Integrated eco-extraction of plant bioactive compounds. Investigation of supercritical fluid extraction (SFE)

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Investigation de l'extraction par fluides supercritiques

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Abbreviation list

- Ace: acetone
- ACN: acetonitrile
- AE: analytical scale-extractor
- ASE: accelerated solvent extraction
- BBD: Box-Behnken design
- BPR: back pressure regulator
- CC-SFE: countercurrent SFE
- CFE: continuous flow extraction
- CNPE: calendula non-polar extracts
- CPE: calendula polar extracts
- d_0: discrimination factor
- DAD: diode array detector
- DCM: dichloromethane
- DE: diatomaceous earth
- DMSO: dimethyl sulfoxide
- DoE: design of experiments
- DPG: dipropylene glycol
- ELSD: evaporative light scattering detector
- EtOH: ethanol
- F: solvent-to-feed ratio (mL/g)
- FA: formic acid
- GC: gas chromatography
- Gly: glycerol
- H_2O: water
- Hept: heptane
- MAE: microwave-assisted extraction
- MeOH: methanol
- NaDESs: natural deep eutectic solvents
- NARP-LC: non-aqueous reversed phase liquid chromatography
- PDA: photodiode Array Detector
- PE: pilot-scale extractor
- PG: propylene glycol
- PHWE: pressurized hot water extraction
- PLE: pressurized liquid extraction
- PPE: pomegranate pericarp extracts
- PTFE: polytetrafluoroethylene
- R^2: determination coefficient
- RSM: response surface methodology
- S^3FE: selective and sequential supercritical fluid extraction
- SBWE: subcritical water extraction
- SC-CO_2: supercritical carbon dioxide
- SFC: supercritical fluid chromatography
- SFE: supercritical fluid extraction
- UAE and USE: ultrasound assisted extraction
- UC: unified chromatography
- UHE-LP/SFC: ultra-high efficiency / low-pressure supercritical fluid chromatography
- UHPLC: ultrahigh performance liquid chromatography
- UV: ultraviolet

Tables of contents

Acknowledgments **4**

Scientific productions **10**

Abbreviation list **14**

Tables of contents **16**

Figures list **24**

Tables list **28**

General introduction **32**

Introduction Générale (FR) **37**

Chapter I: Literature review on eco-extraction of bioactive compounds from plants **42**

1. Bioactive compounds from plants: definition and classification **44**

1.1. Terpenes (Terpenoids and Steroids) 45

1.2. Alkaloids 45

1.3. Phenolic compounds 45

2. Eco-extraction of plants: definition and principles **46**

3. Modern extraction techniques: principles, benefits, and drawbacks **48**

3.1. Ultrasound assisted extraction (UAE) 49

3.2. Microwave-assisted extraction (MAE) 52

3.3. Natural deep eutectic solvents (NaDESs) 53

3.4. Pressurized Liquid Extraction (PLE) 55

4. Supercritical fluid extraction (SFE) application to plant material **60**

4.1. A historical glimpse 60

4.2. Supercritical carbon dioxide (SC-CO_2) 60

4.3. Instrumentation 62

4.4. Parameters influencing the SFE of plant natural compounds. 64

4.4.1. Extraction temperature and pressure 64

4.4.2. Extraction kinetics and flow rate 65

4.4.3. Modifiers or co-solvents 67

4.4.4. The plant material 68

4.5. Selective and sequential extraction applications 68

5. Integrated processes and reduction of unit operations 69

6. References 72

Chapter II: SFE applications on natural polar compounds from plants 81

Part I: Optimization of Supercritical fluid extraction of polar flavonoids from Robinia pseudoacacia L. heartwood 87

Abstract 88

1. Introduction 88

2. Methodology 91

2.1. Plant material 91

2.2. Reference extraction 91

2.3. Chemical and reagents 91

2.4. UHPLC-UV analysis 91

2.5. SFE extraction 92

2.6. Ultra-sound assisted extraction (USE) 93

2.7. Experimental design and Statistical analysis 93

2.8. Extraction time optimization 95

3. Results and discussion 95

3.1. Statistical analysis, model, and factor significance 96

3.2. Optimization of the extraction parameters with Box-Behnken Design. 97

3.3. Extraction kinetics at optimum conditions 99

3.4. Supercritical fluid extraction vs ultrasound extraction 100

3.5. Extraction selectivity 101

4. Conclusion 103

5. References 104

Part II: Supercritical Fluid Extraction (SFE) of polar compounds from *Camellia sinensis* leaves: use of ethanol/water as a green polarity modifier 111

