**.
[68] A. Liazid, R. F. Guerrero, E. Cantos, M. Palma, C. G. Barroso, *Food Chem.*, **2011**, *124*, 1238–1243.
[69] M. Zhao, Y. Luo, Y. Li, X. Liu, J. Wu, X. Liao, F. Chen, *Food Chem.*, **2013**, *141*, 3260–3267.

The obtained theoretical curve and the experimental data for MAE are shown in Figure 12.

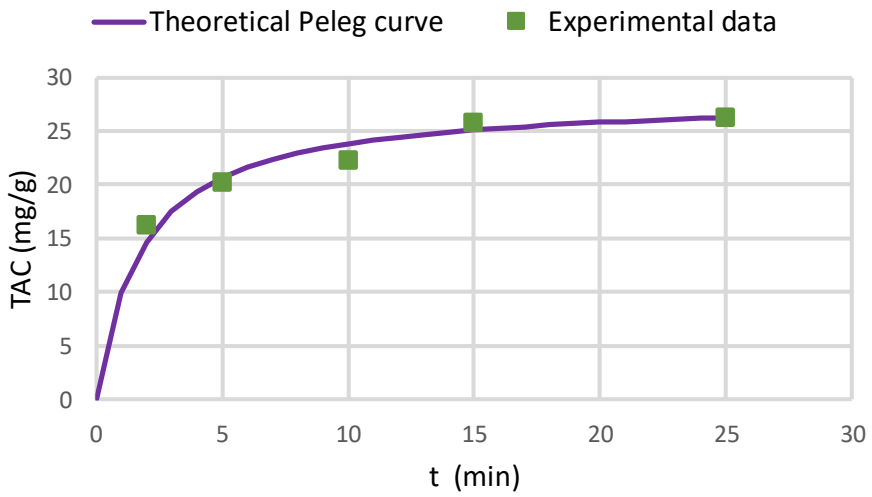

Figure 12. Curve obtained from MAE experimental data, elaboration using Peleg's model.

The extraction rate B_0 was found to be particularly high, leading to 20.30 mg/g TAC after only 5 min of extraction. The maximum TAC yield in $t \to \infty$, $C_{t\to\infty}$ had a significantly higher value than that of the benchmark extraction. Process time optimisation for BP MAE was performed using the theoretical curve, approaching $C_{t\to\infty}$, and the optimised time was found to be 15 min. Afterwards, the TAC did not increase notably, proving that degradation phenomena had a limited impact. The experimental TAC yield at 15 min was 25.83 mg/g, whilst the theoretical yield was 25.03 mg/g. Hence, in summary, the optimal MAE parameters are a BP-to-ChCl:LA ratio of 1 to 30, a temperature of 60 °C and 15 min of extraction.

Conventional comparison – MAE efficiency evaluation

The MAE parameters that led to the highest TAC values were transposed to a heating and stirring system with the aim of evaluating the effect of MW irradiation on BP anthocyanin extraction and comparing it to the conventional extraction methodology. The conventional extractions were performed at 60 °C for 15 min and at a S/L ratio of 1 to 30, in both ChCl:LA and acidified ethanol, to clarify the solvent effect. The results are reported in Table 9.

Table 9. TAC in the ChCl:LA and acidified ethanol conventional extracts and the optimal MW extract.

Extract	TAC (mg/g)
Optimal MAE	25.83
ChCl:LA, conventional	15.88
Acidified EtOH, conventional	19.42

Optimal MAE gave a much higher TAC yield than the conventional extractions that were carried out using NADES and even acidified ethanol. These results strongly confirm the process-intensification capabilities of MW. Conventional extraction performed in ChCl:LA yielded in slightly lower TAC than the extraction performed in acidified ethanol, most probably

due to the ChCl:LA's viscosity, which reduces the diffusion rate. Nevertheless, the combination of MW and NADES offers drastic yield increases and cuts down on process time.

MW degradation test

TAC stability was monitored over extended irradiation because of the observed degradation that occurred during MAE screening (see Figure 11). The phenomenon was therefore investigated by subjecting a known concentration of metabolites to MW irradiation. A starting, anthocyanin-rich fraction was collected from the conventional protocol with ChCl:LA, as described in NADES screening paragraph: this process provided an extract whose components had never been subjected to irradiation. It was therefore possible to avoid underestimating the degradation phenomena, including that of any highly-sensitive compounds. A freshly prepared extract was used to exclude the influence of any possible matrix enzymes or any other matrix compounds that could enhance or reduce anthocyanin stability. After the extraction and subsequent filtration, the extracts were divided into two samples; to replicate ratios of 1 to 20 and 1 to 30. The tests were carried out with MW irradiation for 2 h at 60 °C under an inert N_2 atmosphere to avoid oxidative degradation. The initial and final TAC values were determined, and the results are reported in Figure 13.

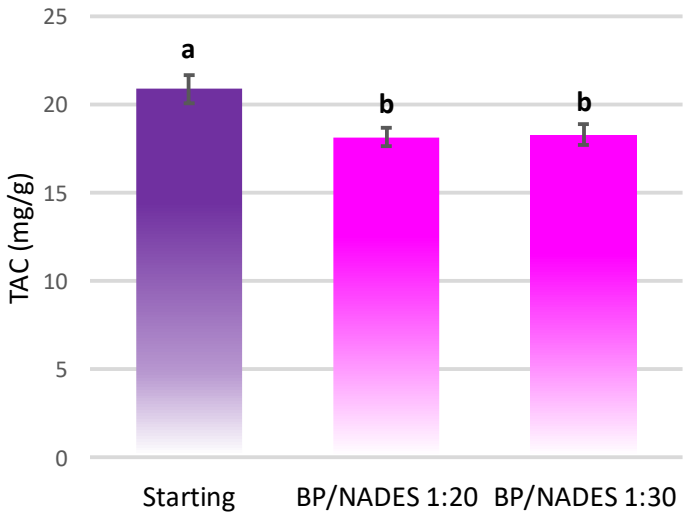

Figure 13. TAC in BP extracts before and after MW irradiation. The presented values followed by different lower-case letters (a–b) are significantly different from each other ($p < 0.05$).

Anthocyanin concentration decreases of 13.06% and 12.38% were observed for the 1-to-20 and 1-to-30 ratios respectively. It can be stated that a higher quantity of ChCl:LA did not have a significant effect on compound stability. When the MW anthocyanin extraction kinetics were monitored between 25 and 40 min, 26.56% of the anthocyanins were degraded. This result suggests that the presence of a matrix may have an effect on anthocyanin stability. For example, Yousefi et al.[70] have investigated anthocyanin degradation in pomegranate juice concentrate. This study confirmed faster colour and anthocyanin alteration when the juice was highly concentrated. In addition, it is well known that enzyme activity and sugar content have an influence on the stability of these metabolites.[64]

[70] S. Yousefi, Z. Emam-Djomeh, S. M. A. Mousavi, G. R. Askari, *Food Bioprocess. Tech.*, **2011**, *5*, 1328–1339.

UAE of BP anthocyanins

Extraction kinetics

The UAE of non-milled BP anthocyanins was performed using two different US probe systems, that supplied 100 W and 500 W, respectively. ChCl:LA, which was previously identified as the optimised solvent, was exploited at a BP-to-NADES ratio of 1 to 20. This S/L amount was introduced as it was the minimum threshold to allow the transmission of acoustic waves through the medium, because of the global system viscosity. Considering the mass-transfer enhancement generated by acoustic cavitation, no other solution consistencies were screened. Sampling was performed every 5 min and the TAC was determined in order to describe the UAE kinetics and evaluate the optimal extraction time. Results are reported in Figure 14.

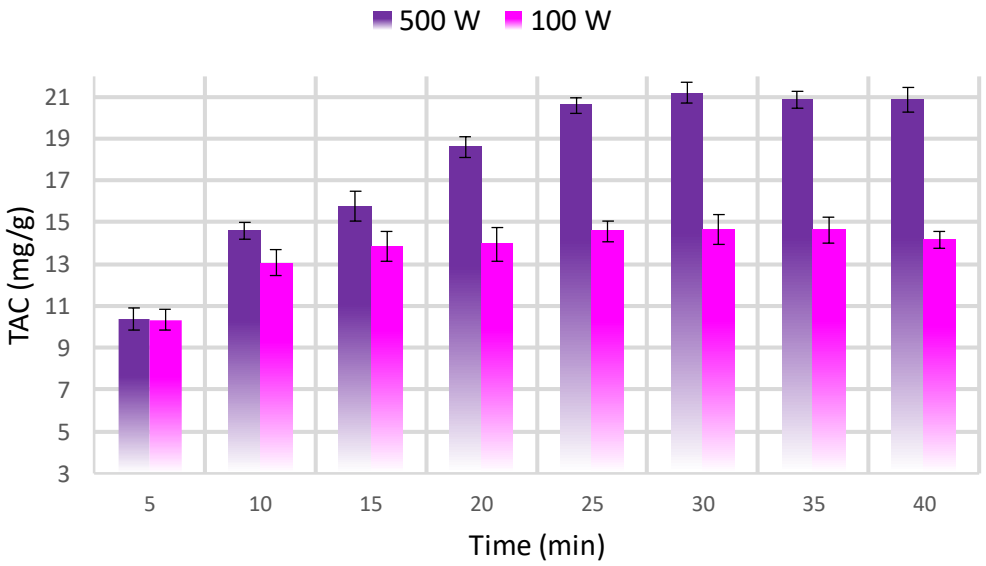

Figure 14. TAC during UAE at different powers (100 W and 500 W).

The results reported for UAE power screening at different times have shown stable extraction trends that are apparently not affected by degradation phenomena. Nevertheless, the extractions were performed at more or less 40 °C, and therefore, based on the literature data, the anthocyanins degradation should not occur. The obtained yields were then processed, according to Peleg's model, to extrapolate kinetics dependencies. For the probe operating at 100 W, the model was defined as follows:

$$TAC(t) = \frac{t}{0.1089+0.0659\times t} \quad (8)$$

B_0 at $t = t_0$ had a value of 9.18 mg/g min. $C_{t\to\infty}$ was 15.17 mg/g.

For UAE operating at 500 W, the obtained model was defined by following equation:

$$TAC(t) = \frac{t}{0.2318+0.0414\times t} \quad (9)$$

B_0 at $t = t_0$ and $C_{t\to\infty}$ were 4.31 mg/g min and 24.15 mg/g, respectively.

The experimental data and theoretical modelled curves are presented in Figure 15, for both US systems.

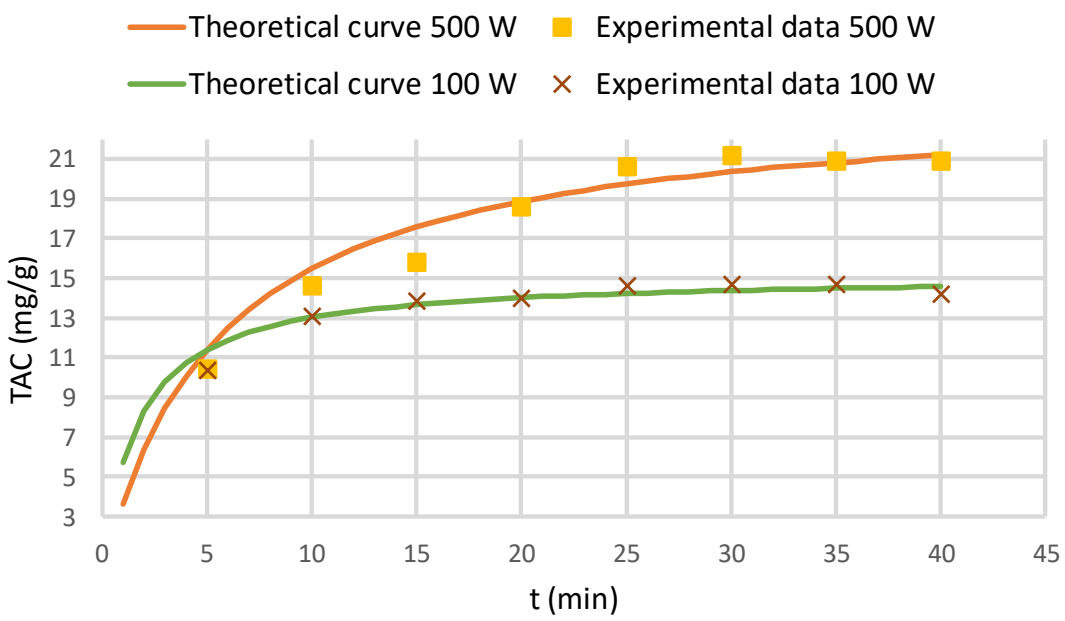

Figure 15. Experimental data and curves resulting from Peleg's model for 100 W and 500 W US systems.

From the theoretical and experimental curves, it is possible to see that the US probe working at 500 W gives a much higher TAC from the first 10 min of the process onwards. The average extraction rate, labelled as B_0, is twice that of the system operating at 100 W (9.18 mg/g min vs. 4.31 mg/g min). These values suggest that the weaker US probe gives a faster increase in TAC yield, but that, on the other hand, it rapidly achieves a lower extraction steady state. In other terms, 500 W intensity led to halved TAC increase over time, but this is counterbalanced by the highest maximum yield. In particular, the parameter that defines maximum TAC ($C_{t\to\infty}$) confirmed that the maximum TAC for the milder UAE is one-third lower than that of the harsher conditions (15.17 mg/g, vs. 24.15 mg/g). The extraction trend is clearly described by the different shapes of the initial section of the curves (first 10 min) in Figure 15. Even though the extraction rate was faster for the 100 W system, the probe operating at 500 W was selected as giving the best performance, due to the final maximum TAC yield in a moderate span of time.

Hence, the optimal process time for the UAE at 500 W was determined from the theoretical curve of Peleg's model. In particular, the closest TAC value to $C_{t\to\infty}$ was achieved at 30 min. At this time, the experimental yield was 21.18 mg/g, which can be compared to the theoretical value of 20.36 mg/g. After this time, no significant TAC increase was detected. Therefore, the optimal UAE was fixed to 30 min of irradiation at 500 W (≈40 °C). The optimised protocol gave a very similar result to that of the benchmark extraction, which was performed for 2 h using acidified ethanol. However, the extraction time of UAE was significantly shorter and the process was carried out using a much greener solvent.

Conventional comparison - UAE efficiency evaluation

The optimal UAE conditions were transposed (extraction temperature 40 °C and time 30 min) to a conventional heating and stirring protocol to evaluate the effect of US irradiation. Silent extractions were performed using both ChCl:LA and acidified ethanol in order to clarify the role of the solvent system. The results, in terms of TAC, are reported in Table 10.

Table 10. TAC in ChCl:LA and acidified ethanol silent extracts and optimal US extract.

Extract	TAC (mg/g)
Optimal MAE	25.83
ChCl:LA, conventional	15.88
Acidified EtOH, conventional	19.42

Silent extraction performed with ChCl:LA gave an approximately halved TAC yield compared to that of the UAE reference. The effect of US is strongly pronounced and can be explained by dramatically enhanced mass transfer. The high viscosity of NADES strongly affects extraction efficiency, and cavitation was demonstrated to be a suitable technology to overcome the issue.

Concerning the evaluation of the solvent system, it was possible to observe how silent extraction with acidified ethanol gave a yield that was quite similar to that of optimal UAE. The explored bio-derived ChCl:LA therefore appears to be competitive with the conventional solvent, without any strong inorganic acid being involved. Moreover, UAE usually provides lower energy consumption than extractions that make use of conventional conductive heating. Furthermore, the removal of a chlorine-based strong inorganic acid appears to be crucial. This environmental friendly extraction protocol is also a cost-effective approach, as dedicated equipment is required to handle and apply hydrochloric acid. Ethanol, besides being a taxed solvent, requires ATEX and anti-flame plants.

US degradation test

The stability issues that were observed during MAE suggest that anthocyanin degradation may even occur during UAE, due to the extreme physical conditions generated by the collapse of cavitation bubbles.[71] Thus, a dedicated investigation was performed at the highest sonication power available (500 W). For this purpose, a starting extract was prepared using the conventional protocol; 2 h using ChCl:LA to avoid US-dependent deterioration during the extraction phase. The extract was filtered and instantly subjected to US irradiation and monitored over the 40-min course, which served as the kinetic screening. During the US degradation test, the temperature was maintained below 40 °C in an effort to reproduce extraction conditions. Sampling was performed every 4 min and the TAC was evaluated.

Nevertheless, no significant TAC alterations were observed throughout the duration of the screening. The TAC during test was 20.91 ± 0.52 mg/g, at all times. Chen et al.[72] have reported that anthocyanin degradation occurred during raspberry UAE when high power was applied, even though the extraction was performed with acidified ethanol, which should act as a stabiliser. The unchanged TAC in our case, despite the high US irradiation being applied, may suggest that ChCl:LA has a stabilisation effect on the BP extract at the working temperature. This hypothesis is supported by Dai et al.,[11] who confirmed the stabilisation activity of eutectic solvents toward anthocyanins. For example, the half-life time of cyanidin (used as a reference) was more than 3 times higher in NADES lactic acid:glucose than in acidified ethanol, at 60 °C.

[71] B. K. Tiwari, A. Patras, N. Brunton, P. J. Cullen, C. P. O'Donnell, *Ultrason. Sonochem.*, **2010**, *17*, 598–604.
[72] F. Chen, Y. Sun, G. Zhao, X. Liao, X. Hu, J. Wu, Z. Wang, *Ultrason. Sonochem.*, **2007**, *14*, 767–778.

Anthocyanins isolation and NADES recycling

The anthocyanins isolation from the ChCl:LA extract and ChCl:LA recycling was performed as described in section 3.1. *Combined ultrasound and microwave irradiation as an efficient technology for grape-stalk anthocyanin recovery*. Briefly, the absorption chromatography was performed where the diluted extract (with 50%) was loaded onto the macroporous resin, which has a high affinity towards polyphenols, and the anthocyanins adsorbed onto this resin, whilst the NADES simply eluted with water.

Finally, 79.48% of the NADES was recovered, and was sufficiently pure for recycling in further extractions. TAC recovery efficiency was 72.55% suggesting TAC loss. The data in the literature also report the loss of some metabolites during the recovery process. Wang et al.[73] have performed polyphenol recovery from fig-leaf extracts obtained in Gly:Xylose:Fructose NADES using a macroporous resin. The recovery of individual polyphenolic compounds varied from 75.3–85.5%. Zhuang et al.[74] used different macroporous resins for the recovery of flavonoids from *Platycladi Cacumen* ChCl:LA extracts. The noted recovery efficiency varied between 77.44% and 98.92% depending on the flavonoid monitored and the resin used. In presented work, the TAC loss during recovery can be explained by the partial elution of anthocyanins together with the NADES/water fraction, as the recycled NADES had a slightly pink colour. However, the presence of anthocyanins in the recycled ChCl:LA should not be a problem, as this solvent should be recycled in the BP extraction process. As LA has shown low cytotoxicity,[59,75] its presence in purified extracts should not be an issue when it comes to implementation in food and cosmetic formulations. This exploratory result encourages further investigation into the use of purification steps with resins.

Extracts antiproliferative activity and cytotoxicity

Blueberries are one of the richest possible sources of anthocyanins,[76] whose chemo-protective properties have been extensively reviewed in *in-vitro* studies and animal models.[6] However, data on the biological activity of polyphenols, including their antiproliferative activity, in NADES are still quite scarce. Generally speaking, DES composed of ChCl and organic acid HBDs display low toxicity.[77] Moreover, both ChCl and LA are substances that are generally recognised as safe (GRAS) by the FDA (Food and Drug Administration).[78] It therefore seems that this NADES could easily be implemented in nutraceutical and pharmaceutical formulations.

In present work, an MTS assay was performed on human tumour cells (HeLa) against human keratinocyte cell line (HaCaT) to investigate the antiproliferative activity of the optimised BP extracts, in accordance with their extraction technologies. The conventional ethanolic sample was taken as a reference. HeLa cells after treatment are shown in Figure 16. The obtained MTS test results are shown on Figure 17.

[73] T. Wang, J. Jiao, Q. Y. Gai, P. Wang, N. Guo, L. L. Niu, Y. J. Fu, *J. Pharm. Biomed. Anal.*, **2017**, *145*, 339–345.
[74] B. Zhuang, L. L. Dou, P. Li, E. H. Liu, *J. Pharm. Biomed. Anal.*, **2017**, *134*, 214–219.
[75] Y. He, W. Wang, J. Ding, *Chin. Sci. Bull.*, **2013**, *58*, 2404–2412.
[76] Z. Diaconeasa, L. Leopold, D. Rugină, H. Ayvaz, C. Socaciu, *Int. J. Mol. Sci.*, **2015**, *16*, 2352–2365.
[77] R. Rafael Alcalde, A. Gutiérrez, M. Atilhan, S. Aparicio, *J. Mol. Liq.*, **2019**, *290*, 110916:1-110916:12.
[78] FDA. Available online: https://www.accessdata.fda.gov/scripts/fdcc/?set=SCOGS.

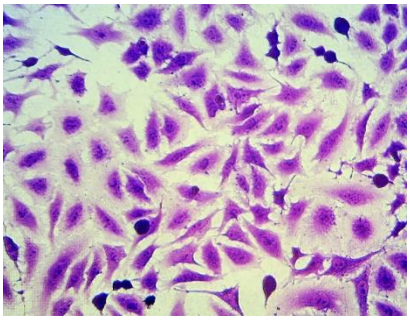

Figure 16. HeLa cells after 72 h treatment with MAE BP extract.

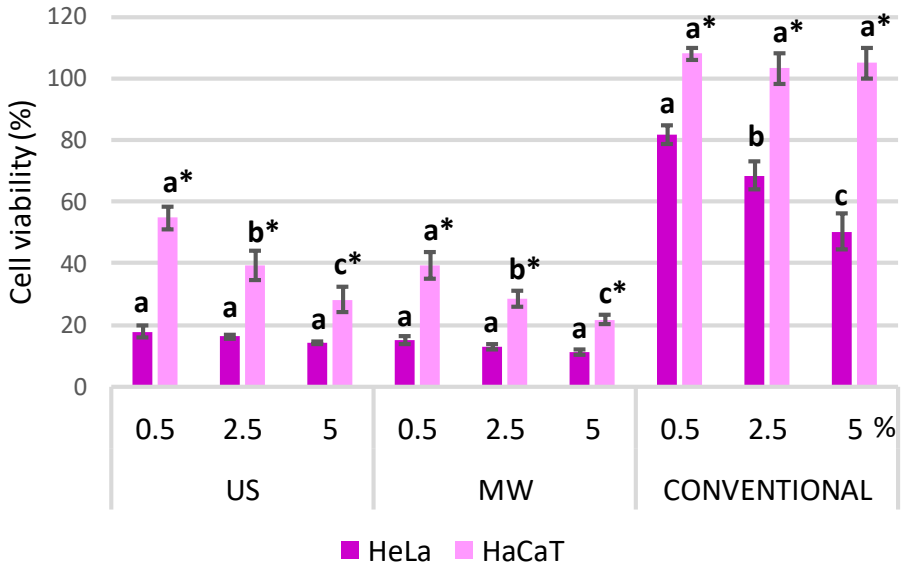

Figure 17. Effect of BP extracts, prepared using the different methodologies, on HeLa and HaCaT cell viability when the extracts were added in final volume ratios of 0.5, 2.5 and 5% (v/v). Statistically different data ($p < 0.05$) are designated by lower-case letters (a–c or a*–c*, HeLa and HaCaT, respectively), according to volume ratios, whereas the statistical difference among the cell lines are marked by a graphical signature (*).

The results reported confirmed that NADES can emphasise the biological activity of the extracts. In comparison with the conventional BP extract, the US and MW extracts possess significantly stronger antiproliferative activities. Moreover, the conventional extract added to the culture medium had a considerably higher TAC concentration (mg anthocayanins/L) in respect to the ChCl:LA extracts, as reported in Table 11, underlining the reported positive effect of NADES on the investigated biological activity.

Table 11. TAC added to the culture medium for HeLa and HaCaT treatment. Reported for every volumetric ratio tested (0.5, 2.5 and 5% v/v).