Abstract 112

1. Introduction 113

2. Results and discussion 116

2.1. Statistical analysis, model, and factor significance 117

2.2. Effects of the extraction parameters assessed with BBD 118

2.3. Extraction optimum of caffeine and catechins 120

2.4. Comparison between supercritical fluid extraction (SFE) and ultrasound assisted extraction (UAE) 120

2.5. Selective extraction of caffeine 122

3. Materials and methods 124

3.1. Plant material 124
3.2. Chemicals and reagents 124
3.3. SFE extraction 125
3.4. Ultra-sound assisted extraction (UAE) 125
3.5 UHPLC-UV analysis 126
3.5.1 Elution gradient and quantification 126
3.6 Sample treatment 127
3.7 Experimental design and statistical analysis 128
4. Conclusion 129
5. References 131

Part III: Integrated supercritical fluid extraction and pre-formulation process of *Punica granatum* L. pericarp polar compounds. 137

Abstract 138
1. Introduction 139
2. Materials and methods 141
2.1. Plant material 141
2.2. Chemical and reagents 141
2.3. UHPLC-DAD analysis 141
2.4. Supercritical fluid extraction (SFE) 143
2.5. Ultrasounds assisted extraction (UAE) 143
2.6. Experimental design and Statistical analysis 144
3. Results and discussion 146
3.1. SFE optimization using BBD 146
3.1.1. Statistical analysis, model, and factor significance 146
3.1.2. Validation of experimental design and extraction kinetics 149
3.2. Comparison between Supercritical Fluid Extraction (SFE) and Ultrasound-Assisted Extraction (UAE) 150
3.3. Integrated process: SFE and pre-formulation of natural cosmetic ingredients 152
3.3.1. Dissolution solvent screening 152
3.3.2. Integrated pre-formulation of pomegranate pericarp SC-CO_2 extracts 154
3.3.3. Stability evaluation of the pre-formulated extracts 155
4. Conclusion 155
5. References 156

Chapter III: Selective-Sequential Supercritical Fluid Extraction (S^3FE) 159

Exploring the Sequential-Selective Supercritical Fluid Extraction (S^3FE) of flavonoids and esterified triterpenoids from *Calendula officinalis* L. flowers ... 165
Abstract ... 166
1. Introduction ... 166
2. Results and discussion ... 169
2.1. Optimization of calendula non-polar extracts (CNPE) ... 171
2.1.1. Statistical analysis, model, and factor significance ... 171
2.1.2. Effects of the extraction parameters on CNPE assessed with Box-Behnken design ... 173
2.1.3. Optimal selective extraction of faradiol esters ... 175
2.1.4. Experimental design validation: extraction kinetics CNPE ... 176
2.1.5. Pilot-scale supercritical extraction of CNPE ... 177
2.1.6. Comparison between supercritical fluid extraction (SFE) and ultrasound assisted extraction (UAE) for CNPE ... 179
2.2. Optimization of marigold polar compounds extraction (CPE) ... 180
2.2.1. Statistical analysis, model, and factor significance ... 180
2.2.2. Extraction parameters influence on CPE assessed with Box-Behnken design ... 183
2.2.3. Optimal selective extraction of narcissin ... 185
2.2.4. Comparison between SFE and UAE for CPE ... 186
3. Methodology ... 188
3.1. Plant material ... 188
3.2. Chemical and reagents ... 188
3.3. SC-CO_2 extraction ... 189
3.4. Ultra-sound assisted extraction (UAE) ... 189
3.5. SC-CO_2 extracts sample treatment ... 190
3.6. Calendula non-polar extracts (CNPE) chromatographic analysis ... 190
3.6.1. Ultra-high efficiency low-pressure / supercritical fluid chromatography (UHLP/SFC-ELSD): terpenoid esters analysis in CNPE . 190
3.6.2. Unified chromatography UC-DAD: analysis of polar compounds in CNPE 191
3.7. Calendula polar extracts (CPE): chromatographic analysis of polyphenols ... 193
3.8. Experimental design and statistical analysis ... 194
4. Conclusion ... 196
5. References ... 197

Chapter IV: Separation of *Calendula officinalis* L esterified triterpenoids with ultra-high efficiency / low-pressure supercritical fluid chromatography (UHE-LP/SFC) 203
1. Introduction 207
2. Methodology 209
2.1. Chemical and reagents 209
2.2. Plant material and extract 209
2.3. Instrumentation 209
2.4. Experimental design and statistical analysis 210
3. Results 210
3.1. Criteria for separation optimization 210
3.1.1. Last peak retention time (LPRT) 211
3.1.2. Discrimination factor - resolution (d_0) 212
3.1.3. Total peak number (TPN) 213
3.1.4. Total resolved peak number (TRPN) 213
3.2. Statistical analysis of the experimental model 213
3.3. Derringer desirability functions and application to experimental design 215
3.3.1. Definition of desirability functions (d_i) 215
3.3.2. Global desirability (D) 218
4. Conclusion 219
5. References 221
Conclusion 223
Perspectives 229
Conclusion et perspectives (FR) 231
Supplementary data 239
Chapter II: Part I (Black locust) 241
Chapter II: Part II (green tea) 245
Chapter II: Part III (pomegranate) 249
Chapter III: Calendula non-polar extracts (CNPE) 253
Chapter III: Calendula polar extracts (CPE) 257
Chapter IV: UHE-LP/SFC-ELSD experimental design 261
Structure of targeted compounds 270

Figures list

Chapter I:

Figure 1: Simplified classification of plants secondary metabolites and some of the compounds of interest investigated in this thesis. 44
Figure 2: Illustration of the eco-extraction principles. 46
Figure 3: Illustration of compression and rarefaction cycles in UAE and the growth of cavitation bubbles reaching their implosion. Recreated from [13] . 49
Figure 4: Implosion of cavitation bubble and the release of plant metabolites. Recreated from [21]. 50
Figure 5: Ultrasonic bath (left) versus an ultrasonic probe (right). 51
Figure 6: Dipolar molecules behavior: (a) without electric field, (b) under continuous electric field, and (c) under high frequency electric field. Recreated from [29]. 52
Figure 7: Phase diagram of a binary system with eutectic point [41]. 54
Figure 8: Chemical structure of commonly used HBAs and HBDs for the preparation of NaDESs [44]. 54
Figure 9: Schematic of a PLE instrument. 55
Figure 10: Change in dielectric constant of water as a function of temperature [62]. 56
Figure 11: illustration of pure carbon dioxide pressure temperature phase diagram. 61
Figure 12: Schematic of a SFE instrument for solid-liquid extraction. Option (1) after BPR direct collection of the extracts. Option (2) fractionation using separators in a cascade of different pressures and temperatures. 62
Figure 13: Graphical representation of factors influencing SFE. 64
Figure 14: Graphical representation of the three periods of extraction kinetics and their correlation to extraction curve [91]. 66
Figure 15: Summarized description of some of the unit operations required for solvent plant extraction, from the plant to the final product. 70

Chapter II: Part I:

Figure 1: Response surface heatmap showing the interactive effect of pressure and co-solvent percentage on the flavonoid yield. 98
Figure 2: SFE Kinetics plot for the extraction of DHR and Rob (total flavonoid yield mg/g) from Robinia Pseudoacaccia L. 99
Figure 3: Comparison between SFE extraction and US extraction. 101
Figure 4: DHR and Rob yield for the selective extraction experiments. 102

Chapter II: Part II:

Figure 1: Chemical structure of green tea polar compounds. _____ 113
Figure 2: Response surface heat-maps showing the interactive effects of the variables on the total molecules yield (mg/g) of green tea leaves extracts. _____ 119
Figure 3: Comparison between supercritical fluid extraction (SFE) and Ultrasound assisted extraction (UAE) for green tea leaves compounds (total catechins represents all the catechins including EGCG). _____ 121
Figure 4: SFE sequential-selective extraction of caffeine from green tea leaves (total catechins represents all the catechins including EGCG). _____ 123
Figure 5: UHPLC separation of green tea extract (210 nm). Peak identifications: (1) GC (Tr=1.91 min), (2) EGC (Tr=4.61 min), (3) Cat (Tr=4.84 min), (4) Caff (Tr=5.29 min), (5) EGCG (Tr=6.58 min), (6) EC (Tr=6.72 min), (7) GCG (Tr=7.35 min), and (8) ECG (Tr=8.38). _____ 126

Chapter II: Part III:

Figure 1: UHPLC-DAD analysis of polar extracts of *Punica granatum* L. pericarp diluted in DMSO:EtOH:H2O at 366 nm. PNG1 (RT= 1.83 min), PNG2 (RT= 2.45 min) and EA (RT= 4.88 min). _____ 142
Figure 2: Response surface heat-maps showing the effects of the variation of experimental design factors on the total compounds of interest yield (mg/g) from pomegranate pericarp. (a) Interaction between temperature (X_1) and total modifier percentage (X_2) and (b) interaction between total modifier percentage (X_2) and H_2O% in modifier (X_3). _____ 148
Figure 3: SFE kinetics for the total sum of compounds of interest (EA and PNGs) concentration in mg/g. Constant: 15 MPa, 1g of plants, 3 mL/min flow rate, modifier composition EtOH:H_2O 80:20 *v:v*. _____ 149
Figure 4: Comparison between supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) for polar compounds of *Punica granatum* L. pericarp. _____ 151
Figure 5: UHPLC-DAD analysis of pomegranate pericarp polar extracts solubilized in cosmetic solvent (composition solvent:H_2O mixture) at 366 nm. Glycerol (Gly) red, pentanediol purple, propylene glycol (PG) green, dipropylene glycol (DPG) orange and 1,3-propanediol in blue. _____ 153
Figure 6: illustrated scheme of the integrated SFE (step one) and pre-formulation of the final extracts with cosmetic solvents (step two). The stability study evaluation of the preformulated extract (step three). _____ 154

Chapter III:

Figure 1: Response surface heat-maps showing the effect of the factors variation on the total faradiol esters (faradiol myristate and faradiol palmitate) peak area (mAU×min). (a) Interaction between pressure (X_1) and temperature (X_2), (b) interaction between temperature (X_2) and EtOH percentage (X_3). _____ 174

Figure 2: SFE kinetics of total fardiol esters (faradiol myristate and faradiol palmitate). (a) Variation of EtOH percentage (5 and 15%) at constant pressure (15 MPa) and temprature (80°C). (b) Variation of pressure (10, 15 and 20 MPa) at constant temperature 60°C and 15% EtOH. (c) Variation of temperature (40, 60 and 80°C) at constant pressure (15 MPa) and 15% EtOH_____ 176
Figure 3: Schematic representation of the analytical scale extractor (AE) and pilot-scale extractor used in this study. _____ 178
Figure 4: Comparison of the peak areas (mAU×min/g) of major faradiol myristate and faradiol palmitate, between analytical-scale extraction (AE) and pilot-scale extraction (PE) for CNPE. (a) Extraction kinetics of the total yield of faradiol (b) variation of the total yield in function to solvent-to-feed-ratio. _____ 178
Figure 5: Comparison between supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) for CNPE faradiol esters (myristate and palmitate). _____ 180
Figure 6: Response surface heat-maps showing the effect of the factor's variation on the narcissin yield (mg/g). Variation of total modifier percentage (X'2) and water percentage (X'3). _____ 184
Figure 7: SFE kinetics of narcissin (mg/g) from CPE, variation of temperature 40,60 and 80°C constant conditions: 30% EtOH:H2O 80:20 v:v as a modifier, 15 MPa, 3 mL/min _____ 185
Figure 8: Comparison between supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) for polar compounds of CPE (total polar molecules represent all polar compounds quantified at 354 nm including narcissin). _____ 186
Figure 9: Extraction kinetics of the S3FE of non-polar and polar compounds from *Calendula officinalis* L. represented in yield percentage for 60 min of extraction (total polar molecules represent all polar compounds quantified at 354 nm including narcissin)._____ 187
Figure 10: UHLP/SFC-ELSD analysis of calendula non-polar extract (CNPE). Peak identification (1) faradiol-3-O-myristate (tr =19.78 min), (2) fardiol-3-O-palmitate (tr=23.46 min). _____ 191
Figure 11: (a) UC-DAD gradient of polar compounds in CNPE. Bleu: flow (mL/min), yellow: MeOH+0.1% MSA (%) and red: pressure (MPa). (b) UC-DAD analysis of polar compounds in CNPE (354 nm)/ SFE extract solubilized in MeOH:DCM 1:1 v:v. narcissin (tr=3.15 min). _ 192
Figure 12: UHPLC-DAD analysis of polar extracts (354 nm); narcissin (tr=1,93 min)._____ 194

Chapter IV:

Figure 1: List of commonly found triterpenoids in *Calendula officinalis* L. non-polar compounds. _____ 207
Figure 2: representation of co-eluted peaks with the hv (valley above the baseline) and hp (hight of small peak). _____ 212
Figure 3: Derringer functions representation. (a): LPRT, (b) d_0P_1, (c) d_0P_2, (d): TPN and (e): TRPN._____ 217
Figure 4: UHE-LP/SFC-ELSD chromatograms for exp n°10 (green) representing highest D mean and exp n°2 (bleu) with the lowest D mean. FM (faradiol myristate) and FP (faradiol palmitate)_____ 219