Extract	TAC in 0.5% (mg/L)	TAC in 2.5% (mg/L)	TAC in 5% (mg/L)
US	5.30	26.48	52.95
MW	4.31	21.53	43.05
Conventional	7.57	37.83	75.67

However, the conventional extract is not cytotoxic for human skin cells (HaCaT), while the US and MW extract demonstrate cytotoxicity in HaCaT cells. Nevertheless, the effects of the US and MW extracts on tumour-cell growth are considerably greater than their effect on keratinocytes. Different activity trends have been seen in different cell lines:

- In HeLa cells, the US and MW extracts showed almost unvaried inhibitory effects regardless of the applied volume ratio, while the conventional sample showed dose-dependent behaviour.

- The HaCaT cell line was characterised by less pronounced growth inhibition than in Hela cells, which is dose-dependent. The hydroalcoholic extract did not affect cell growth.

Comparable results were obtained by Radošević et al.,[27] who evaluated the antiproliferative activity of grape pomace extracts that were prepared using different NADES and MeOH. The conventional MeOH extract demonstrated very low antiproliferative activity, unlike the sample prepared with ChCl:MA. Similar results were achieved in tumour cells by Panić et al.,[24] who investigated grape and olive pomace extracts, obtained by means of NADES systems. Both substrates were prepared in ChCl:CA, using EtOH as the benchmark. The latter showed much lower inhibitory effects than the eutectic/extract mixtures.

Some further considerations as to the acidic pH of the tested BP samples are required. When the extract is added, the culture medium is not able to maintain physiological pH, even at the lowest volume ratios. In order to verify that the observed activity arose from the extract composition and not from its pH, dedicated test was performed. Hence, to stabilize the culture medium the pH was adjusted up to 6 and the viability of HeLa cells was measured. Cell treatment with these precautions gave comparable results as the ones with the original pH, as reported in Figure 18. The viability variations were slightly dissimilar, but not statistically significant.

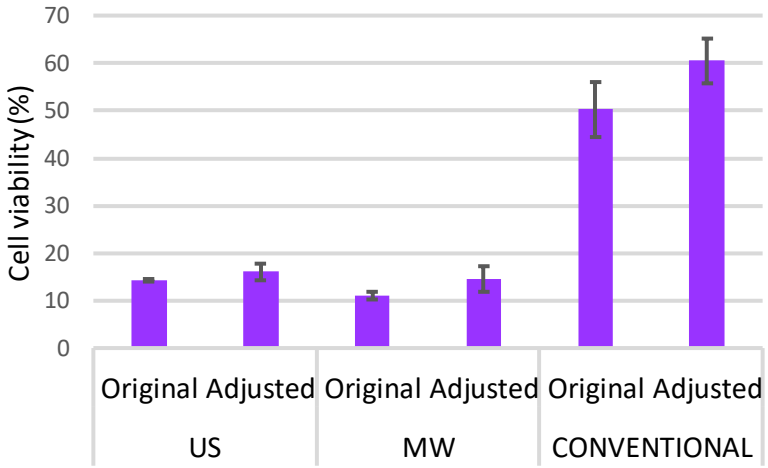

Figure 18. HeLa cell viability. Original extracts pH vs. pH-adjustment, for different extraction techniques. There is no statistical difference between the results, as determined by Tukey's HSD test.

In support of these results, several studies have shown that an *in-vivo* extracellular matrix of the tumour-cell microenvironment has a more acidic pH than normal tissue.[79,80] For normal

[79] K. Smallbone, D. J. Gavaghan, R. A. Gatenby, P. K. Maini, *J. Theor. Biol.*, **2005**, *235*, 476–484.
[80] M. Stubbs, P. M. J. McSheehy, J. R. Griffiths, C. L. Bashford, *Mol. Med. Today*, **2000**, *6*, 15–19.

human cells, p53-dependent apoptosis pathways are activated in environments with pH lower than 7, and this finally results in cell death. Tumour cells, on the other hand, have mutant p53 genes, and usually exhibit a maximum proliferation rate in a relatively acidic medium, at around pH 6.8.[79] Since the results suggest that extracts with adjusted pH still have an anti-proliferative effect on tumour cell line, it can be concluded that extract pH is not involved in the cell growth inhibition mechanism.

Cell-death evaluation

Mammalian cell-death types are most widely defined into two major classes: apoptosis and necrosis.[81] Necrosis is an un-regulated (accidental) type of cell death and is caused by acute physicochemical injuries. Apoptosis is the most commonly studied programmed cell death. It maintains the equilibrium between growing and dead cells and prevents damaged and malignant cell growth. Nevertheless, other programmed cell-death types, autophagy and programmed necrosis, are known.[82,83,84,85] Besides uncontrolled cell proliferation, one of the prominent characteristics of cancer cells is their resistance to apoptosis, making it one of the most essential factors for the monitoring of chemo-protective effects.[54]

Whereas the antiproliferative evaluation demonstrated the activity of the BP extracts, the cell-death type was evaluated on a Muse® Cell Analyser by a flow cytometry using a Muse™ Annexin V & Dead Cell Kit. The aim was to discover whether BP extracts act *via* a predominant cell-death mechanism. The results are depicted in Figures 19 and 20 for both HeLa and HaCaT cell lines, respectively.

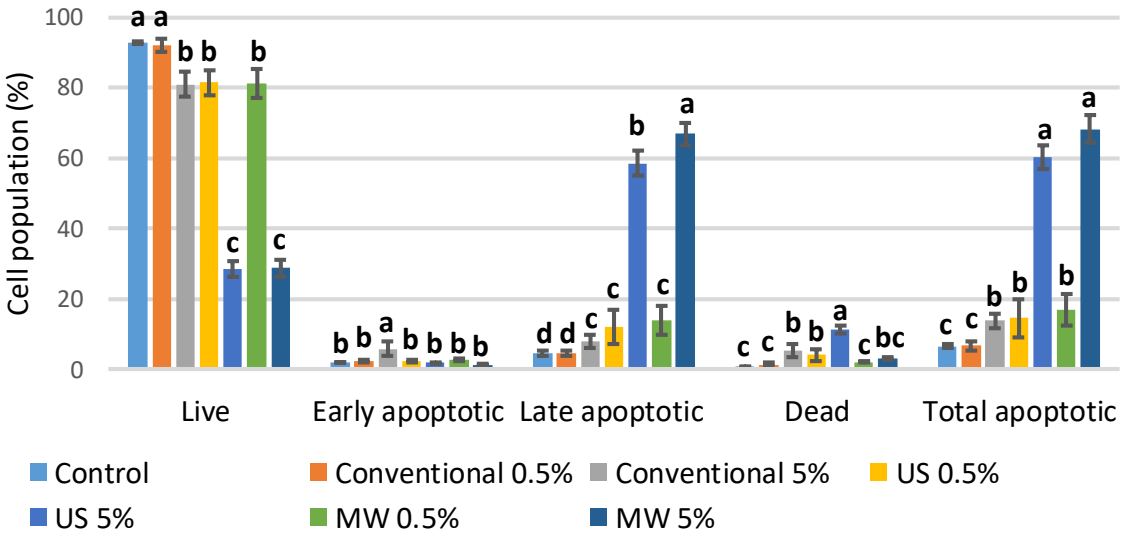

Figure 19. Cell-death evaluation for HeLa cells after treatment with BP extracts. Statistically different data according to cell population type among control and obtained extracts ($p < 0.05$) are designated by lower-case letters (a–d).

[81] R. S. Hotchkiss, A. Strasser, E. Jonathan J. E. McDunn, P. E. Swanson, *N. Engl. J. Med.*, **2009**, *361*, 1570–1583.
[82] O. Krysko, T. Løve Aaes, V. E. Kagan, K. D'Herde, C. Bachert, L. Leybaert, P. Vandenabeele, D. V. Krysko, *Immunol. Rev.*, **2017**, *280*, 207–219.
[83] L. Ouyang, Z. Shi, S. Zhao, F. T. Wang, T. T. Zhou, B. Liu, J. K. Bao, *Cell Prolif.*, **2012**, *45*, 487–498.
[84] Y. Sun, Z. L. Peng, *Postgrad. Med. J.*, **2009**, *85*, 134–140.
[85] M. C. Lopez de las Hazas, J. I. Mosele, A. Macia, A. I. Ludwig, M. J. Motilva, *J. Agric. Food Chem.*, **2016**, *65*, 6477−6487.

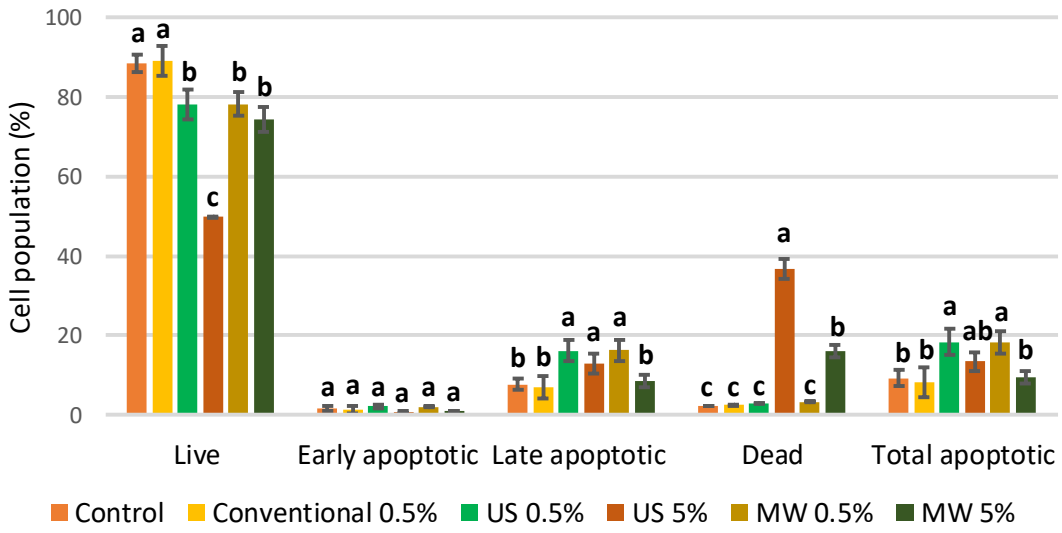

Figure 20. Cell-death evaluation for HaCaT cells after treatment with BP extracts. Statistically different data according to cell population type among control and obtained extracts ($p < 0.05$) are designated by lower-case letters (a–c).

For the HeLa cell line, the conventional EtOH extract, added at a 0.5%, did not have a significant effect on cell population, while the higher concentration (5%) caused a slight increase in apoptosis. For the US and MW extracts prepared in ChCl:LA, low concentrations (0.5%) led to a negligible increase in apoptotic calls. Nevertheless, the apoptotic cell population was significantly increased, up to 60%, when these extracts were added at a 5%. Therefore, it can be concluded that apoptosis plays the main role in tumour-cell death when BP extracts are added.

Similarly to tumour cells, low-ratio extract additions did not have an effect on the HaCaT cell population, regardless of the extract source (conventional, ChCl:LA in US and MW, see Figure 19). However, cell death by accidental necrosis occurred when the US and MW extracts were added to the culture medium at a 5%, v/v. Specifically, US prompted a great increase in the necrotic cell population, whereas a slight increase was observed for the MW sample.

Such difference in type of cell death between tumour and normal cells can be explained by already mentioned fact that tumour cells are less sensitive to acidic pH of its surrounding media, whereas for normal HaCaT cells acidification of culture media due to addition of 5% volume US and MW is probably a physicochemical injury which led to necrosis.

The cell-death evaluation results confirmed that the conventional BP extract does not possess antiproliferative activity that is as strong as that of the BP extracts prepared in NADES. Moreover, the primary cause of tumour cell-death is apoptosis. Unfortunately, the BP extracts prepared using enabling technologies prompted an increase in necrotic cells in normal skin cells, which was much more prominent in the extracts prepared using US. However, the apoptotic cell population in the HeLa cell line was much higher than the necrotic cell population in normal skin cells, especially with the MW extract, implying that these extracts could serve as innovative agents in cancer treatment. Certainly, accurate *in-vivo* tests are required to furtherly evaluate the chemo-protective and anti-cancer effects of the investigated systems.

3.2.2. Conclusions

Bioactive compounds from plant materials are currently gaining increasing amounts of attention mainly because of their health-promoting benefits. At the same time, since environmental pollution has become a global problem, the exploitation of wastes and by-products has become a favourable alternative to their disposal. Hence, various plant residues can be exploited as sources of phytochemicals. In this study, the residues of blueberry processing have therefore been used as a source of anthocyanins, which are valuable metabolites that possess a wide range of biological activity.

Academia and industry are both challenged to develop new green extraction methodologies that can reduce environmental impact. The present study addressed this topic with two strategies:

- Investigating the use of NADES, a novel class of sustainable solvents;
- Evaluating the process intensification of two enabling technologies, specifically MW and US.

Specifically, five NADES have been tested in conventional BP anthocyanin extraction, with an acidified hydroalcoholic solution as the benchmark. The shelf-life of the extracts was monitored over 9 months at between +4 and +8 °C, which confirmed that all the samples have similar stability, except the glucose-based one. Moreover, a supplementary evaluation of solvent-dependent degradation was performed by PPC. This test showed that ChCl:LA, ChCl:MA and ChCl:CA provide similar anthocyanin stability compared to the hydroalcoholic reference, which highlights them as promising candidates to replace conventional solvents. ChCl:LA was finally selected as the most suitable NADES based on extraction efficiency, cost, viscosity and toxicity.

In order to pursue higher process efficiency, two non-conventional technologies, namely MAE and UAE, have been explored for use with ChCl:LA. The extraction kinetics were described using Peleg's model and the optimal parameters were determined. MAE led to 25.83 mg/g TAC in 15 min at 60 °C, with a BP-to-ChCl:LA ratio of 1:30. UAE achieved 21.18 mg/g TAC after 30 min of sonication at 500 W power. The role played by the technologies and NADES was verified via comparisons with conventional procedures. The results supported the innovative approaches and showed that they were able to enhance productivity and save time. A preliminary study on the MW- and US-mediated degradation of anthocyanins was included, and a matrix effect, which is predominant in MAE, was observed.

In order to fulfil the requirements of the circular economy approach, an explorative test on anthocyanin concentration and NADES recycling was performed using a macroporous resin. A final TAC recovery of 72.55% was achieved, together with solvent reuse of 79.48%.

Finally, the antiproliferative activity of conventional and ChCl:LA (MAE and UAE) extracts was determined *in-vitro* in human tumour cell line. The HeLa cell line was tested and compared to human skin cells (HaCaT) in MTS assays, and it was found that the MAE and UAE samples possess significantly stronger antiproliferative activity than the conventional BP extract. Moreover, the growth inhibition effect is considerably greater in tumour cells than in skin cells. These results confirm that NADES are able to emphasise the biological effects of recovered phytochemicals. Cell-death type was determined by flow cytometry, using a Muse™ Annexin V & Dead Cell Kit, to provide insight into the cell-growth inhibition mechanism. The

experiment detected that apoptosis was the primary tumour cell-death cause indicating the possibility to induce destruction of tumour cells by obtained extracts. Further *in-vivo* tests are required to better understand and verify the proposed biological activity.

In conclusion, this study has developed two new extraction methods for the recovery of anthocyanin-rich extracts with enhanced antiproliferative activity. Moreover, the results demonstrated how US and MW can provide extensive extraction efficiency intensification compared to the conventional extraction protocol. These results pave the way for the development of new pharmacologically active compounds that are prepared using innovative green procedures.

3.2.3. Experimental part

Plant material

Frozen blueberry (*Vaccinium myrtillus* L.) peels were provided by INDENA S.p.a. (Settala, MI - Italy) as industrial residues. The matrix was stored at -18 °C.

Water content in plant material

The water content in frozen BP was determined by freeze drying. The plant material was freeze-dried for 24 h using a LyoQuest–85 lyophilizer (Telstar, Madrid, Spain). The analyses were performed in triplicate.

Plant material cryo-milling

BP were frozen with liquid nitrogen and subsequently milled in a professional blender (HGBTWTS360, Waring Blender). The milled BP were stored at -18 °C.

Conventional BP anthocyanins extraction

The conventional GS anthocyanin extraction protocol was performed using acidified ethanol (0.8% of HCl in 60% hydroalcoholic solution) according to Dai et al.[11] and Panić et al.[38], as described in Experimental section of *3.1. Combined ultrasound and microwave irradiation as an efficient technology for grape-stalk anthocyanin recovery* part. Only modification was S/L ratio, which in this case was 1 to 15.

NADES preparation

NADES were prepared as described in Experimental section of *3.1. Combined ultrasound and microwave irradiation as an efficient technology for grape-stalk anthocyanin recovery* part. However, the NADES tested in this study were prepared with 22% (v/v) of water.

NADES screening

All the NADES with water contents of 22% were tested for anthocyanin extraction from cryo-milled BP to find the optimal HBD. The BP-to-NADES ratio was 1 to 15 and the final water content in NADES was 25% (v/v).

The process parameters from a previous investigation into the use of NADES in anthocyanin extraction[11,38], were adapted. In particular, extractions were performed with conventional heating and stirring approach for 2 h at 55 °C and at 200 rpm. The obtained

extract was filtered. Every extraction was performed in triplicate and stored at -18 °C before TAC determination.

NADES systems shelf-life

The shelf-life of the NADES BP extracts was evaluated in order to identify the optimal NADES. The products of the NADES screening were stored between +4 and +8 °C and the TAC was measured in triplicate after 1 week, 3 weeks and 9 months. Results are expressed as the average ± SD.

PPC determination

Percentage polymeric colour (PPC) determination was used to evaluate the monomeric/polymeric ratios of anthocyanins and anthocyanin-like compounds. The acidified ethanol, ChCl:LA, ChCl:MA and ChCl:Gly extracts that were obtained *via* the conventional extraction method were evaluated using the method described in Giusti, Wrolstad.[36] 2.8 mL of the diluted sample was placed in the test tubes, and 200 µL 20% (w/v) of potassium metabisulfite solution was added to one parallel set, and 200 µL of water was added to the other. After 15 min, the absorbance of the resulting solutions was measured at 420 nm, λvis-max, and 700 nm in a 1 cm quartz cuvette, using a Cary 60 UV-Vis spectrophotometer, against a blank. The colour density (CD) of the control sample (treated with water) was calculated as follows:

$$CD = [(A_{420\,nm} - A_{700\,nm}) + (A_{\lambda\,vis-max} - A_{700\,nm}) * DF \quad (10)$$

where DF is the dilution factor. The polymeric colour (PC) of the bisulphite bleached sample is defined as follows:

$$PC = [(A_{420\,nm} - A_{700\,nm}) + (A_{\lambda\,vis-max} - A_{700\,nm}) * DF \quad (11)$$

Finally, PPC is calculated according to the following equation:

$$PPC = \frac{PC}{CD} * 100 \quad (12)$$

All analyses were performed in triplicate and results expressed as the average.

MAE of BP anthocyanins

MAE was performed in SynthWAVE (Milestone, Italy), which is a pressure-resistant multimode microwave (MW) reactor that is capable of feeding inert gas (N_2) into the system. For each test, appropriate purging with nitrogen was carried out three times in order to reduce oxidising degradation. The reaction chamber was finally pressurised with 2 bars of N_2. All screenings were carried out at 500 W of irradiation, with a heating step of 3 min.

MAE was firstly optimised in terms of extraction temperature and non-milled-BP-to-optimal-NADES ratio. Tests at 40, 60, 80 and 100 °C were performed at BP-to-NADES ratios of both 1:20 and 1:30, and the extraction time was kept at 15 min. The crude solutions were filtered and TAC was determined.

After the temperature and BP-to-NADES ratio screenings, MAE kinetics were evaluated under the optimal conditions. The instrumental set-up (cool-down required before the reactor could be opened) meant that on-going sampling could not be performed. Dedicated extractions were therefore carried out at each sampling time; starting with 2 and 5 min and then progressive increases of 5 min up to 40 min. Crude solutions were filtered under vacuum.

Every extraction was performed in triplicate and stored at -18 °C before TAC determination. The obtained results, expressed as the average ± SD, were used to describe MAE kinetics using Peleg's model.

Conventional comparison – MAE efficiency evaluation

In order to be able to evaluate the role of MW irradiation, the MAE that was optimised was reproduced using conventional heating and stirring extraction, with both the optimal NADES and acidified ethanol. In detail, the extraction was performed for 15 min at 60 °C and the non-milled-BP-to-solvent ratio was 1 to 30. The extracts were than filtered under vacuum. Every extraction was performed in triplicate and stored at -18 °C before TAC determination. The obtained results were expressed as the average.

MW degradation test

The stability of the anthocyanins was monitored during MW irradiation because of their high sensitivity.[86,87] Firstly, conventional heating and stirring BP extraction was performed as previously described (*Conventional BP anthocyanins extraction*), using the optimal NADES as the solvent. The extracts were then filtered and the TAC was determined. The obtained extract was then divided in three samples and subjected to MW irradiation. The test lasted for 2 h and the temperature was set at 60 °C. The TAC was then evaluated again expressing the results as the average ± SD.

UAE of BP anthocyanins

UAE was performed using two different probe systems, which were two immersion horns. The first device (Danacamerini sas, Turin, Italy) was set at 100 W (20 kHz), whilst the second (HNG-20500-SP, Hainertec Suzhou, China) supplied 500 W (20 kHz). In both extraction processes, the non-milled-BP-to-optimal-NADES ratio was 1 to 20, the average temperature was around 40 °C and the sampling of roughly 1 mL was performed every 5 min. Total extraction time was 40 min. Every sample was filtered under vacuum. Every extraction was performed in triplicate and stored at -18 °C before TAC determination. The obtained results, expressed as the average ± SD, were used to describe the UAE's kinetics using Peleg's model.

Conventional comparison - UAE efficiency evaluation

To evaluate the role of US irradiation, comparison tests were performed under silent conditions. The UAE that was optimised was reproduced using conventional heating and stirring extraction, with both optimal NADES and acidified ethanol. In detail, the extraction was performed for 30 min at 40 °C with a non-milled-BP-to-solvent ratio of 1 to 20. Both extracts were then filtered under vacuum. Every extraction was performed in triplicate and stored at -18 °C before TAC determination. The obtained results were expressed as the average.

US degradation test

The stability of anthocyanins under sonication was monitored because of the risk of degradation.[86,87] Firstly, conventional heating and stirring BP extraction was performed (as described in *Conventional BP anthocyanins extraction*), using optimal NADES as the solvent. The obtained extract was filtered and the TAC was evaluated. The same extract was divided

[86] E. V. Petersson, J. Liu, P. J. R. Sjöberg, R. Danielsson, C. Turner, *Anal. Chim. Acta*, **2010**, *663*, 27–32.
[87] L. G. D'Alessandro, K. Dimitrov, P. Vauchel, I. Nikov, *Chem. Eng. Res. Des.*, **2014**, *92*, 1818–1826.

in three samples and then submitted to an US degradation test using the harshest conditions available, namely 500 W immersion-horn irradiation. The temperature was maintained at around 40 °C, mimicking that of UAE. Sampling of roughly 1 mL was performed every 4 min. The total test time was 40 min. The TAC was then evaluated again for every sample, expressing the results as the average ± SD.

Peleg's model

As mentioned above, UAE and MAE kinetics were expressed using Peleg's model,[88] as described in *Chapter 2: 2.1. Green extraction technologies for cocoa been shell polyphenols recovery*.

TAC determination

TAC was determined as described in Experimental section of *3.1. Combined ultrasound and microwave irradiation as an efficient technology for grape-stalk anthocyanin recovery* part.

Anthocyanins isolation and NADES recycling

Anthocyanins were isolated from the ChCl:LA extract and NADES was recycled according to the protocol described in Experimental section of *3.1. Combined ultrasound and microwave irradiation as an efficient technology for grape-stalk anthocyanin recovery* part.

Antiproliferative activity and cytotoxicity determination

The antiproliferative activity and cytotoxicity of the BP extracts that were prepared in ChCl:LA, both using the above-described enabling technologies and the conventional method, were evaluated in vitro using the CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay, as described in Panić et al..[24] Two human adherent cell lines were used for this test; tumour HeLa cells derived from the cervical adenocarcinoma, and normal human keratinocyte cells (HaCaT). Both cell lines were cultivated in DMEM supplemented with 5% (v/v) FBS and 1% (v/v) antibiotic/antimitotic solution, and were kept in BioLite petri dishes (Thermo Fisher Scientific, USA) in an incubator with a humidified atmosphere and 5%, v/v CO_2 at 37 °C. Single tests on the antiproliferative activity and cytotoxicity of the extracts were performed in 96-well plates (Thermo Fisher Scientific, USA) that have been seeded with exponentially growing cells at an initial concentration of 3×10^4 cells per well in 100 µL of culture media. After 24 h of incubation, under the cell cultivation conditions, the cells were treated with the extracts. The raw BP extracts that were prepared with ChCl:LA under US and MW, and the conventional BP extract were all diluted in the culture medium and then applied to the cells, resulting in final volume ratios of 0.5%, 2% and 5% (v/v). A double control-set was performed: i) the vehicle-treated cells, for ChCl:LA and acidic ethanol in same volume ratio as the extracts; ii) non-treated cells. In order to verify that the biological activity was not affected by low solvent pH, dedicated screening was performed on tumour cells adjusting the extract pH, testing the higher volume ratio previously applied (5% v/v). Treatment lasted for 72 h in the incubator, and was followed by the MTS assay. The assay was performed according to the manufacturer's instructions with a few modifications. 10 µL of MTS reagent was added to each well and the cells were incubated for 3 h, and the absorbance was measured at 492 nm on the microplate reader (Tecan, Manneford, Switzerland). Cell-viability percentage was expressed as the ratio

[88] M. Peleg, *J. Food Sci.*, **1988**, *53*, 1217–1219.

between the absorbances of the treated versus control cells. The tests were performed in triplicate with four parallels for each volume ratio.

Cell-death evaluation by flow cytometry

The quantitative analysis of live, apoptotic and dead cells that had been treated with the BP extracts, those obtained in ChCl:LA under US and MW and the conventional extract, was carried out on a Muse® Cell Analyser (EMD Milipore Corporation, Massachusetts, USA) using the Muse™ Annexin V & Dead Cell Kit according to the manufacturer's specifications. HeLa and HaCaT cells were seeded individually into 6-well culture plates at an initial concentration of 5×10^4 cells mL^{-1} (2 mL per well). After incubation overnight, the cells were treated with BP extracts that had been prepared with ChCl:LA under US and MW and the conventional BP extract, individually, for 72 h. HeLa cells were treated with 0.5 and 5% v/v of all the aforementioned extracts. The treatment of HaCaT cells was performed with 0.5 and 5% v/v of US and MW extracts individually, and with a 0.5% v/v of the conventional extract. After treatment, both the floating and adherent cells were collected using a trypsin-EDTA solution to dissociate the cells from the culture plates and give single-cell suspensions. The collected suspensions were centrifuged (30,000 g) for 15 min and the separated cells were suspended in the cell culture medium to adjust the cell concentration according to the manufacturer's protocol. 100 µL aliquots of the cell suspension were then added to 100 µL of Muse™ Annexin V & Dead Cell Reagent and incubated in the dark at room temperature (RT) for 20 min. Subsequently, the cells were analysed using the Muse® Cell Analyser. The Muse™ Annexin V & Dead Cell Assay detects phosphatidylserine on the external membranes of apoptotic cells via Annexin V-PE binding, while 7-aminoactinomycin D (7-AAD) is used as a dead-cell marker. Therefore, this assay is able to detect four distinctive cell populations: live (Annexin V negative and 7-AAD negative), early apoptotic (Annexin V positive and 7-AAD negative), late-stage apoptotic (Annexin V positive and 7-AAD positive) and dead cells (Annexin V negative and 7-AAD positive). Each sample was tested in duplicate and each experiment was performed twice.

Statistical analysis

Statistical analysis was performed as described in *Chapter 2: 2.1. Green extraction technologies for cocoa been shell polyphenols recovery*.

General conclusions

The present PhD thesis showcased successful green phytochemicals extraction processes. Emerging technologies, in particular US and MW, were combined with different green solvents aiming to achieve a green process intensification. In most cases these novel protocols showed to be promising alternatives to traditional procedures, even in a pilot scale. Their implementation to industrial processes could reduce the environmental impact, ensure economic benefits in the form of lower energy consumption, and reduced operating time, providing safer extracts with higher biological activity. In addition, as suggested by circular economy concept, cocoa, wine and juice industry by-products were also tested for the extraction of valuable bioactive compounds. It is hoped that the promising case studies herein presented could help the diffusion on non-conventional technologies also at industrial scale, paving the way to zero waste productions.

Supporting materials

1. Cannabis volatiles GC-MS analysis

Herein are reported acquired GC-MS chromatogram both for optimal MAHD test and CHD volatile fractions, respectively (Figure 1 and 2). All identifications with relative retention times are reported in Table 1. Fragmentation of each detected component is reported in Figure 3-58. In addition, in Figures 59 and 60 are shown CBD and THC mass fragmentation spectra.

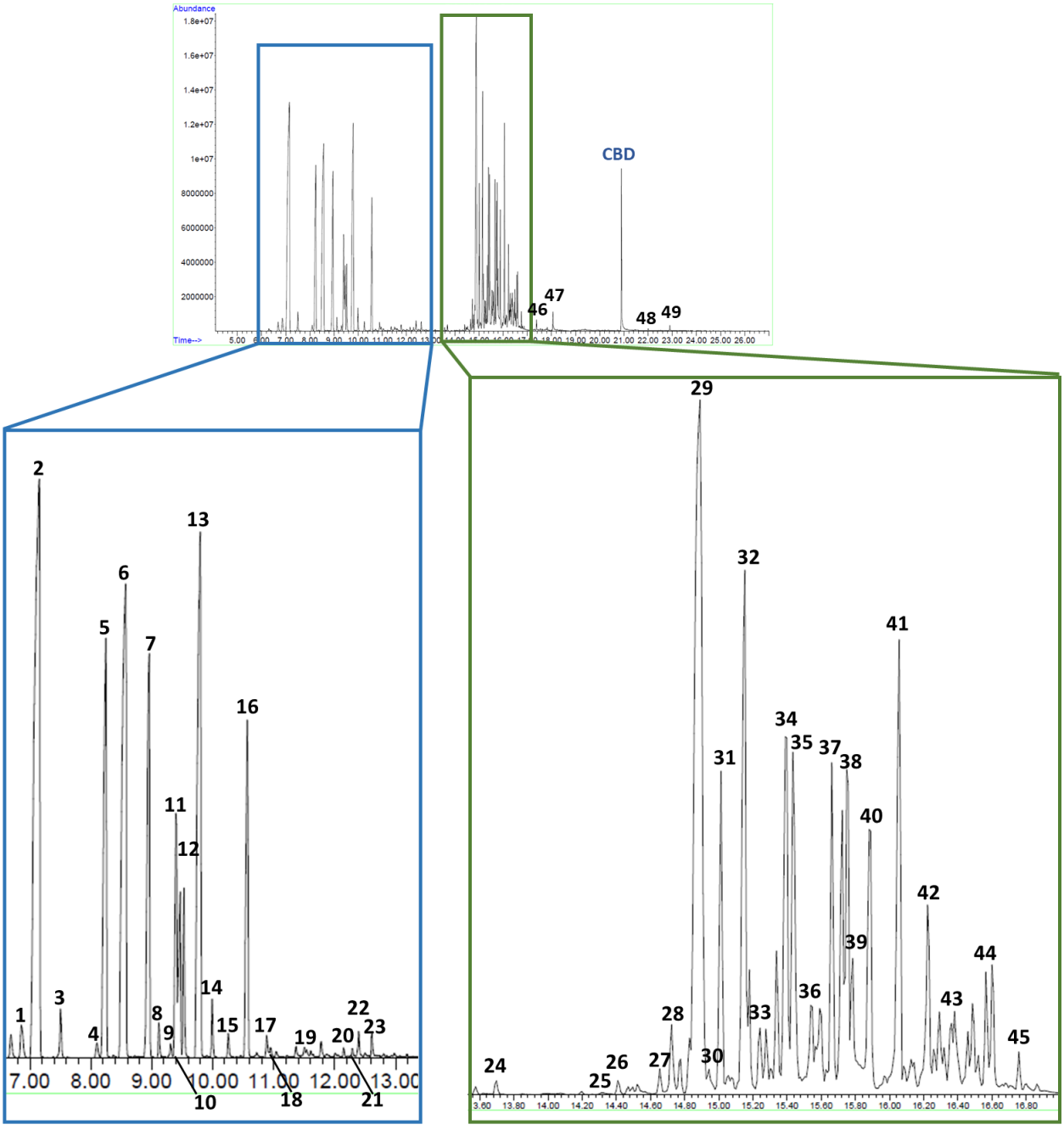

Figure 1. GC-MS analysis of MW-assisted hydrodistillate of *Cannabis sativa* L.. TIC detail from 6 to 14 min and from 14 to 17 min.

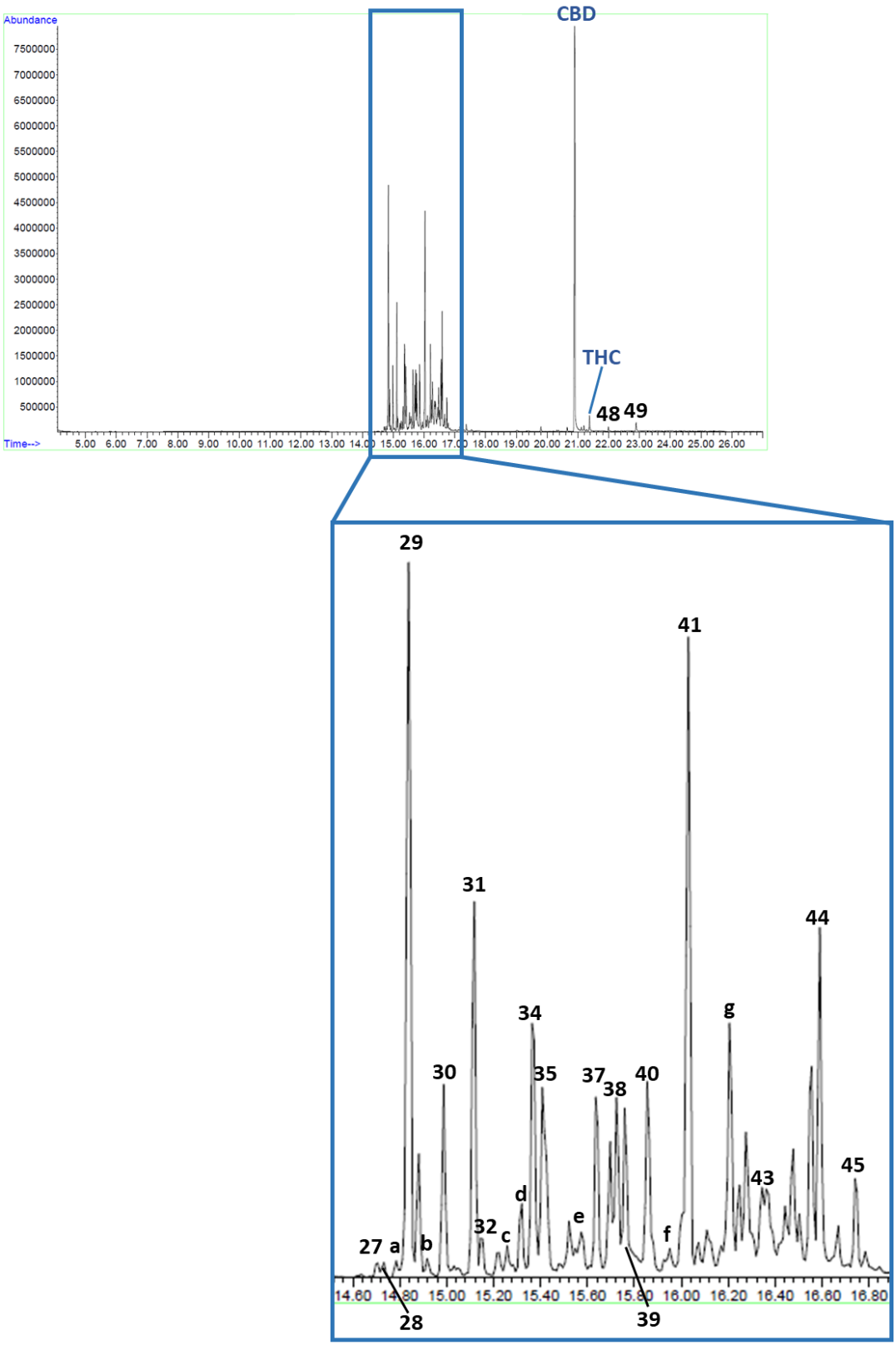

Figure 2. GC-MS analysis of conventional hydrodistillate of *Cannabis sativa* L.. TIC detail from 14 to 17 min.

Table 1. GC-MS identification with relative retention times of volatile fraction from *Cannabis sativa* L. MW-assisted hydrodistillation. Labels refer to Figure 1 and 2.

Label	Compound	Retention time (min)	Figure Reference to Mass fragmentation
1	α-thujene	6.821	3
2	α-pinene	7.101	4
3	Camphene	7.460	5
4	Sabinene	8.056	6
5	β-pinene	8.170	7
6	β-myrcene	8.512	8
7	δ-3-carene	8.924	9
8	α-terpinene	9.072	10
9	p-cymene	9.248	11
10	o-cymene	9.256	12
11	Limonene	9.353	13
12	1,8-cineole	9.405	14
13	β-ocimene	9.730	15
14	γ-terpinene	9.949	16
15	Trans-sabinene hydrate	10.203	17
16	α-terpinolene	10.492	18
17	Dehydro-Linalool	10.834	19
18	Cis-sabinene hydrate	10.895	20
19	Fenchol	11.316	21
20	Pinocarvone	12.113	22
21	Borneol	12.244	23
22	Terpinen-4-ol	12.358	24
23	α-terpineol	12.612	25
24	n-tridecane	13.664	26
25	α-ylangene	14.373	27
26	α-copaene	14.435	28
27	Z-caryophyllene	14.689	29
28	α-trans-bergamotene	14.741	30
29	E-caryophyllene	14.812	31
30	β-farnesene	14.978	32
31	α-humulene	15.101	33
32	Aromadendrene	15.153	34
33	β-patchoulene	15.214	35
34	β-selinene	15.364	36
35	α-selinene	15.416	37

36	δ-cadinene	15.512	38
37	α-gurjunene	15.635	39
38	Selina-3,7(11)-diene	15.723	40
39	Nerolidol	15.767	41
40	Germacrene B	15.863	42
41	Caryophyllene oxide	16.021	43
42	7-epi-α-selinene	16.275	44
43	Caryophylla-4(12), 8(13)-dien-5-β-ol	16.362	45
44	α-bisabolol	16.573	46
45	Eudesm-7(11)-en-4-ol	16.739	47
46	Allo-aromadendrene	17.020	48
47	Hexahydrofarnesyl acetone	17.361	49
48	Heptacosane	21.979	50
49	Nonacosane	22.864	51
	Compounds present only in CHD volatile fraction:		
a	α-santalene	14.785	52
b	α-guaiene	14.917	53
c	β-gurjunene	15.259	54
d	γ-selinene	15.320	55
e	β-guaiene	15.574	56
f	γ-muurolene	15.951	57
g	Valencene	16.284	58

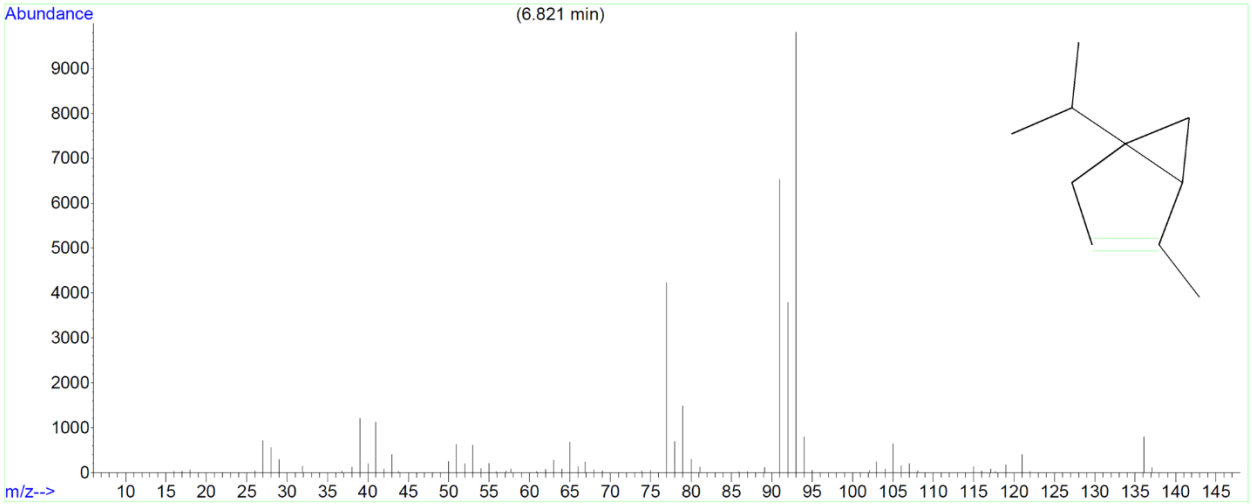

Figure 3. α-thujene

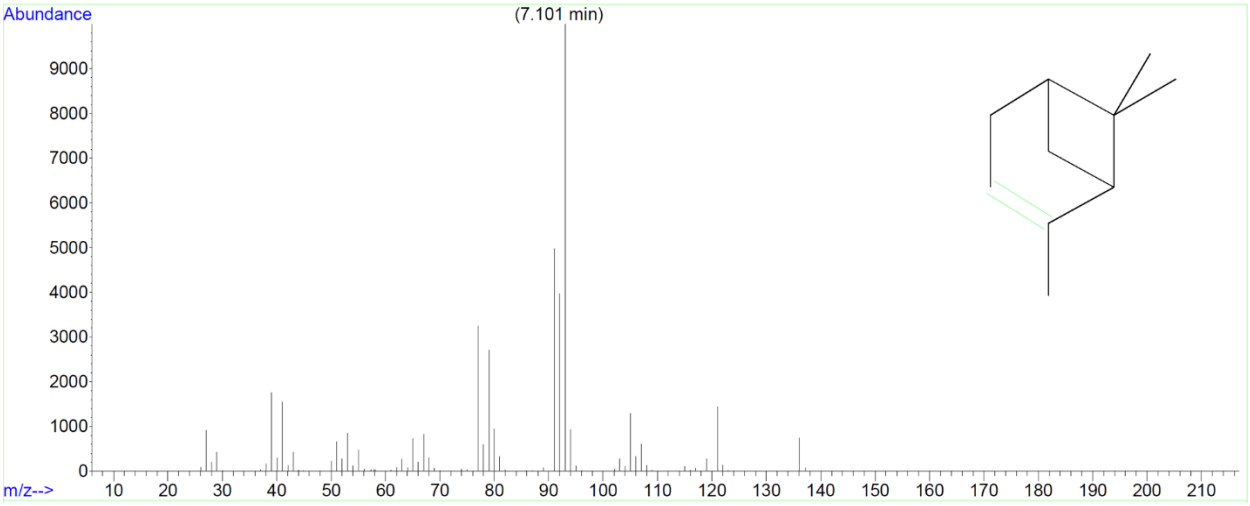

Figure 4. α-pinene

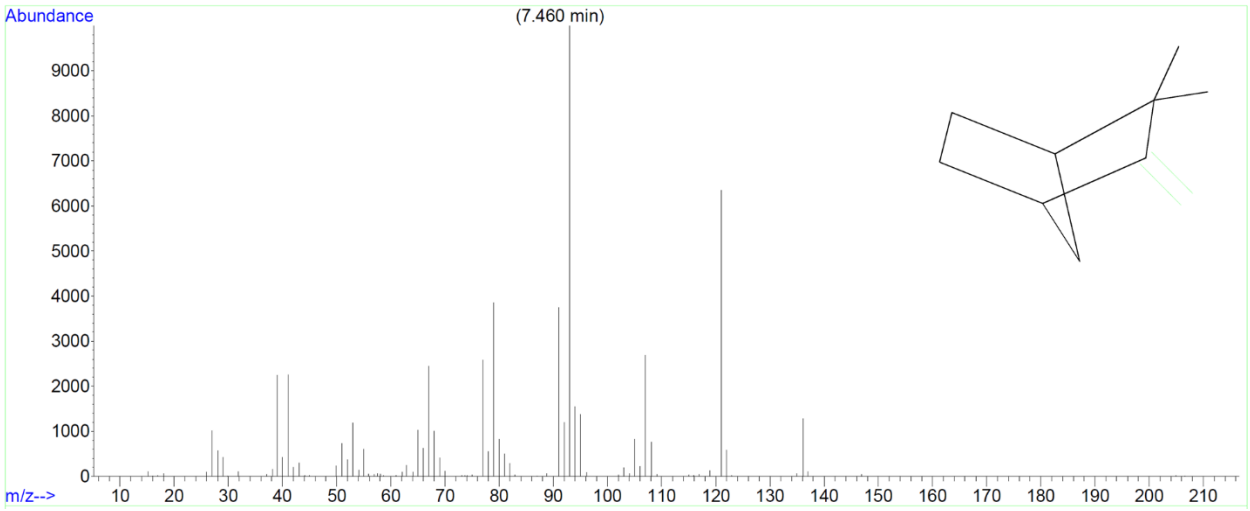

Figure 5. Camphene

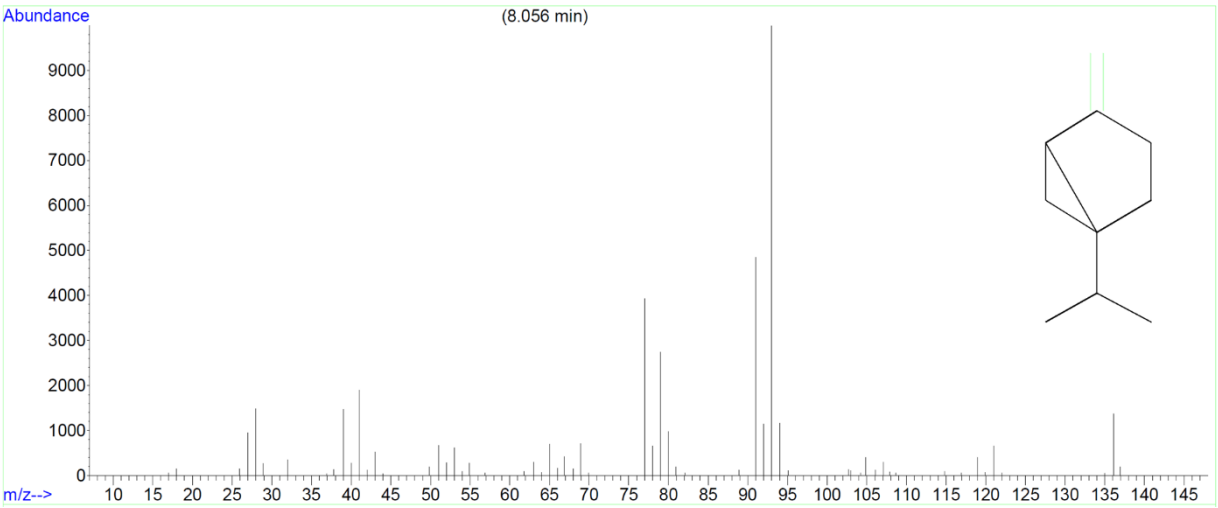

Figure 6. Sabinene

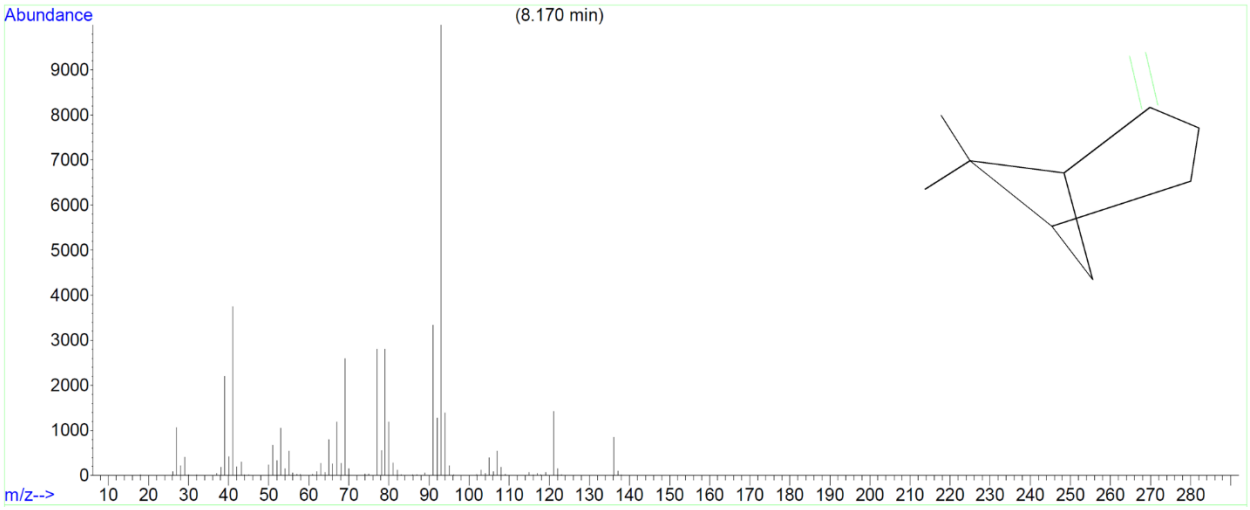

Figure 7. β-pinene

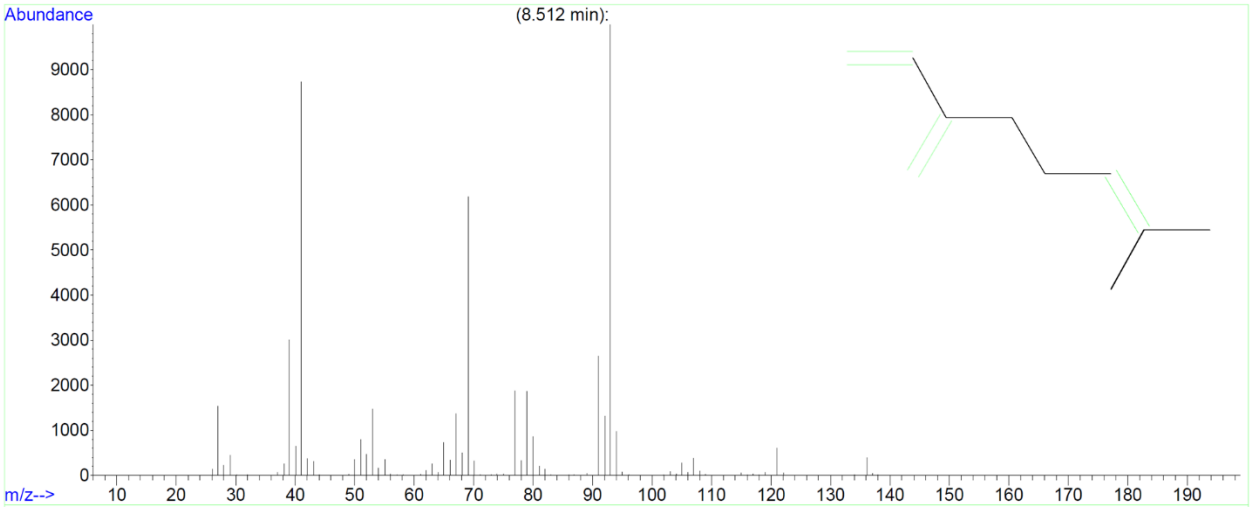

Figure 8. β-myrcene

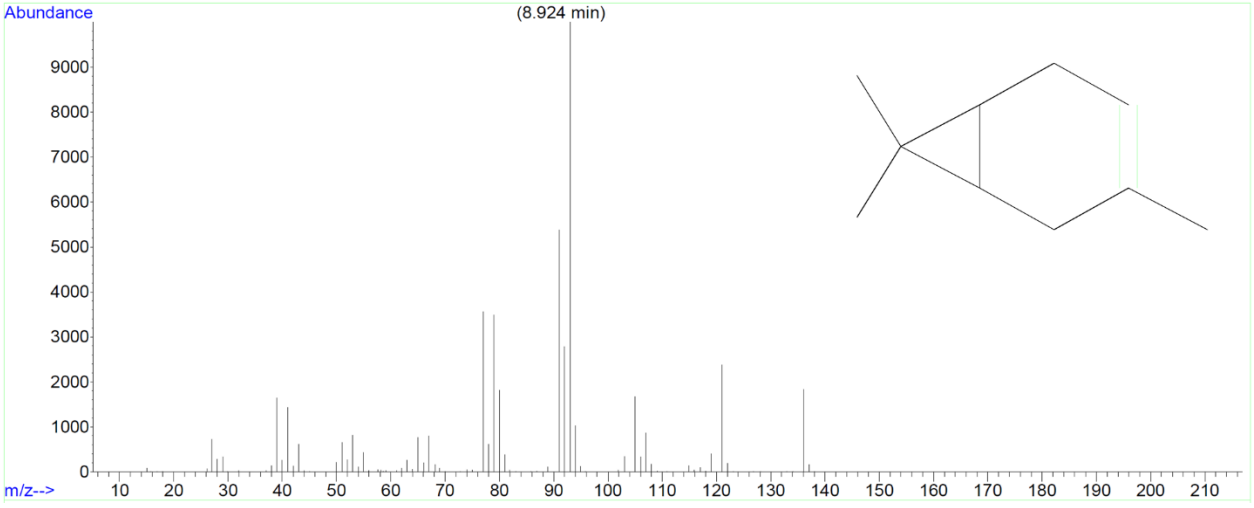

Figure 9. δ-3-carene

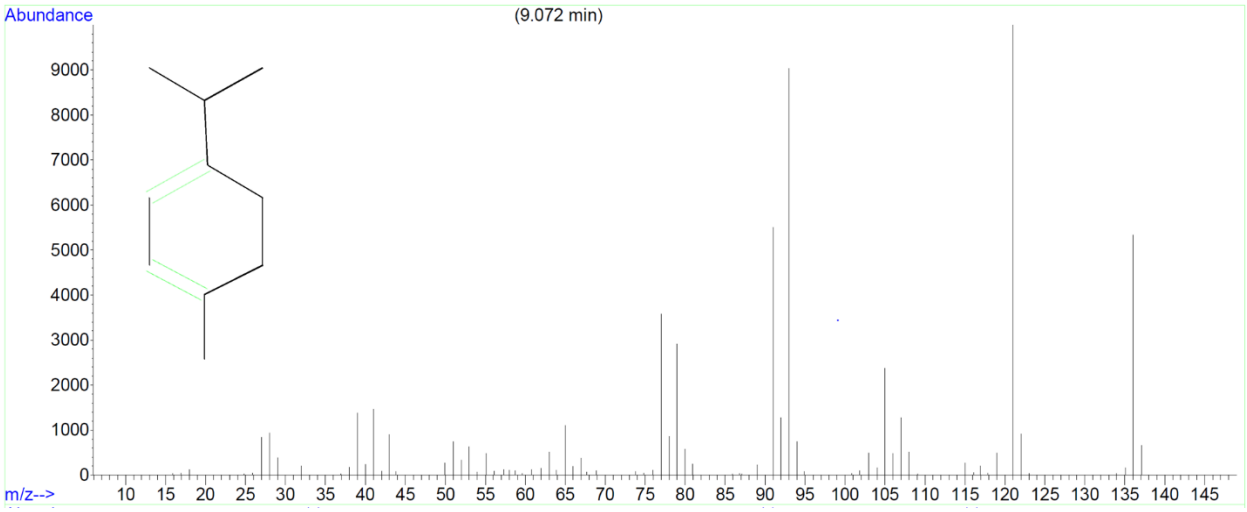

Figure 10. α-terpinene

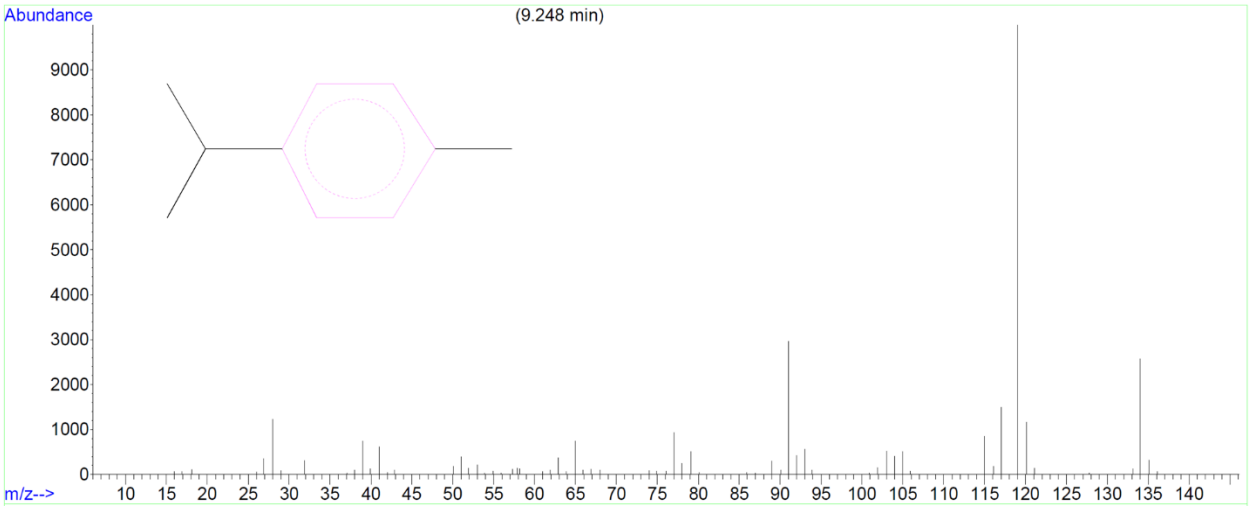

Figure 11. p-cymene

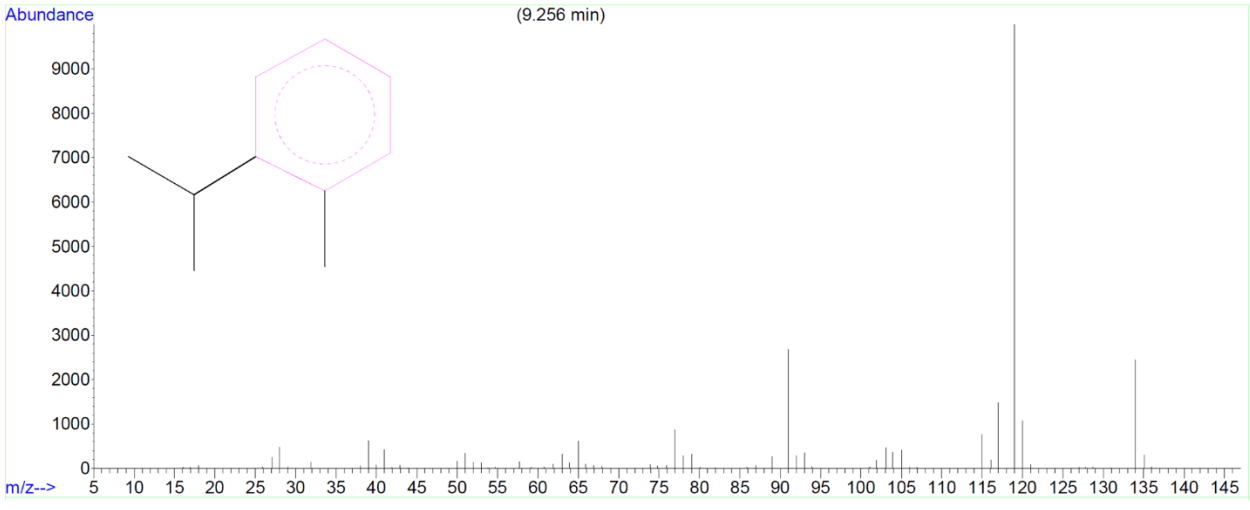

Figure 12. o-cymene

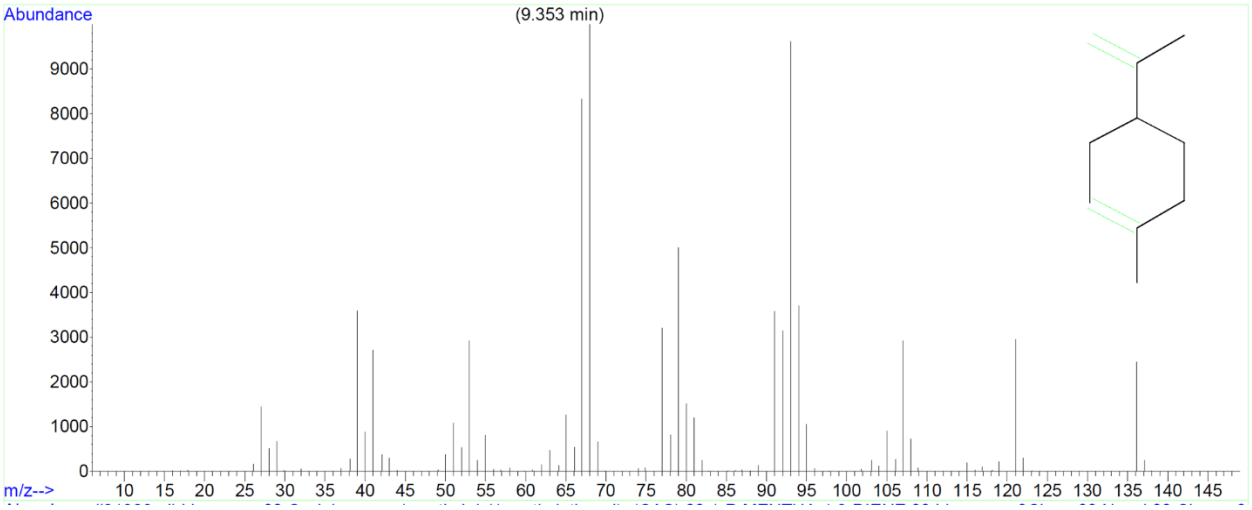

Figure 13. Limonene

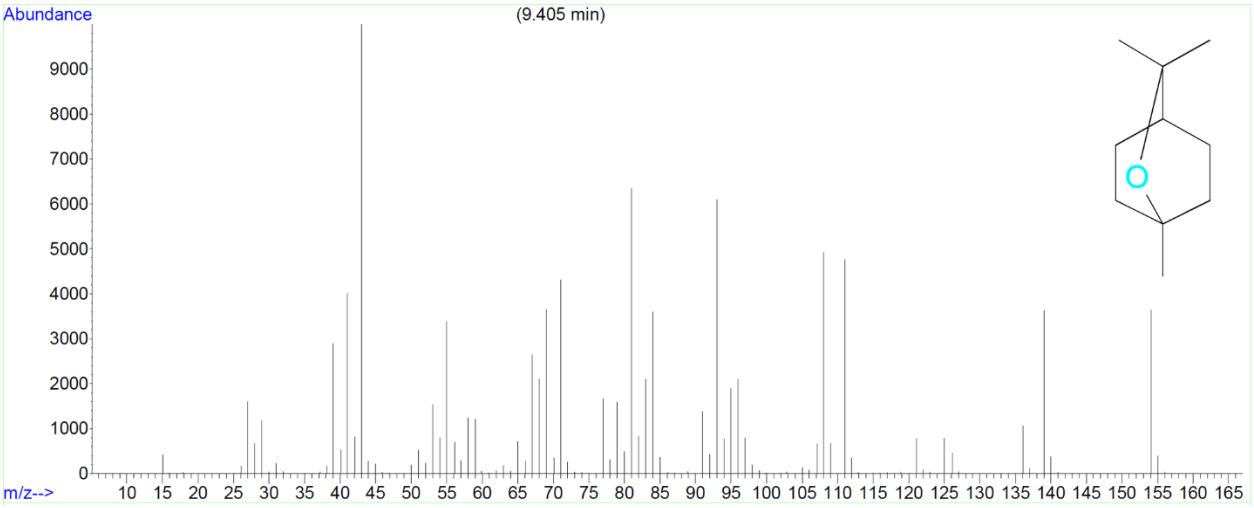

Figure 14. 1,8-cineole

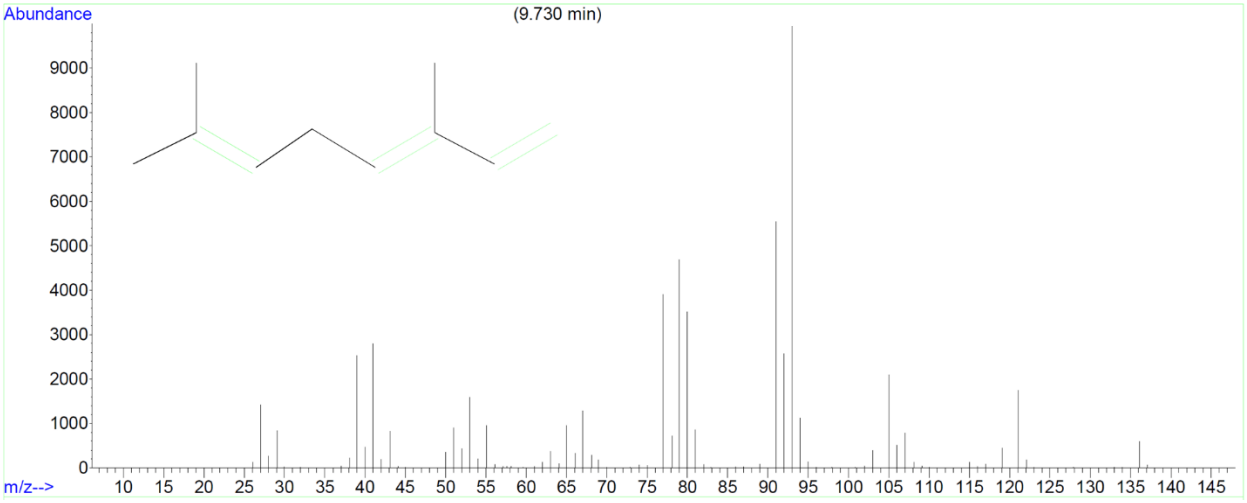

Figure 15. β-ocimene

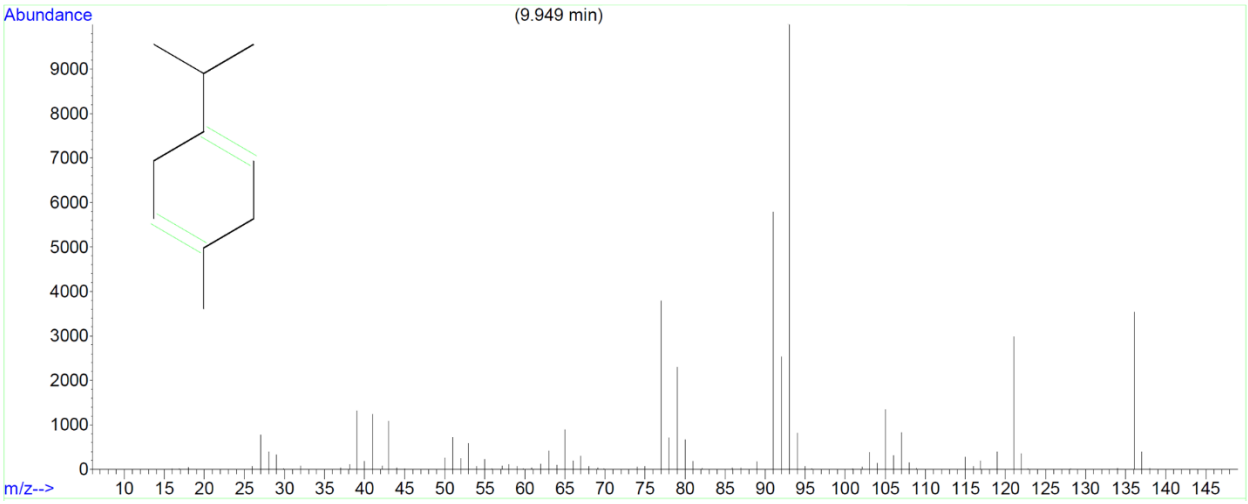

Figure 16. γ-terpinene

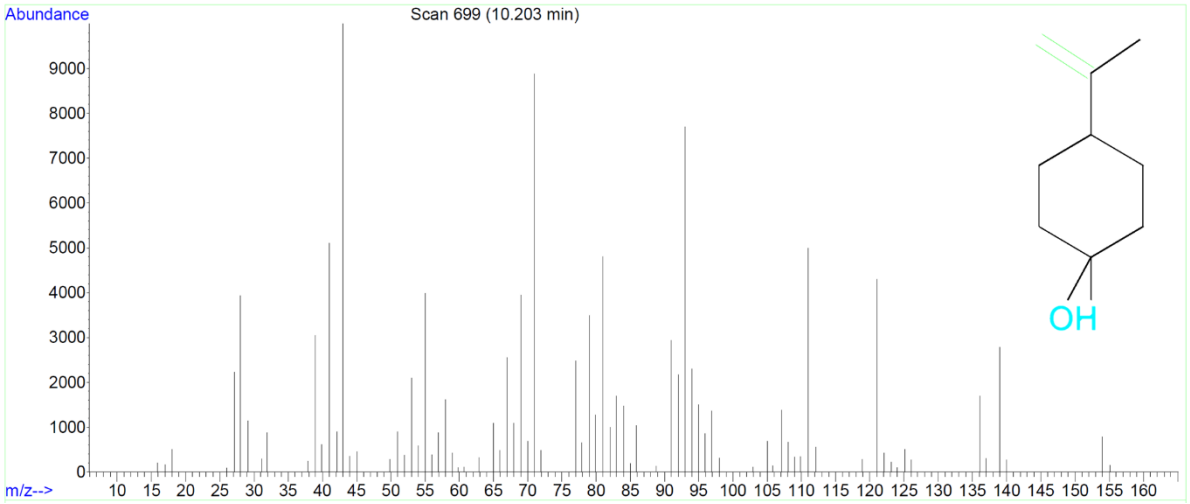

Figure 17. Trans-sabinene hydrate

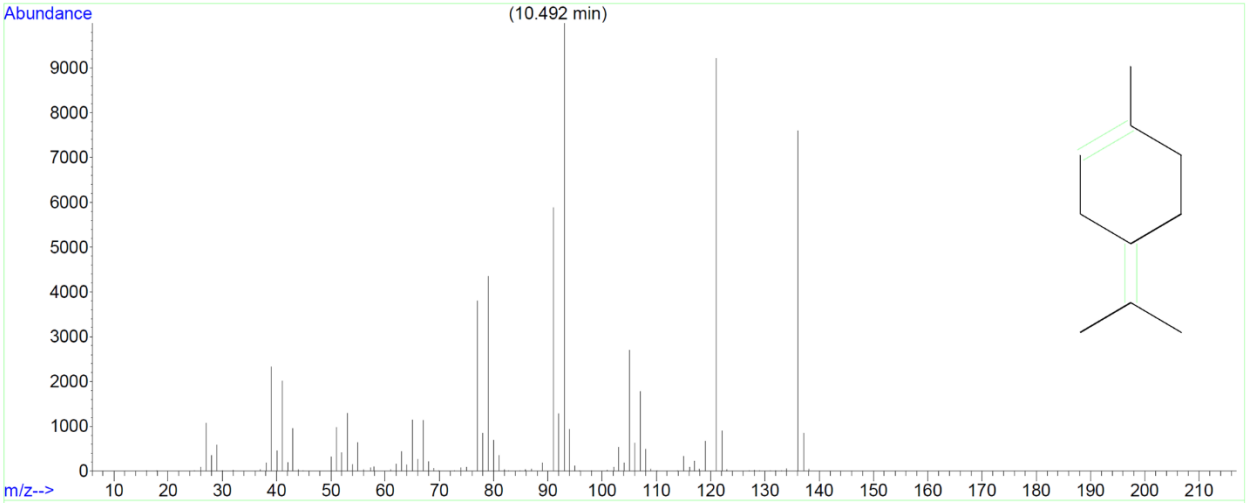

Figure 18. α-terpinolene

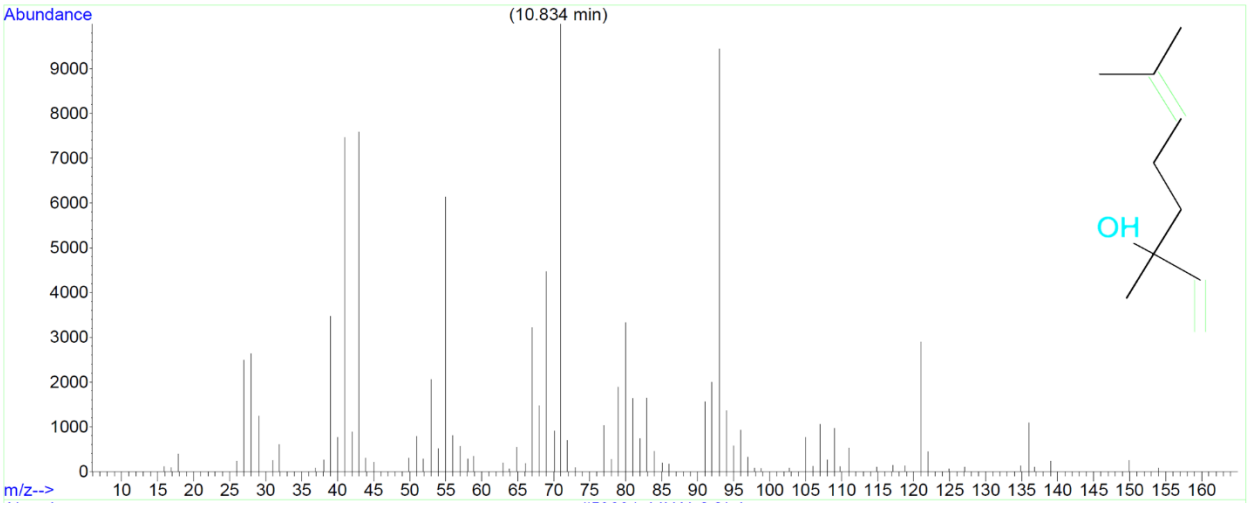

Figure 19. Dehydro-Linalool

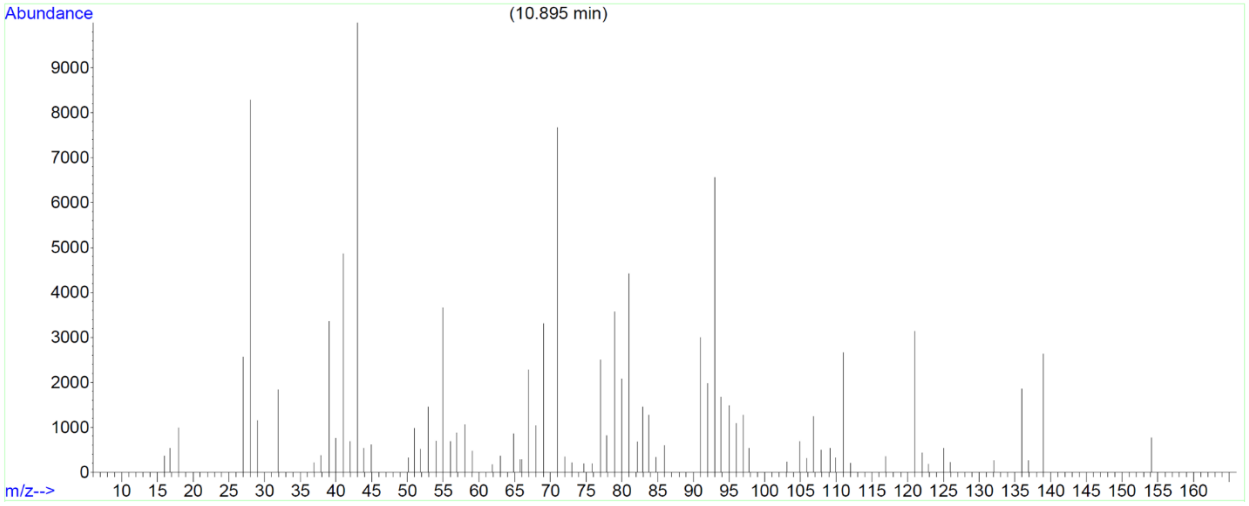

Figure 20. Cis-sabinene hydrate

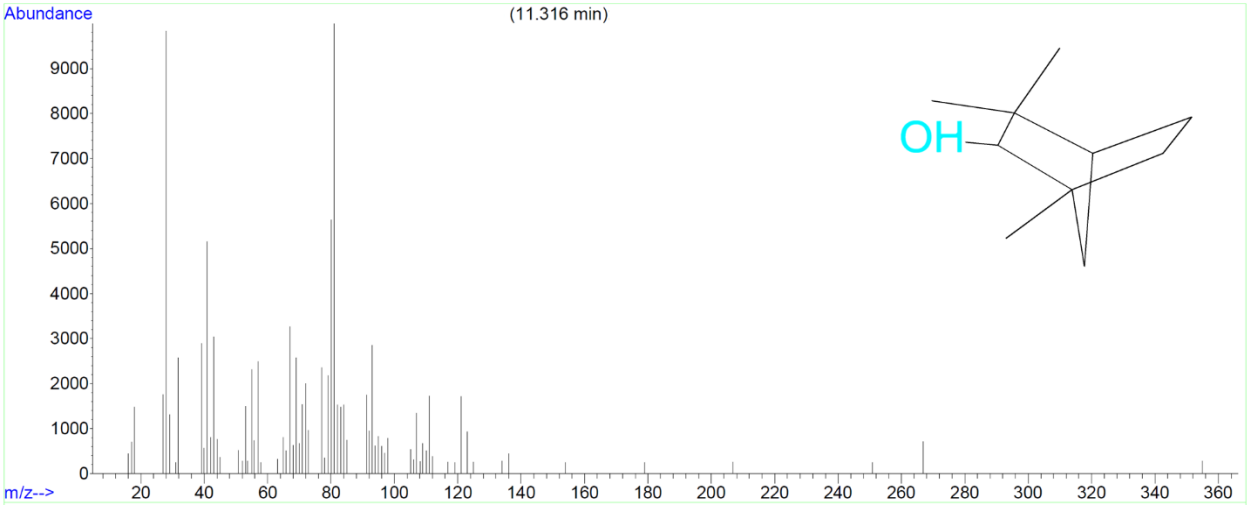

Figure 21. Fenchol

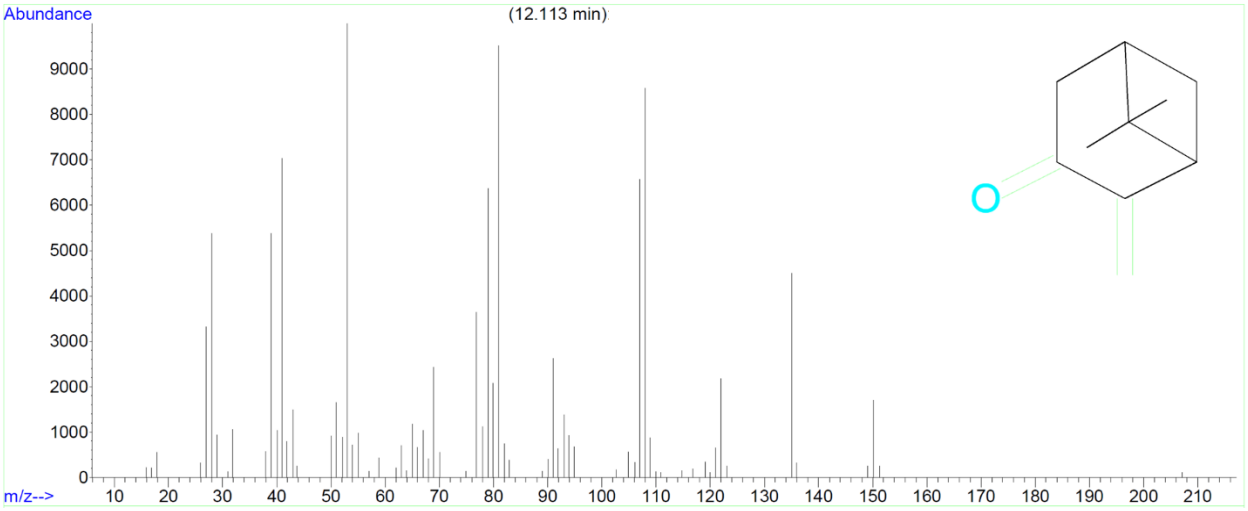

Figure 22. Pinocarvone

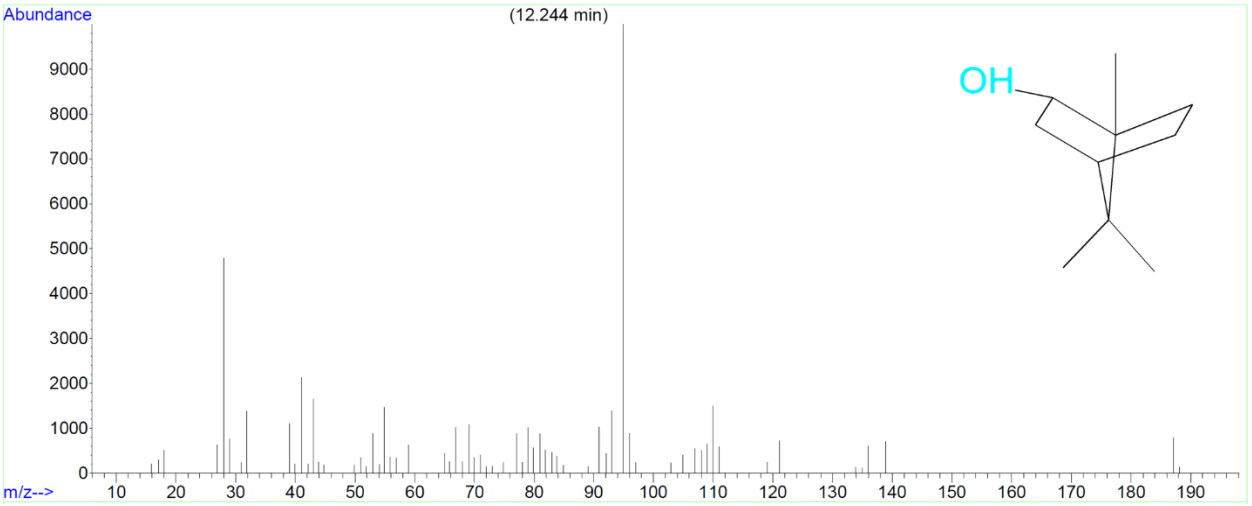

Figure 23. Borneol

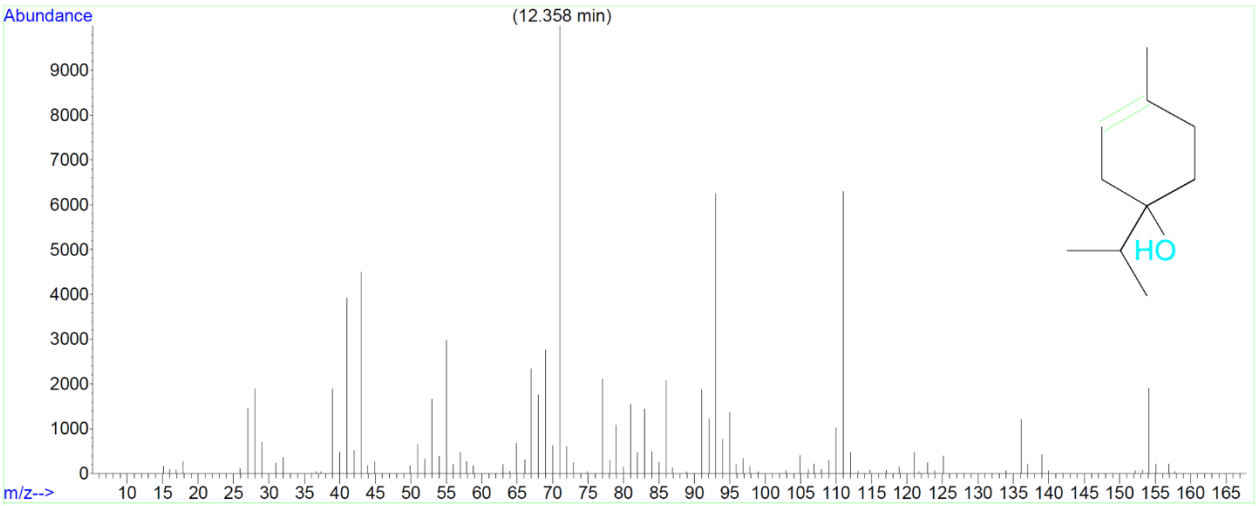

Figure 24. Terpinen-4-ol

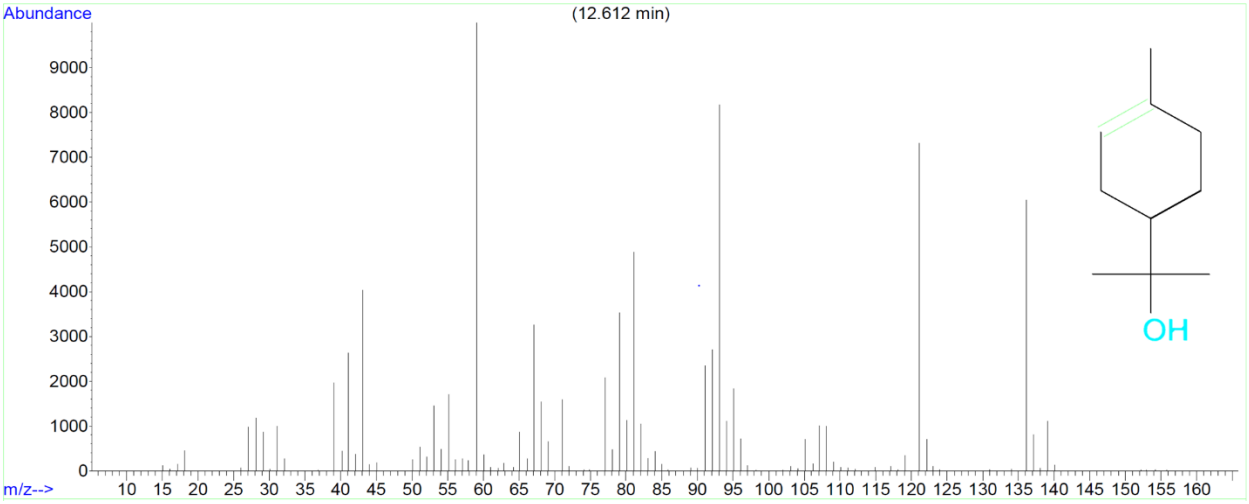

Figure 25. α-terpineol

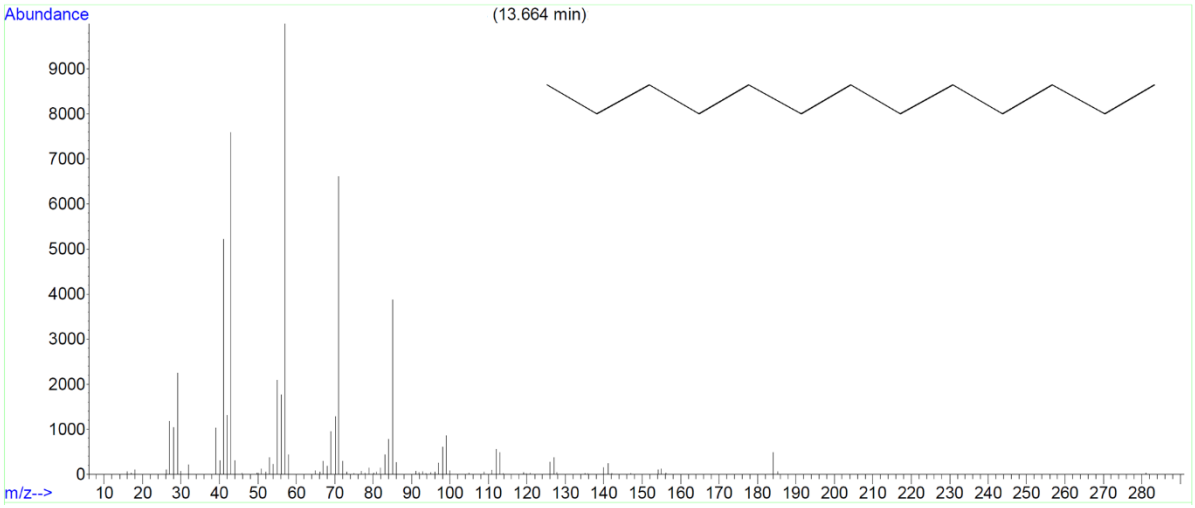

Figure 26. n-tridecane

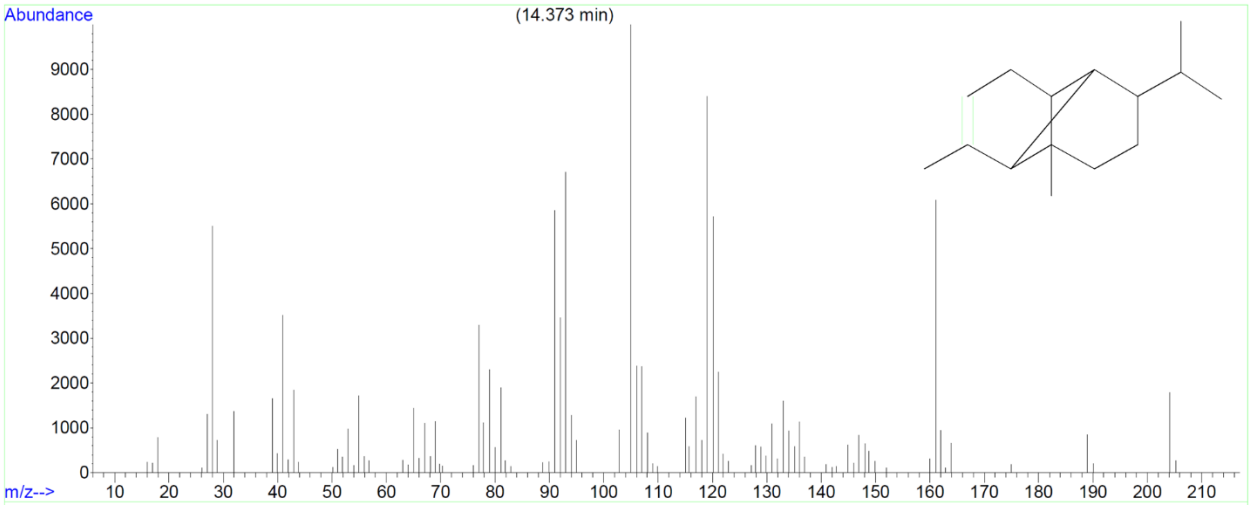

Figure 27. α-ylangene

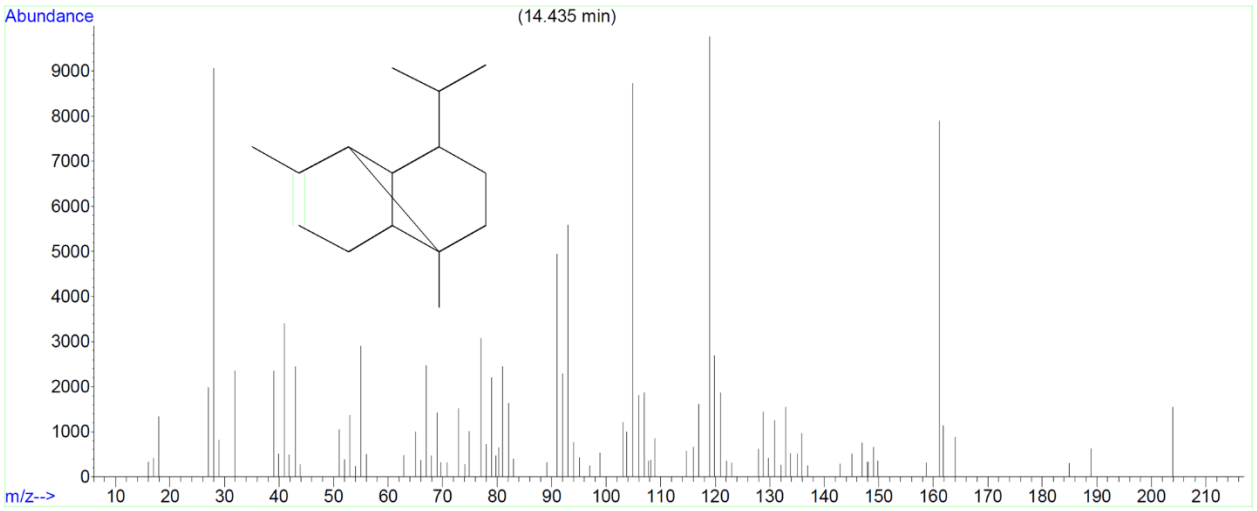

Figure 28. α-copaene

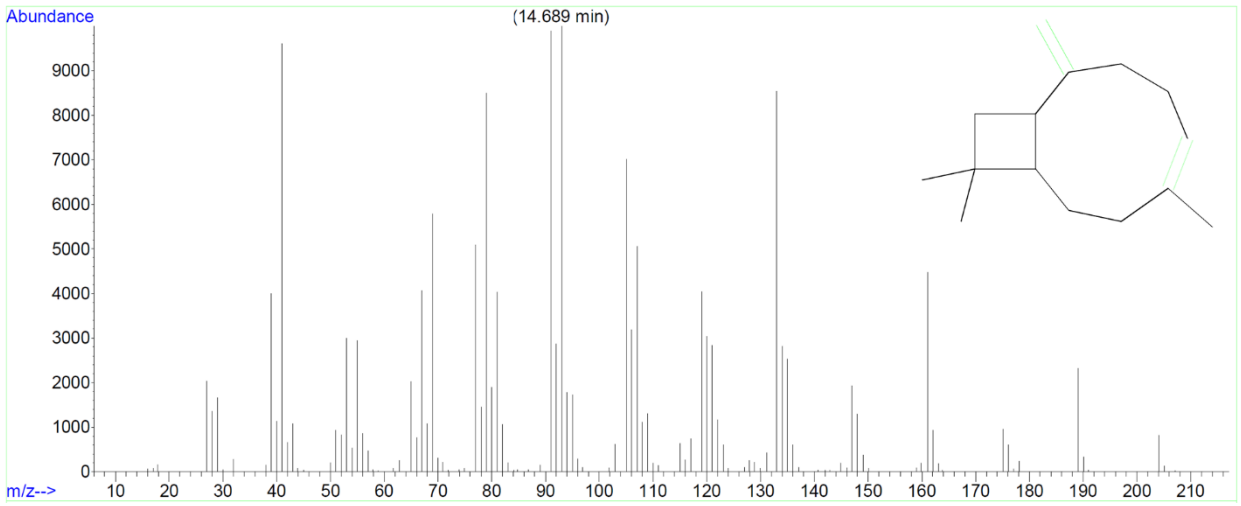

Figure 29. Z-caryophyllene

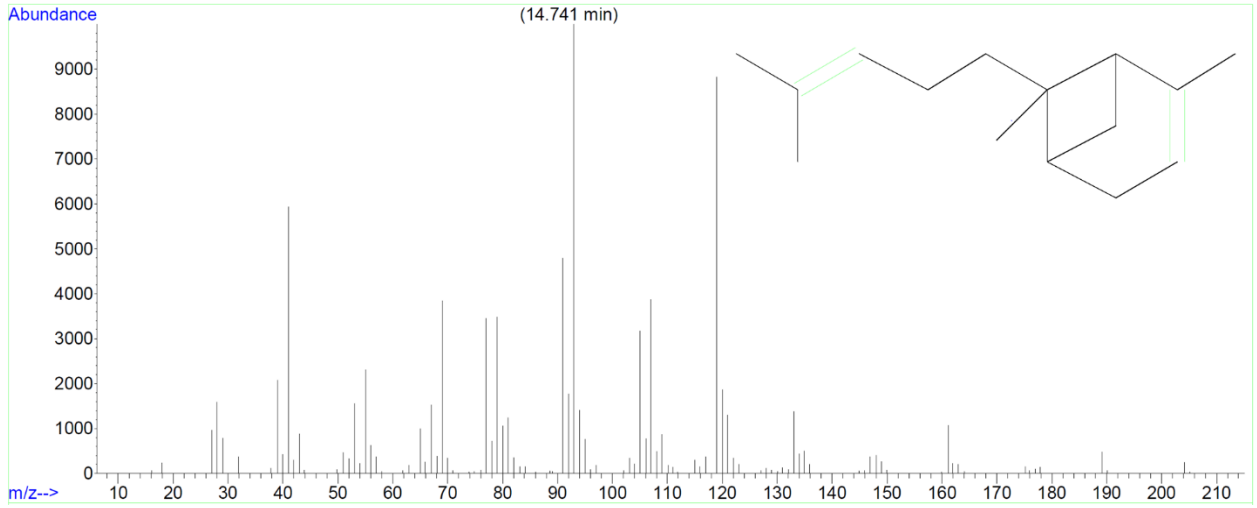

Figure 30. α-trans-bergamotene

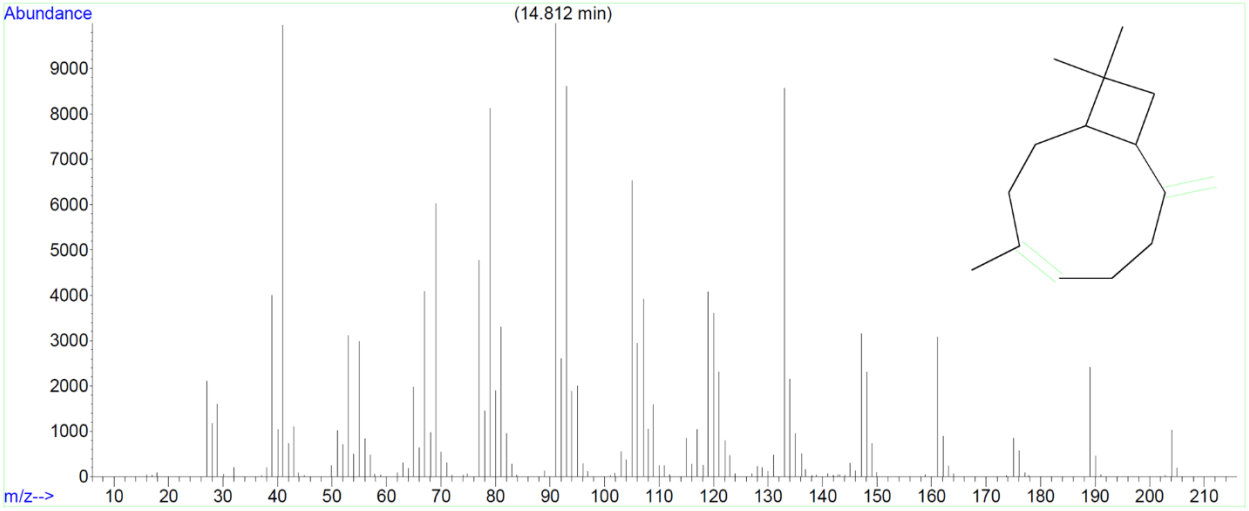

Figure 31. E-caryophyllene

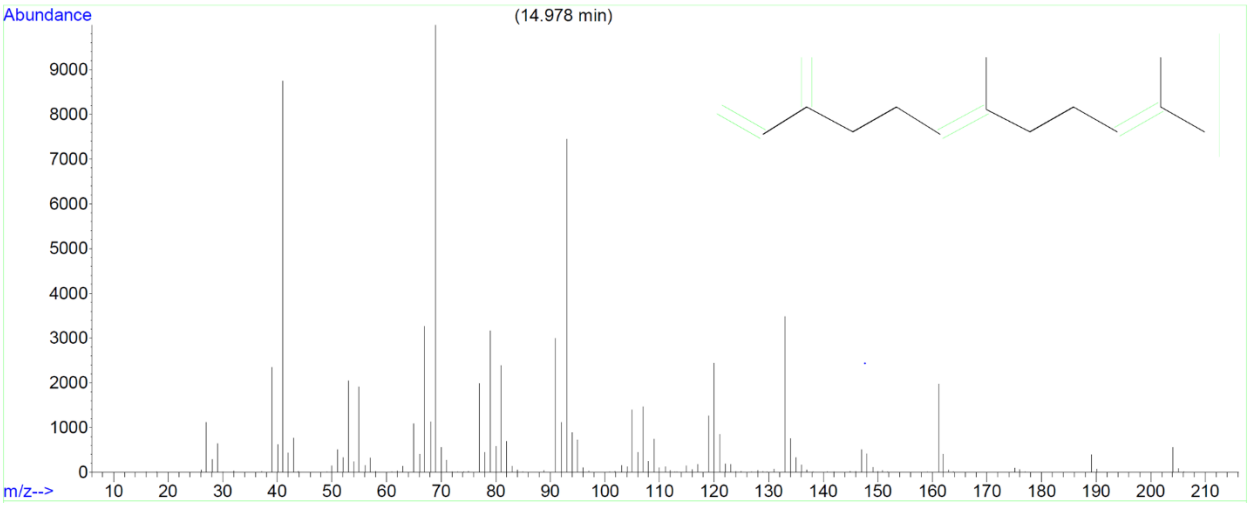

Figure 32. β-farnesene

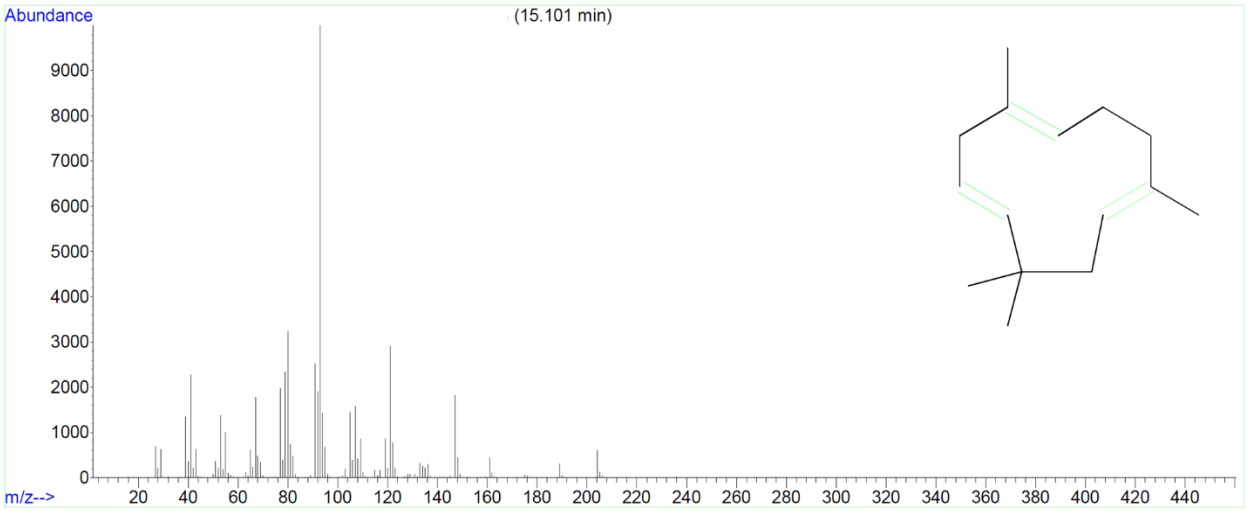

Figure 33. α-humulene

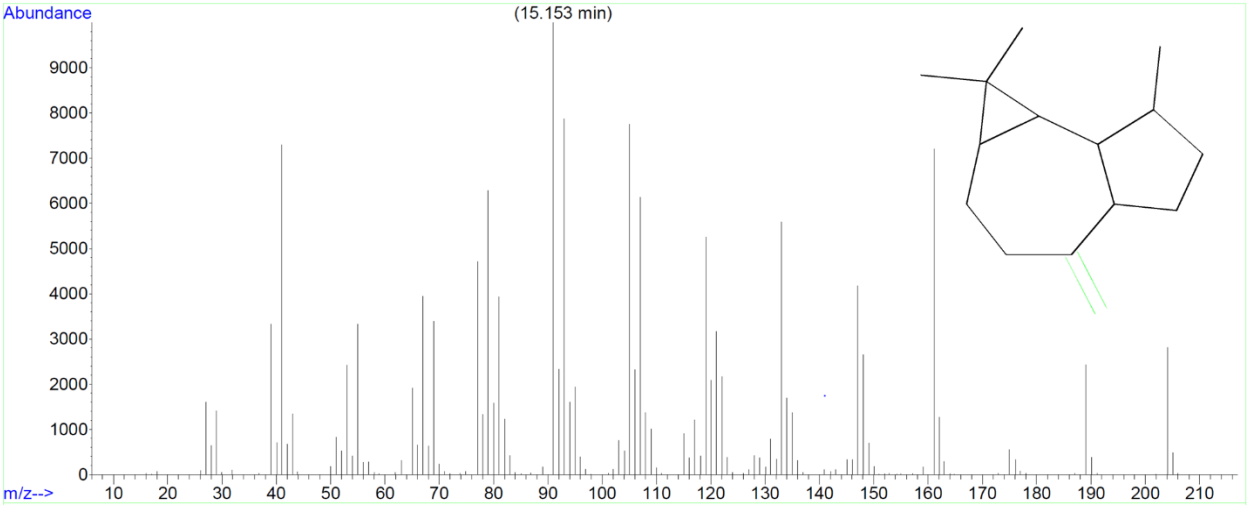

Figure 34. Aromadendrene

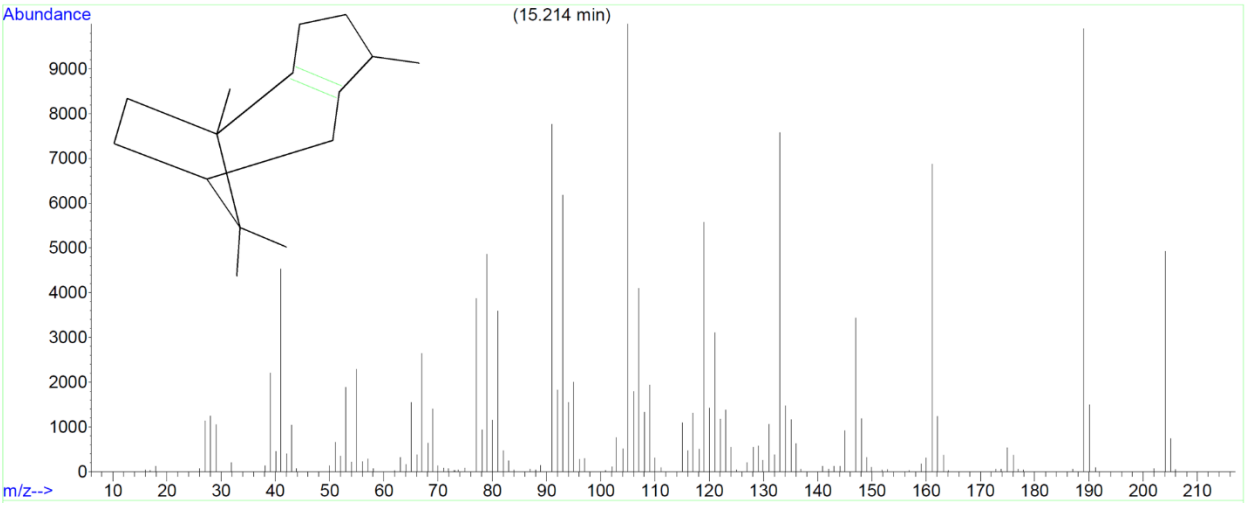

Figure 35. β-patchoulene

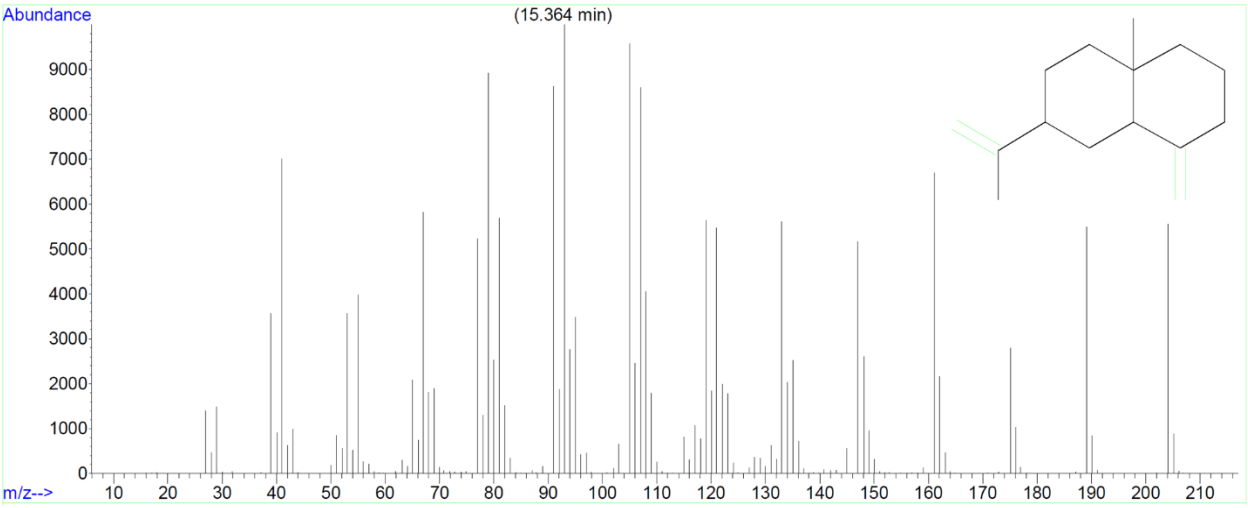

Figure 36. β-selinene

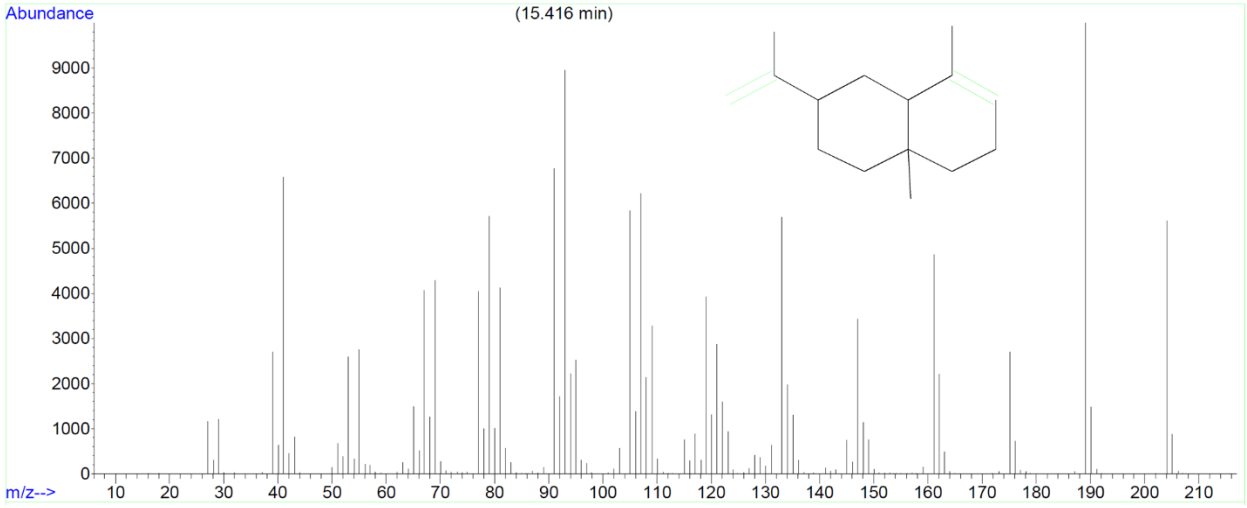

Figure 37. α-selinene

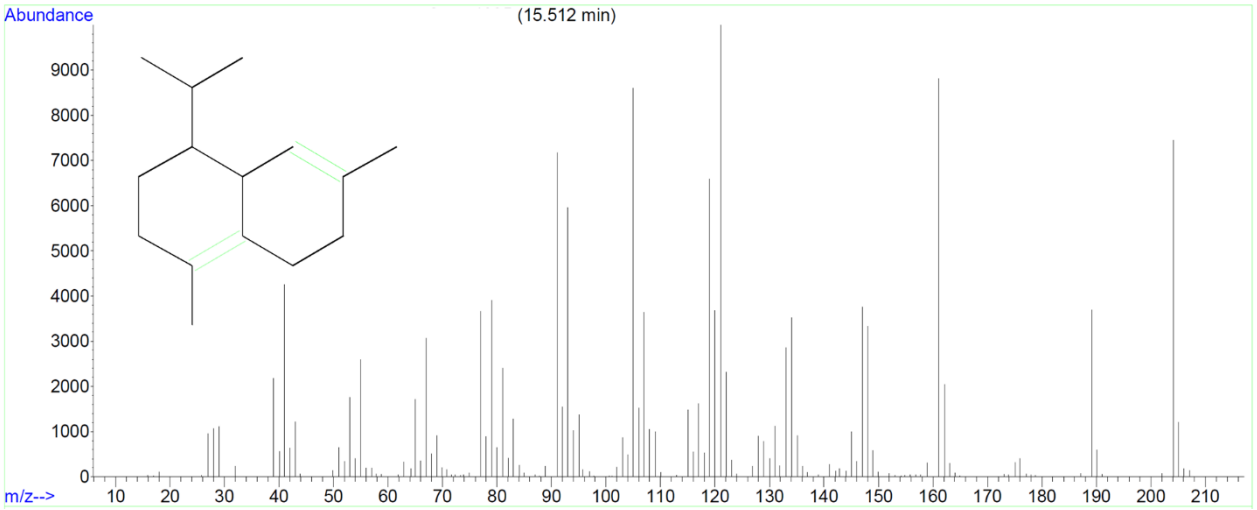

Figure 38. δ-cadinene

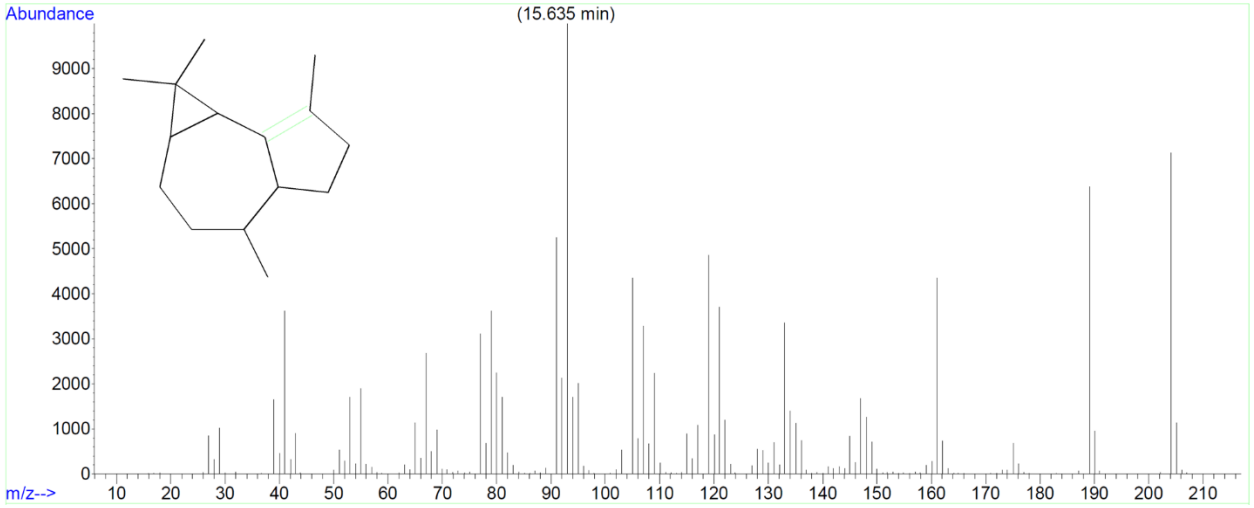

Figure 39. α-gurjunene

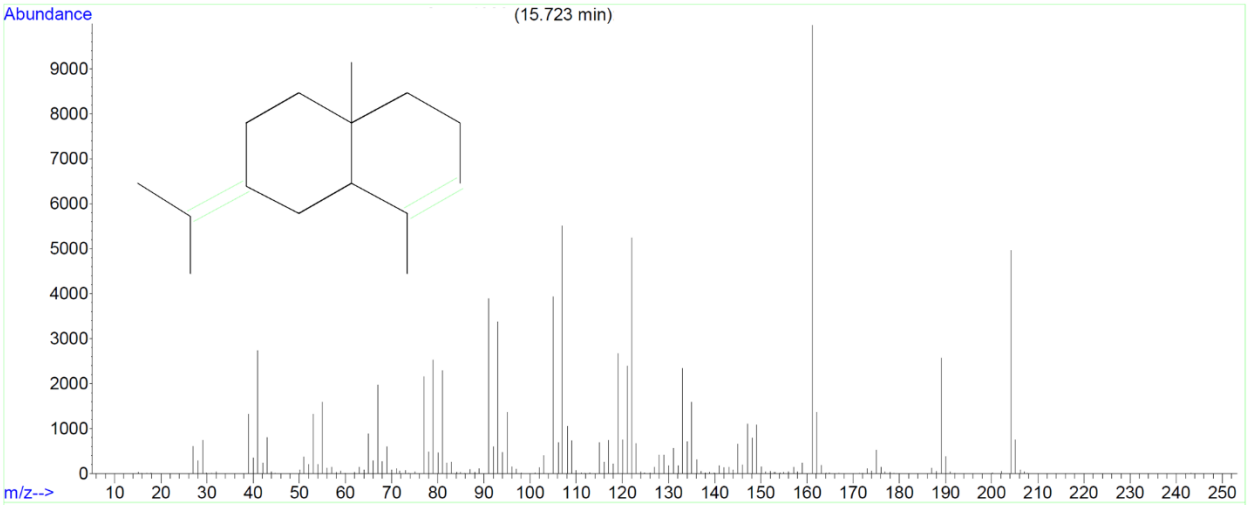

Figure 40. Selina-3,7(11)-diene

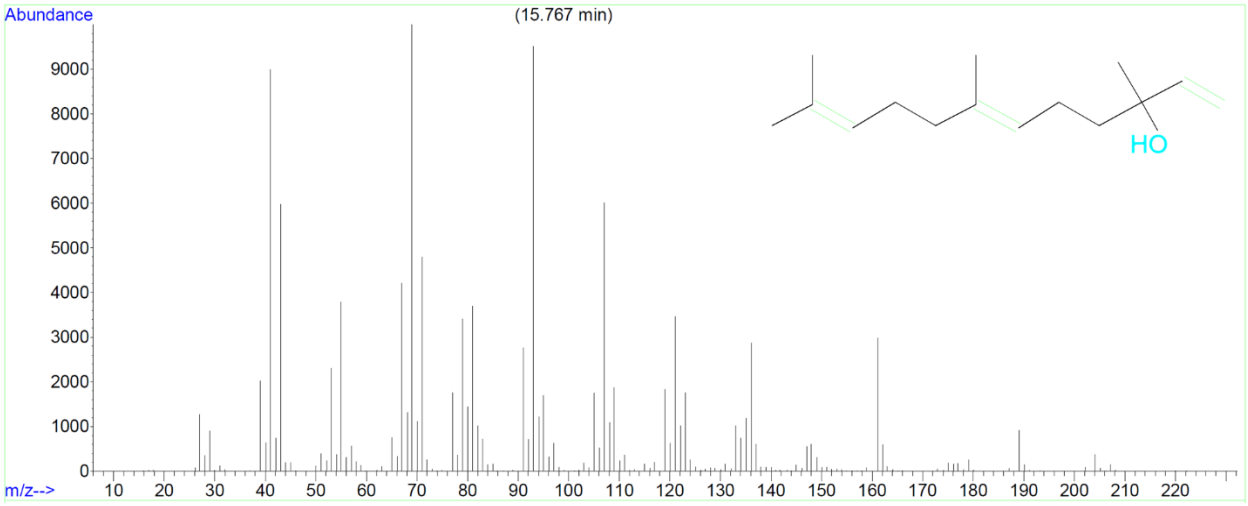

Figure 41. Nerolidol

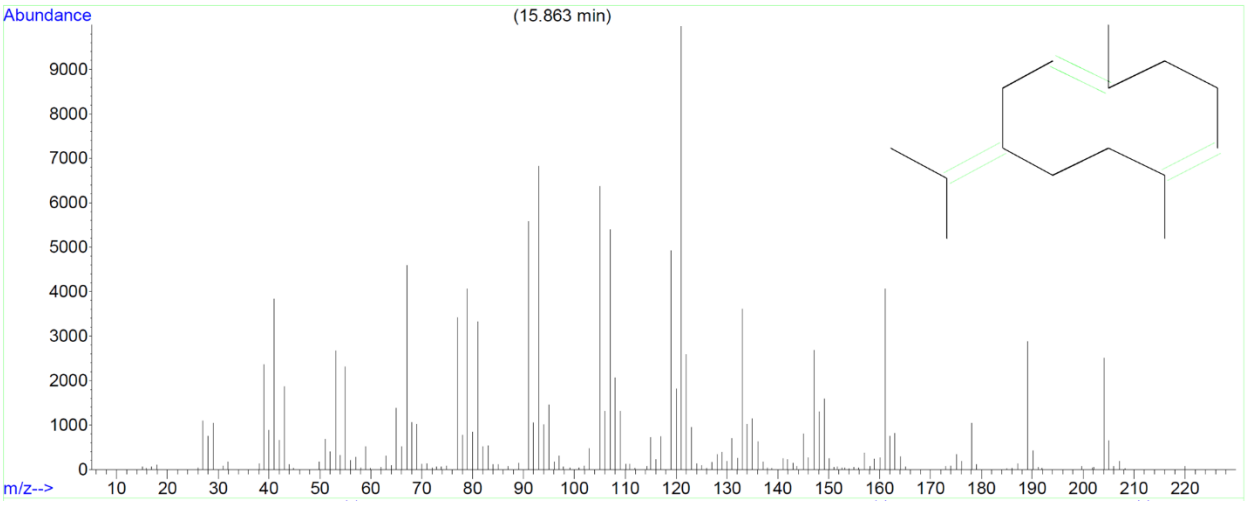

Figure 42. Germacrene B

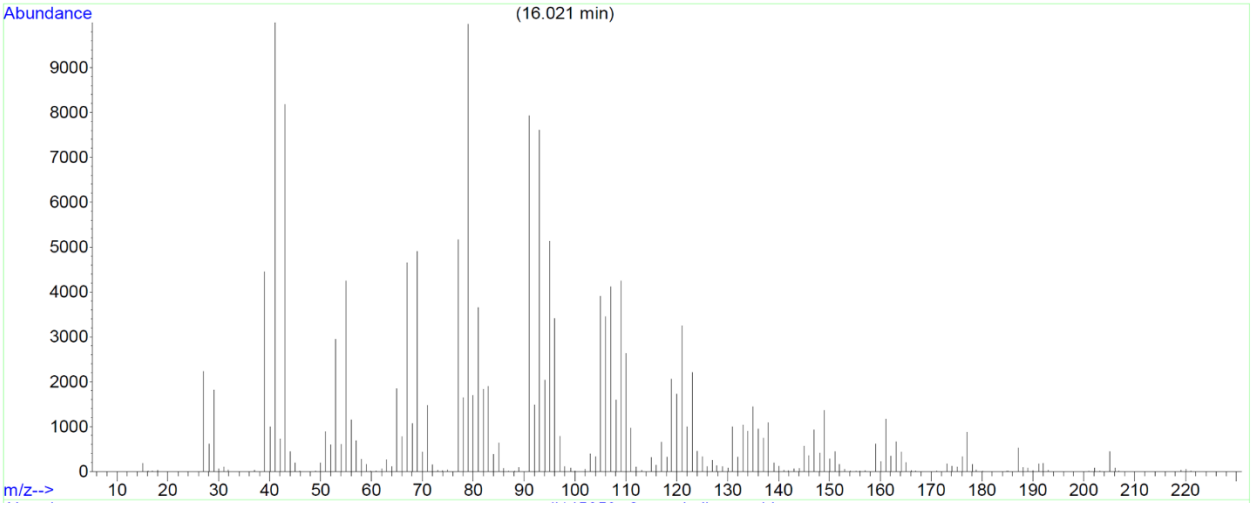

Figure 43. Caryophyllene oxide

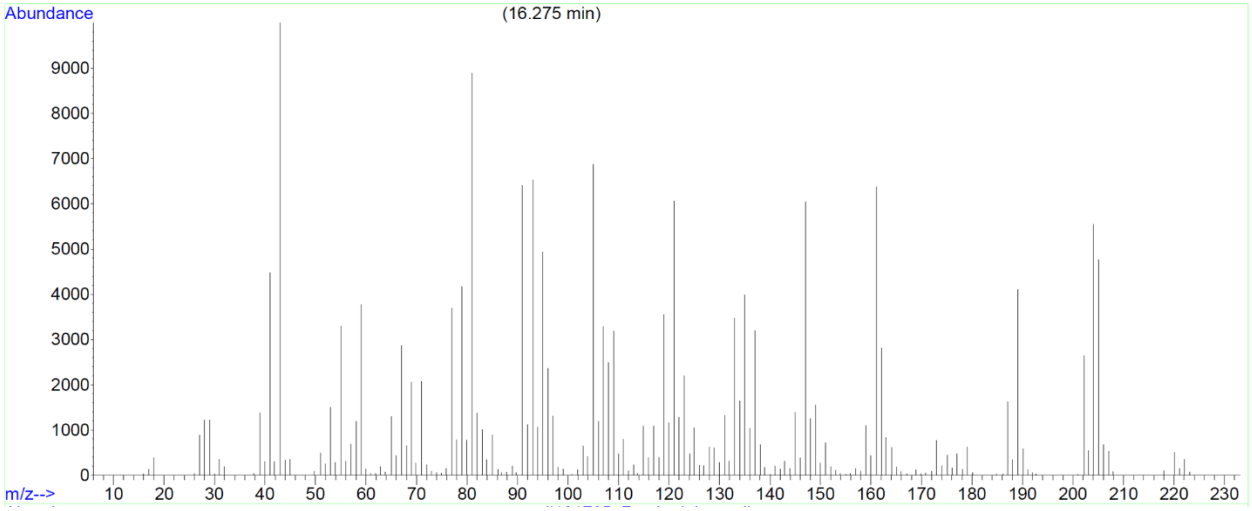

Figure 44. 7-epi-α-selinene

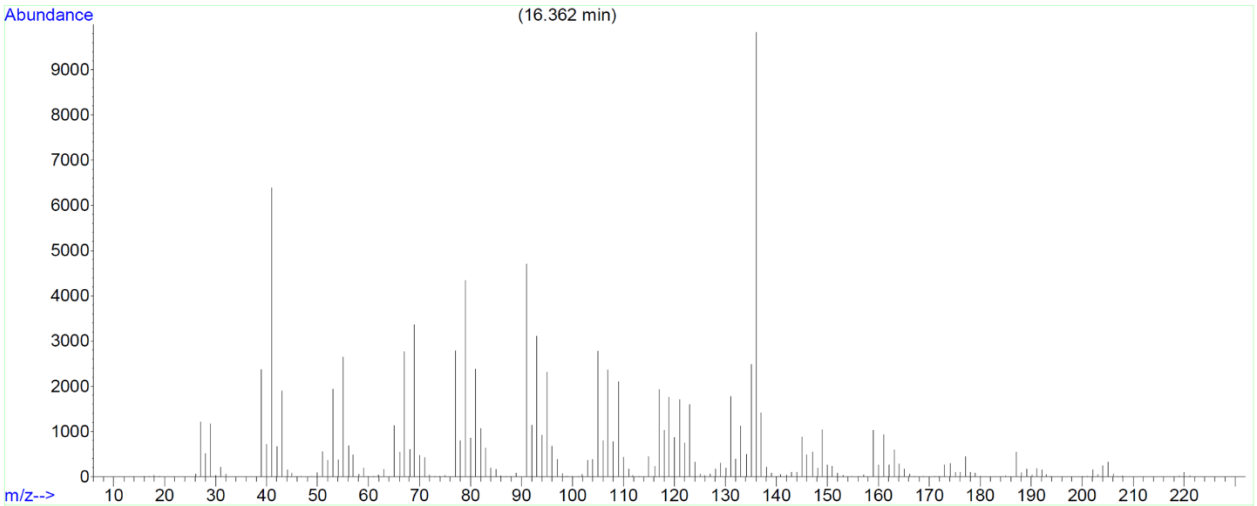

Figure 45. Caryophylla-4(12), 8(13)-dien-5-β-ol

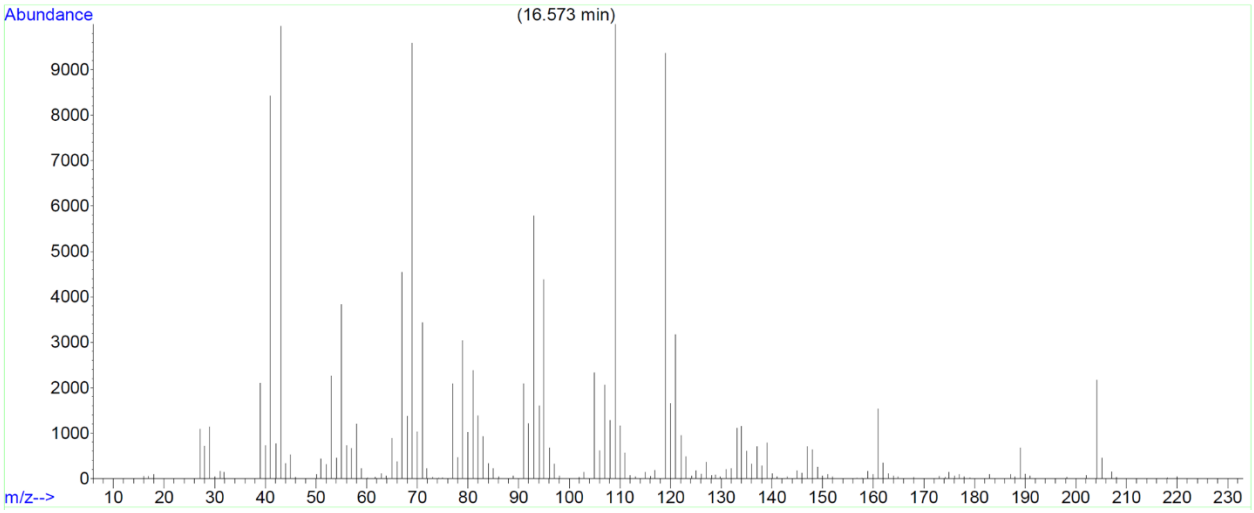

Figure 46. α-bisabolol

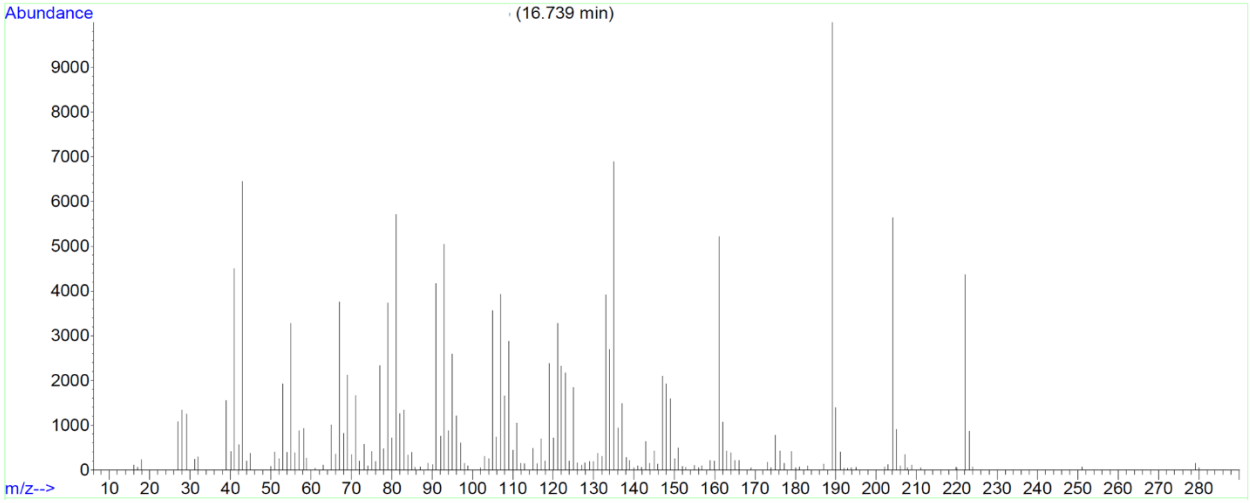

Figure 47. Eudesm-7(11)-en-4-ol

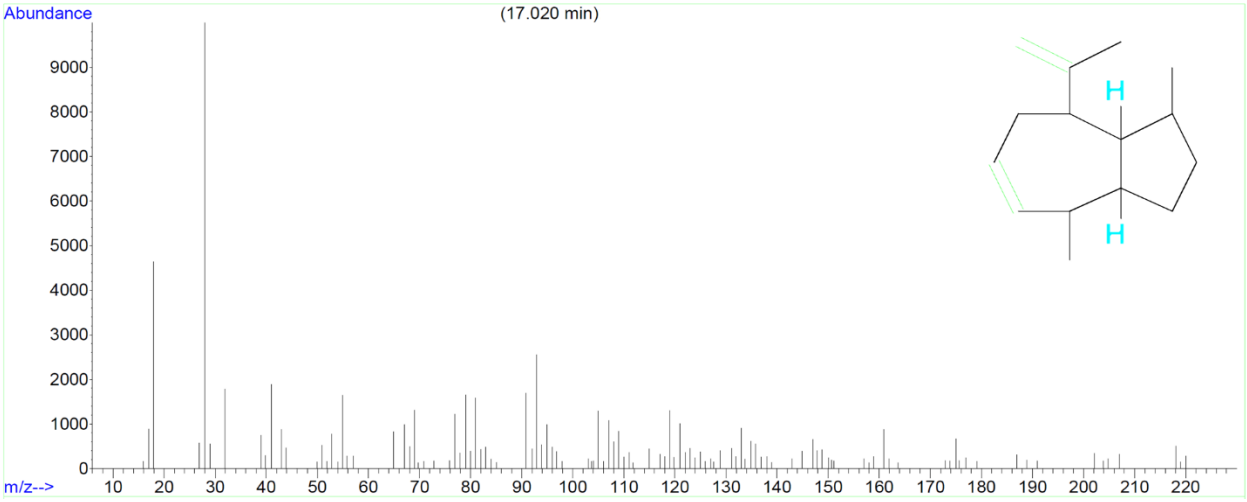

Figure 48. Allo-aromadendrene

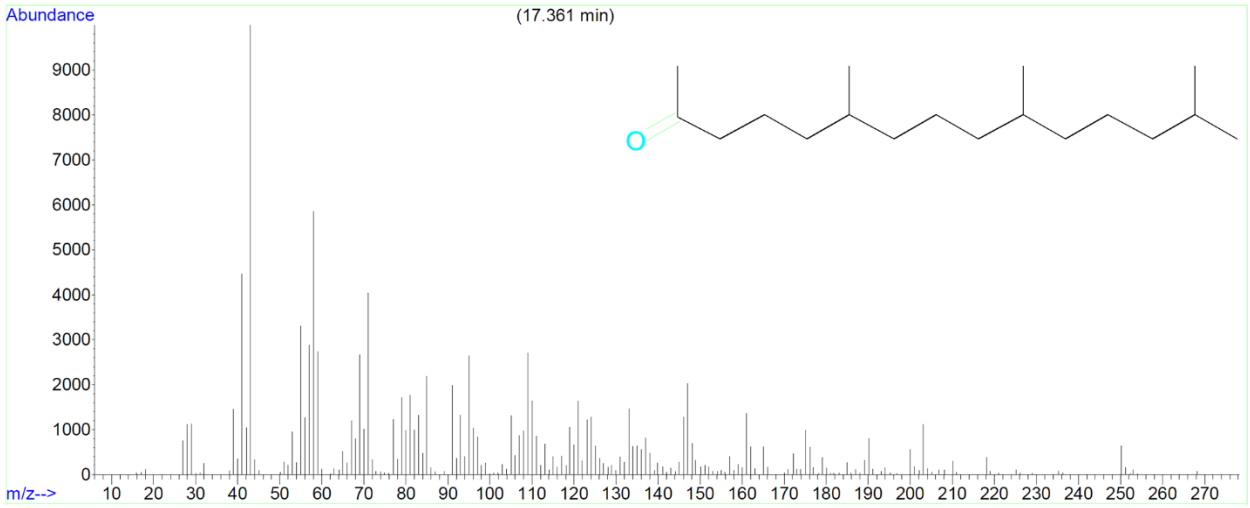

Figure 49. Hexahydrofarnesyl acetone

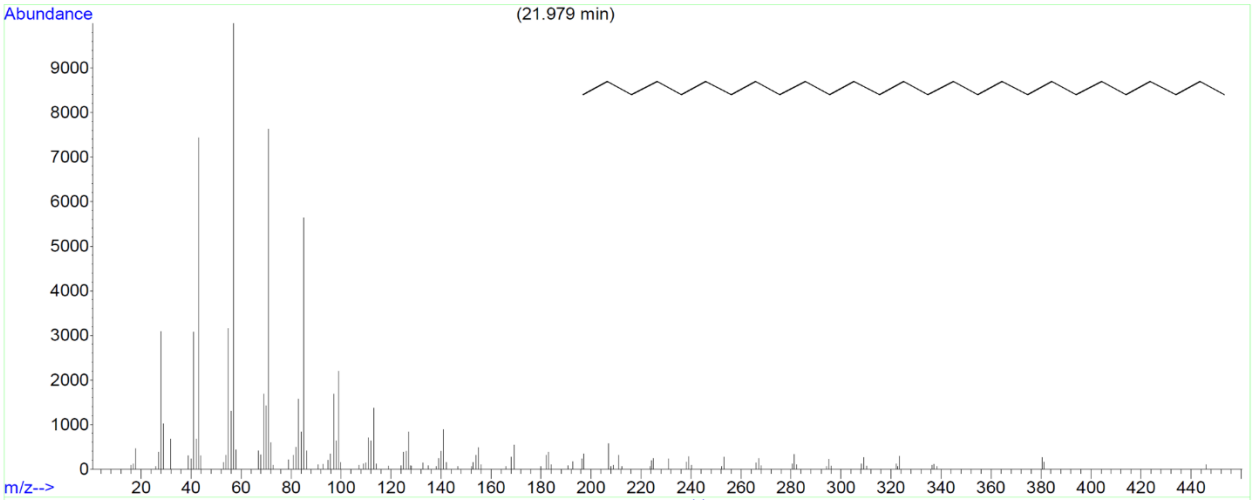

Figure 50. Heptacosane

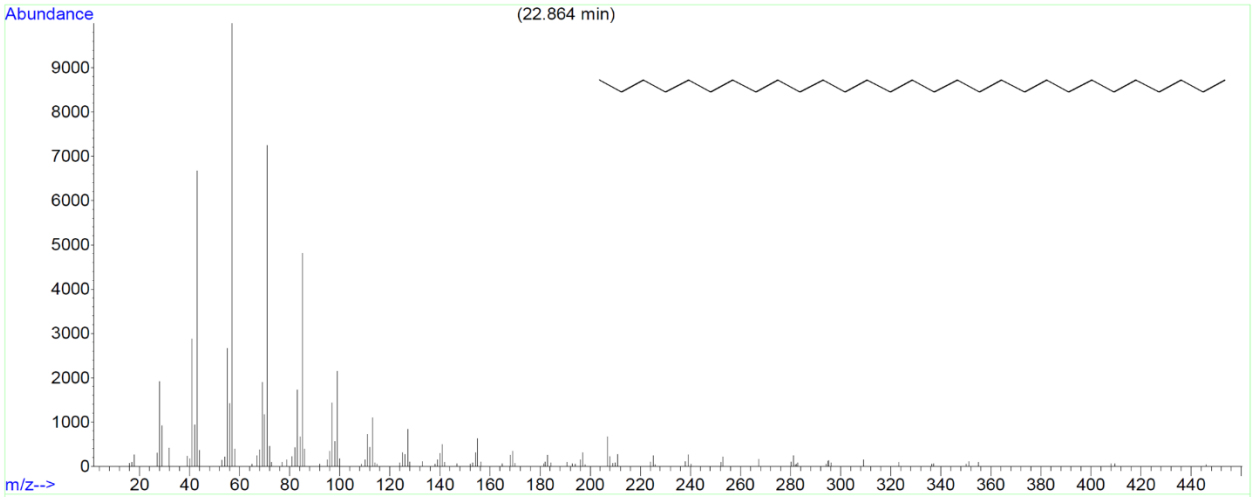

Figure 51. Nonacosane

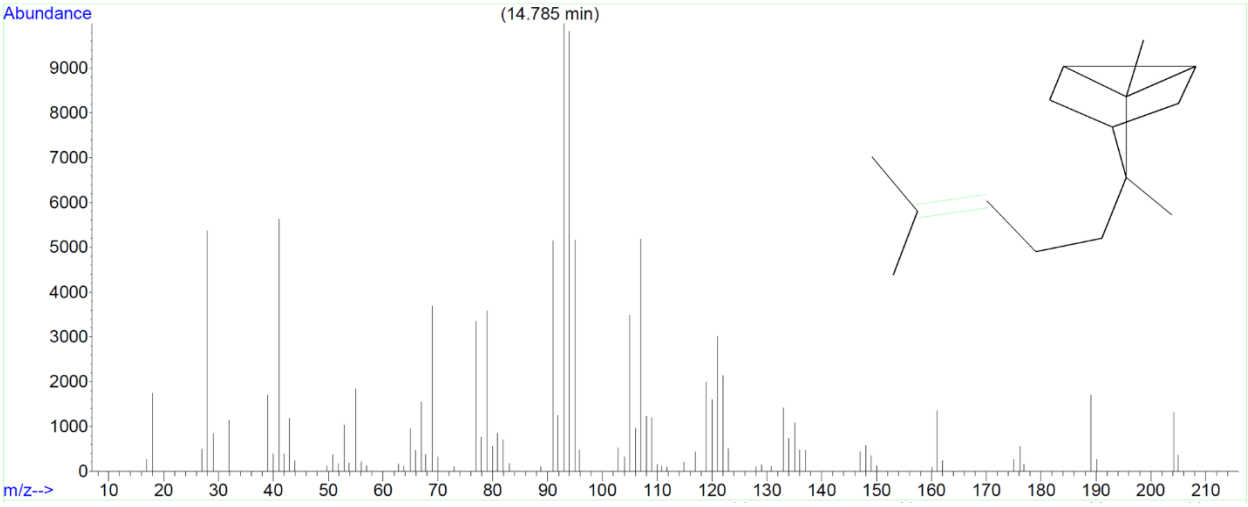

Figure 52. α-santalene

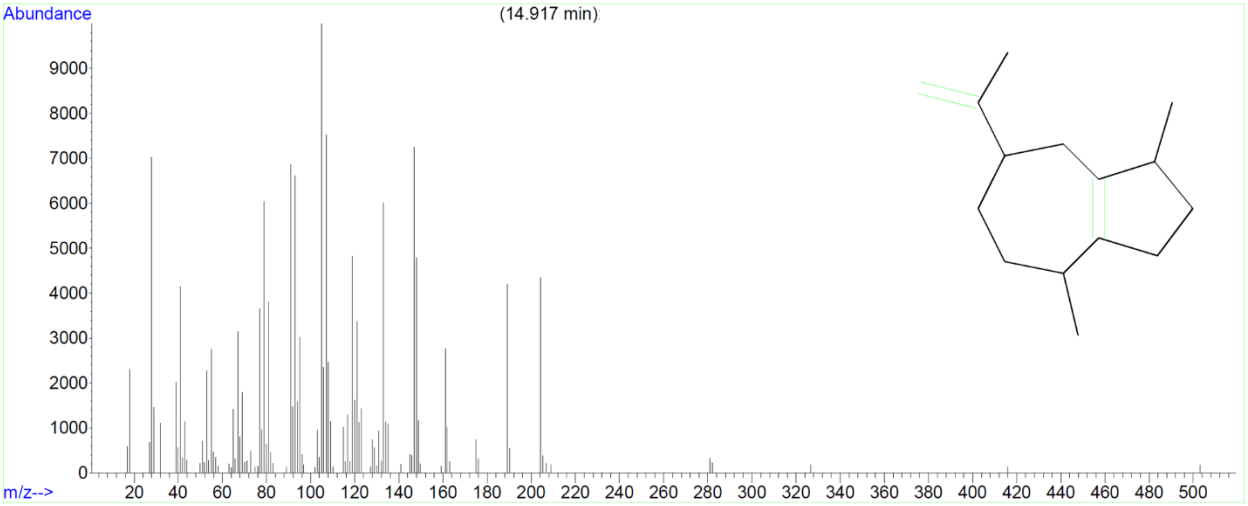

Figure 53. α-guaiene

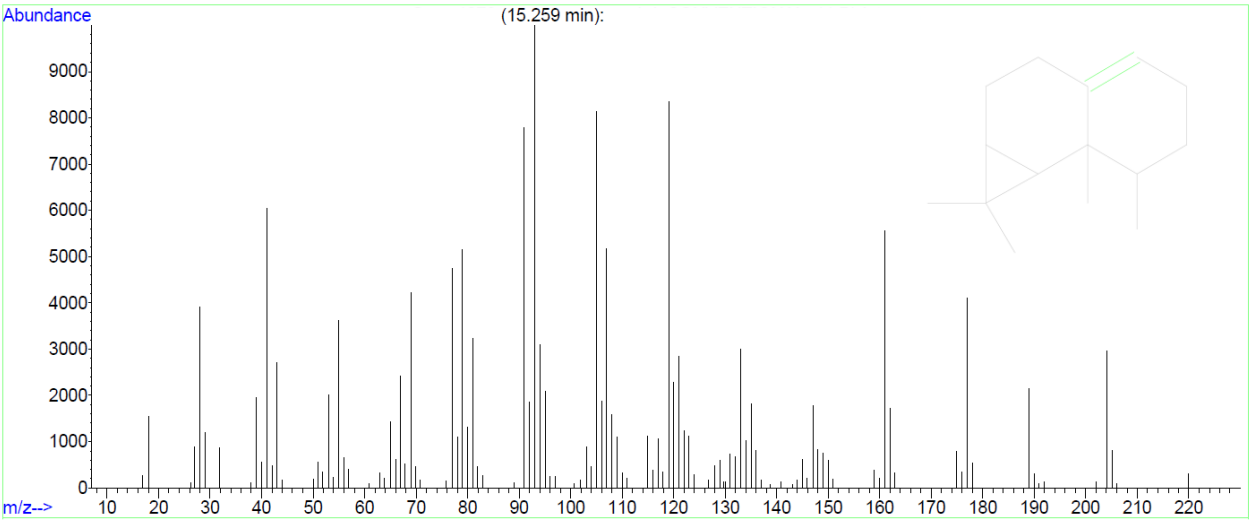

Figure 54. β-gurjunene

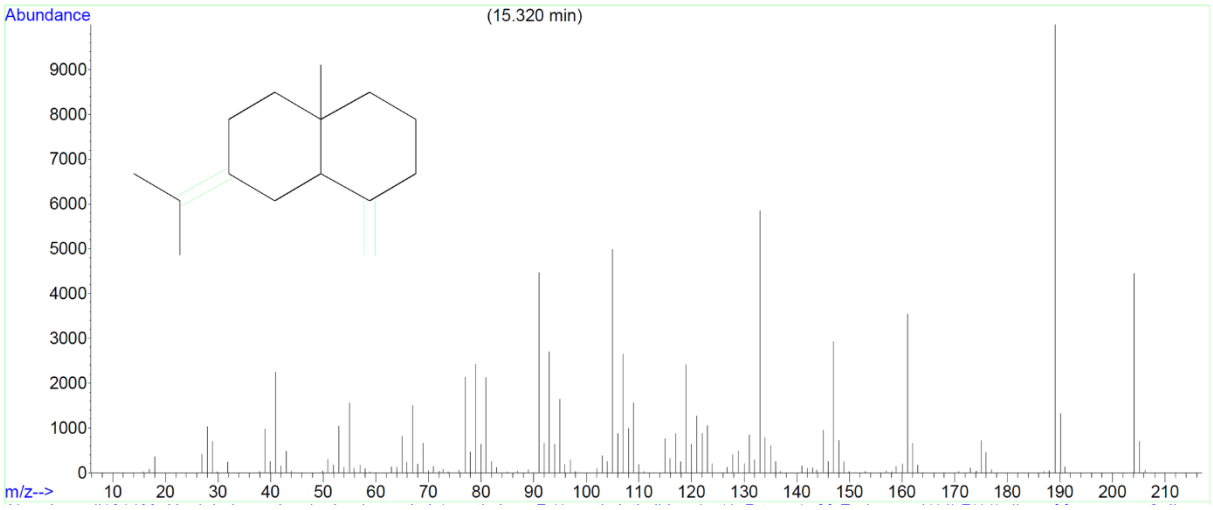

Figure 55. γ-selinene

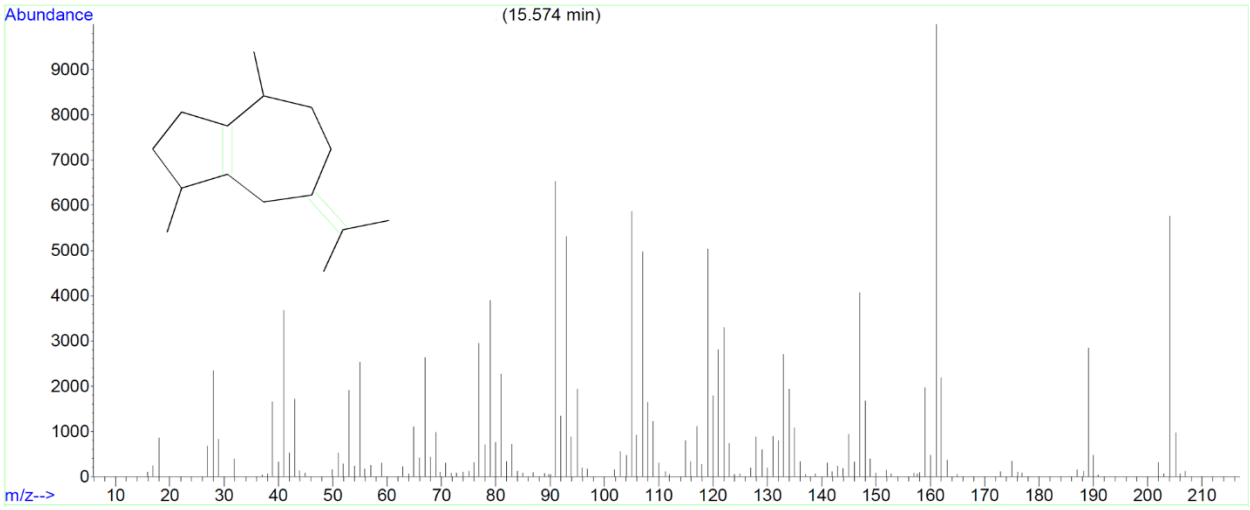

Figure 56. β-guaiene

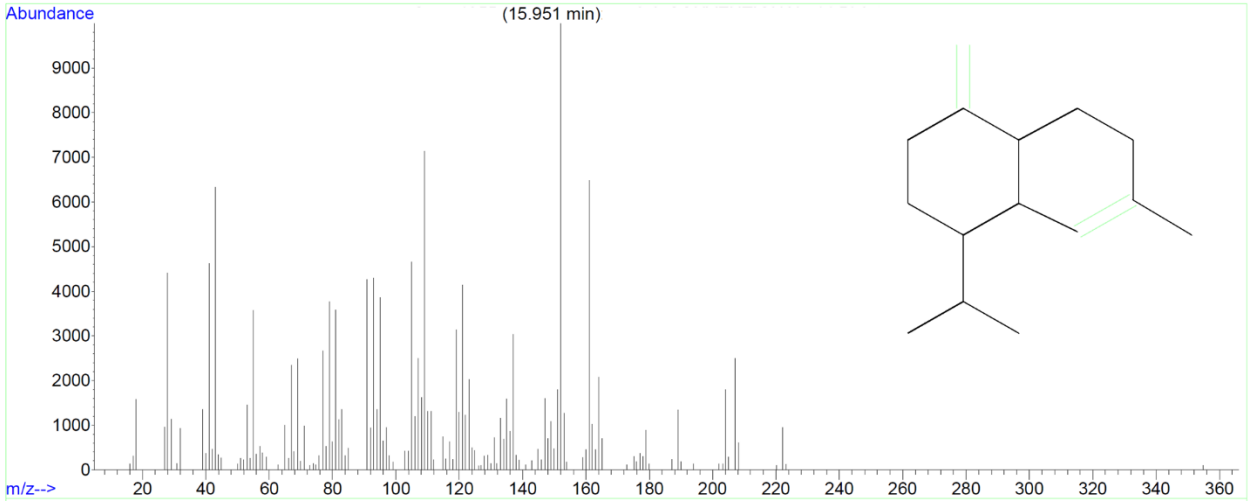

Figure 57. γ-muurolene

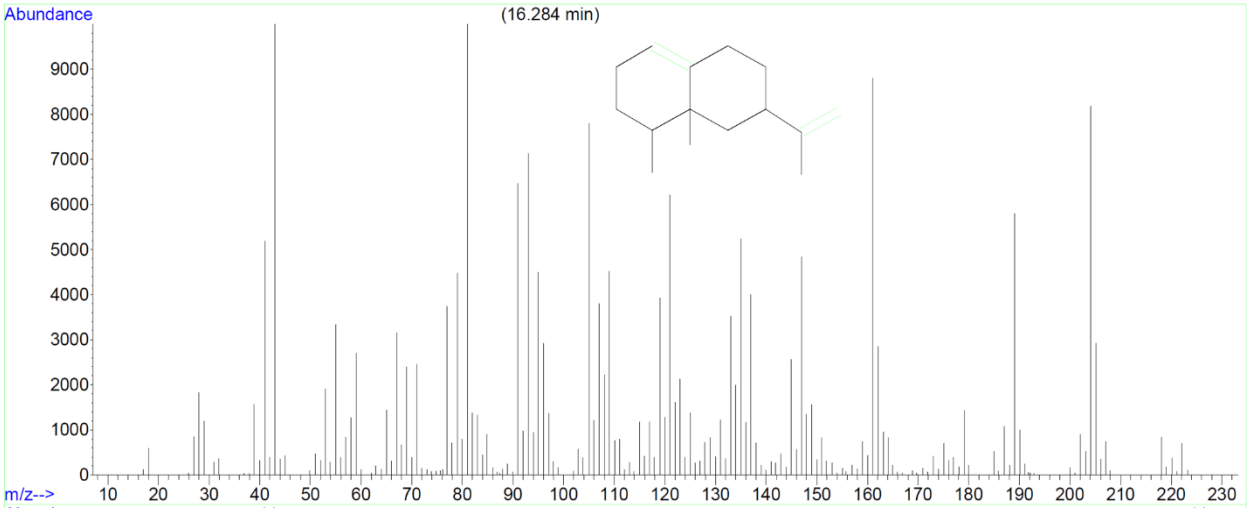

Figure 58. Valencene

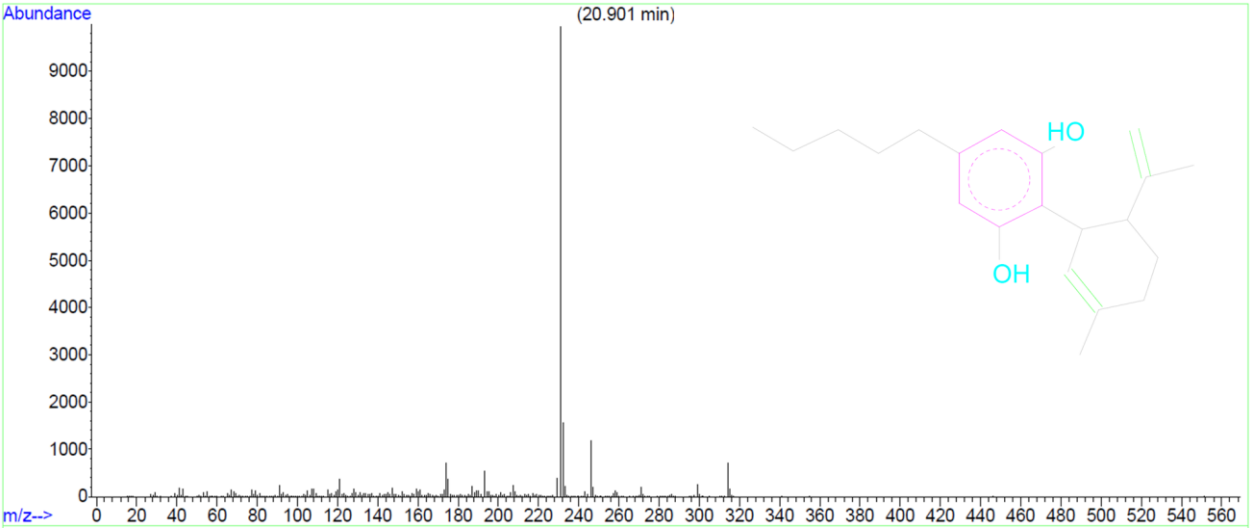

Figure 59. CBD

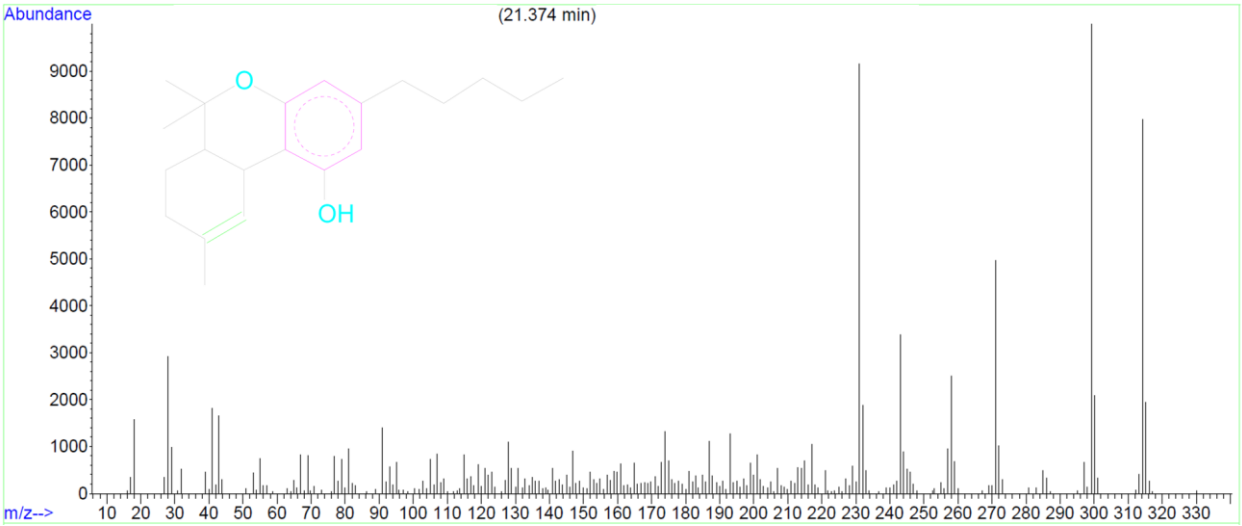

Figure 60. THC

2. Polyphenols analysis on HPLC-DAD

In Table 2 are reported retention times, λ_{max}, λ for quantification, calibration curves, linearity ranges, r^2, LOD and LOQ for all Standard polyphenolic compounds used in this thesis.

Table 2. Retention time, absorption maximum (λ_{max}), λ used for detection, calibration line, linearity range, linear equation fit (r^2), LOD and LOQ of the standard compounds used as external standards.

Compound	Retention time (min)	λ_{max} (nm)	λ for detection (nm)	Linear equation (mg/mL)	Linearity range (mg/mL)	r^2	LOD (mg/mL)	LOQ (mg/mL)
Luteolin-7-*O*-glucoside	23.97	203.4; 254.2; 348.3	340	$y = 3 \times 10^6 \times x - 14330$	0.001-0.500	1.000	0.0005	0.001
Apigenin-7-*O*-glucoside	25.52	266.0; 337.6	340	$y = 2 \times 10^6 \times x - 4724.6$	0.001-0.500	0.999	0.0005	0.001
Catechin	16.56	233.5; 280.6	280	$y = 1.6 \times 10^7 \times x + 568$	0.002-0.100	0.999	0.0010	0.002
Epicatechin	21.55	204.6; 279.1	280	$y = 9.6 \times 10^5 \times x + 754.2$	0.001-1.000	0.999	0.0008	0.001
Chlorogenic acid	20.07	216.4; 240.0; 325.6	340	$y = 2 \times 10^6 \times x + 10241$	0.001-1.000	0.999	0.0006	0.001
Caffeic acid	22.14	217.5; 240.0; 324.4	340	$y = 5 \times 10^6 \times x - 40824$	0.001-1.000	0.999	0.0005	0.001
Protocatechiuic acid	17.10	204.6;258.9;293.4	280	$y = 1 \times 10^6 \times x - 4880.9$	0.001-1.000	0.999	0.0005	0.001
Quercetin-3-*O*-glucoside	28.7	257.0;355.4	340	$y = 4.1 \times 10^7 \times x - 14146.1$	0.001-0.100	1.0000	0.0006	0.001

3. THC and CBS analysis on UPLC-MS/MS

TBC and CBD retention times, r^2, limit of detection (LOD), and limit of quantification (LOQ) are listed in Table 3. In addition, on Figure 61 and 62 are reported UPLC-MS/MS chromatograms for both external standards.

Table 3. THC and CBD standards retention times, r^2, LOD and LOQ.

Compound	Retention time (min)	r^2	LOD (mg/mL)	LOQ (mg/mL)
THC	2.55	0.999	0.0004	0.0006
CBD	2.23	0.999	0.0004	0.0006

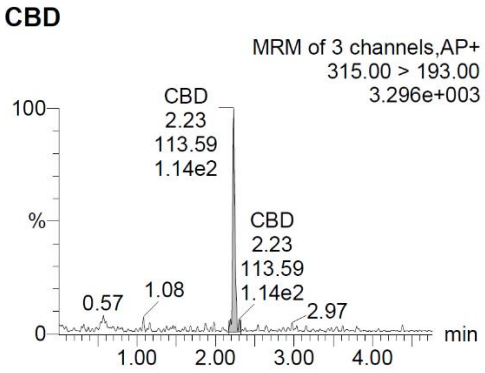

Figure 61. UPLC-MS/MS chromatogram for CBD quantification.

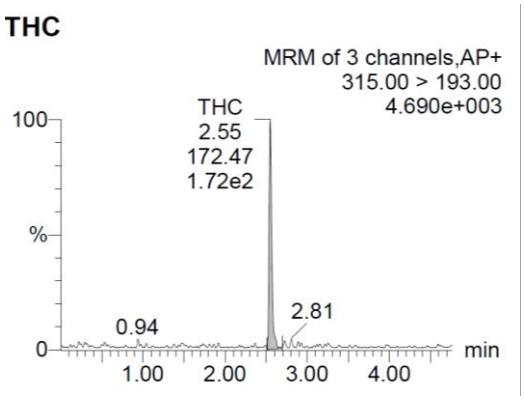

Figure 62. UPLC-MS/MS chromatogram for THC quantification.

Appendix

Publications:

1) G. Cravotto, F. Mariatti, V. Gunjević, M. Secondo, M. Villa, J. Parolin, G. Cavaglià, Pilot Scale Cavitational Reactors and Other Enabling Technologies to Design the Industrial Recovery of Polyphenols from Agro-Food By-Products, a Technical and Economical Overview. *Foods*, **2018**, *7*, 130; doi:10.3390/foods7090130.

2) F. Chemat, M. Abert-Vian, A. S. Fabiano-Tixier, J. Strube, L. Uhlenbrock, V. Gunjević, G. Cravotto, Green extraction of natural products. Origins, current status, and future challenges. *Trac-Trend Anal. Chem.*, **2019**, *118*, 248–263; https://doi.org/10.1016/j.trac.2019.05.037.

3) M. Panića, V. Gunjević, G. Cravotto, I. Radojčić Redovniković, Enabling technologies for the extraction of grape-pomace anthocyanins using natural deep eutectic solvents in up-to-half-litre batches extraction of grape-pomace anthocyanins using NADES. *Food Chem.*, **2019**, *300*, 125185; https://doi.org/10.1016/j.foodchem.2019.125185.

4) G. Grillo, V. Gunjević, K. Radošević, I. Radojčić Redovniković, G. Cravotto, Deep Eutectic Solvents and Nonconventional Technologies for Blueberry-Peel Extraction: Kinetics, Anthocyanin Stability, and Antiproliferative Activity. *Antioxidants*, **2020**, *9*, 1069; doi:10.3390/antiox9111069.

5) V. Gunjević, I. Radojčić Redovniković, L. Stevanato, G. Cravotto, Combined Ultrasound and Microwave Irradiation as an Efficient Technology for Grape-Stalk Anthocyanin Recovery. *Int. J. Green Tech.*, **2020**, *6*, 51-63; https://doi.org/10.30634/2414-2077.2020.06.05.

6) F. Mariatti, V. Gunjević, L. Boffa, G. Cravotto, Process intensification technologies for the recovery of valuable compounds from cocoa by-products. *Innov. Food Sci. Emerg. Technol.*, **2021**, *68*, 102601; https://doi.org/10.1016/j.ifset.2021.102601.

7) V. Gunjević, G. Grillo, D. Carnaroglio, A. Binello, A. Barge, G. Cravotto, Selective recovery of terpenes, polyphenols and cannabinoids from *Cannabis sativa* L. inflorescences under microwaves in kg-scale, *Ind. Crop. Prod.*, **2021**, *162*, 113247; https://doi.org/10.1016/j.indcrop.2021.113247.

8) V. Gunjević, G. Grillo, D. Carnaroglio, A. Binello, A. Barge, G. Cravotto, Analytical dataset of terpenes, cannabinoids and polyphenols form *Cannabis sativa* L. by pilot-scale microwave-assisted extraction, *Data Brief*, **2021** (in press).

Poster presentations at congresses:

1) M. Panić, K. Radošević, V. Gunjević, M. Cvjetko Bubalo, K. Kraljić, M. Obranović, D. Škevin, I. Radojčić Redovniković, Green extraction of phenolic compounds from food by-products and their biological activity. *19th International Conference on Food Processing & Technology*, 23rd–25th October **2017**, Paris, France.

2) V. Gunjević, M. Panić, I. Radojčić Redovniković, G. Cravotto, Green extraction of grape marc and pomace polyphenols. *Natural resources, green technology and sustainable development/3-GREEN2018*, 5th–7th June **2018**, Zagreb, Croatia.

3) V. Gunjević, L. Boffa, G. Cravotto, Optimization of grape marc anthocyanins extraction assisted by simultaneous ultrasound and microwave irradiation. *Green Extraction of Natural Products GENP2018 – III Edition*, 12th–13th November **2018**, Bari, Italy.

4) C. M. Cova, L. Boffa, V. Gunjević, K. Martina, R. Luque, G. Cravotto, Green paths for the valorization of *Chichorium intybus* and *Ocimum basilicum*: microwave/ultrasound-assisted extraction of polyphenols. *Green Extraction of Natural Products GENP2018 – III Edition*, 12th–13th November **2018**, Bari, Italy.

5) M. Panić, V. Gunjević, I. Radojčić Redovniković, G. Cravotto, Green extraction of polyphenols from whole cocoa beans. *Green Extraction of Natural Products GENP2018 – III Edition*, 12th–13th November **2018**, Bari, Italy.

6) V. Gunjević, G. Grillo, L. Boffa, A. Binello, S. Mantegna, G. Cravotto, Cocoa shell waste valorization – extraction from lab to pilot-scale cavitational reactors. *SCI Giovani Merck & Elsevier: Young Chemist Symposium*, 25th–27th November **2018**, Rimini, Italy.

7) V. Gunjević, G. Grillo, K. Radošević, I. Radojčić Redovniković, G. Cravotto, Deep eutectic solvents and non-conventional technologies for blueberry peels extraction: kinetics, anthocyanins stability and antiproliferative activity. *SCI 8° Workshop Nazionale gruppo interdivisionale di green chemistry- chimica sostenibile*, 29th September **2020** [online].

Oral presentations at congresses:

1) G. Cravotto, V. Gunjević, F. Mariatti, E. Calcio Gaudino, A technology platform based on cavitation to link lab research to industry. *Workshop on cavitation exploitation*, 27th– 28th September **2018**, Ljubljana, Slovenia.

2) V. Gunjević, A. Gimenez, G. Cravotto, Estudio quimico y actividad biolgica antiparasitica de *Salacia impressifolia* (Chuchuasi). *Conferencias de Facultad de Farmacia, Universidad de Torino*, 18th June, **2019**, La Paz, Bolivia.

3) G. Grillo, V. Gunjević, D. Carnaroglio, M. Taurisano, G. Cravotto, Estrazione assistita con microonde (MAE): Potenziali applicazioni per la canapa. *Utilizzo dei prodotti di scarto della canapa industriale come fonte di biopesticidi*, 6th December **2019**, Camerino, Italy.

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