Tables list

Chapter II: part I:

Table 1: Design matrix in the Box-Behnken model. and the flavonoid yield as response. 94
Table 2: ANOVA for response surface regression model for DHR and Rob extraction yield. 96

Chapter II: part II:

Table 1: Design matrix in the Box-Behnken model and responses (represented with SD values for each response) for green tea extracts (total catechins represents all the catechins including EGCG) 116
Table 2: ANOVA for response surface regression model for total yield of target molecules (mg/g) from green tea leaves extracts. 118

Chapter II: part III:

Table 1: Design matrix for the SFE design of experiment (BBD) and responses for polar compounds of interest from *Punica granatum* L. pericarp. 144
Table 2: ANOVA for response surface regression model for total compounds of interest (EA and PNGs) yield from *Punica granatum* L. pericarp SFE extracts 147

Chapter III:

Table 1: Design matrix for the selective SFE design of experiment (BBD) of CNPE with responses for non-polar compounds of interest (faradiol esters) and narcissin (UV response 354 nm UC-DAD). 170
Table 2: ANOVA for response surface regression model for total peak area of faradiol esters (faradiol myristate and faradiol palmitate) in selective CNPE 173

Table 3: Design matrix for the selective SFE design of experiment (BBD) and responses for polar compounds of interest from marigold flowers. (total polar molecules represent all polar compounds quantified at 354 nm including narcissin). 181
Table 4: ANOVA for response surface regression model for narcissin yield from selective CPE. 183

Chapter IV:

Table 1: Coded levels and independent variables (factors) for the experimental model to optimize the SFC separation of *Calendula officinalis* L non-polar compounds. 210
Table 2: BBD experimental design list with coded level, values, factors. The results or dependant responses. With Y_1 -LPRT (last peak retention time), Y_2 - d_0P_1 (discrimination factor of faradiol myristate), Y_3 - d_0P_2 (discrimination factor of faradiol palmitate), Y_4 - TPN (total peak number) and Y_5 - (total resolved peak number). Maximal and minimal responses are in bold. 211
Table 3: summary of the statistical analysis of BBD experimental design for each response (Y). 214
Table 4: BBD experimental design list with coded level and Derringer values for each experiment. Experiment de highest (exp n° 10) and lowest (exp n°2) global Derringer score are in bold. 218

General introduction

The relationship between humans and plants has been tightly knit since the beginning of existence. Originally, it started as a source of nourishment. After that came the discovery of nature flora's medicinal and cosmetic properties, that offered health improvements, skin care benefits and illness remedies. These advantages are closely linked to the compounds produced by the plant and defined as secondary metabolites; they exist in a multitude of structures and categories and offer various biological and physiological activities needed for the daily lives of many people. And thus, early civilizations created extraction techniques to produce medicine, beauty elixirs and perfumes. The extraction methods that we now consider conventional were groundbreaking in their day and paved the way for ones that followed [1].

To extract bioactive compounds, after plant harvest, numerous unit operations are needed: grinding, mixing with extraction solvent, filtration, concentration, evaporation and drying. All these steps are needed to transfer the compounds found inside the initial plant to a liquid solvent, creating a final extract ready to be formulated in cosmetic, food or pharmaceutical products. In the case of conventional extraction techniques, certain drawbacks are fairly known. For example, large amounts of potentially toxic to the environment or human health solvents are used, that needs to be eliminated by extensive evaporation. In addition, long extraction periods and several cycles could be required, increasing energy and manufacturing costs.

This is why in the last fifty years, focus on sustainability and innovative processes have been highlighted, uniting technological progress and natural resources, bending their physio-chemical properties, and allowing the reduction of the above-mentioned drawbacks while in some cases offering higher yields.

The need for sustainable plant extraction techniques that respect both humans and their environment has been the motor for the thesis research in every step, from the extraction of the plants to the analysis and finally to the concentration and pre-formulation.

The thesis aims are the following:

- Replacement of conventional extraction techniques and optimization of bioactive hydrophilic and hydrophobic natural compounds recovery using supercritical carbon dioxide extraction.
- Development of standardized selective SFE approach for compounds of different polarity.
- Development and optimization of analytical methods to reduce analysis time and/or solvent consumption *i.e.*, ultrahigh performance liquid chromatography

(UHPLC), supercritical fluid chromatography (SFC) and unified chromatography (UC).
- Integrated extraction process allowing the reduction of unitary steps reaching the final cosmetic product.

During this project many plants with established activities were investigated: the black locust heartwood, green tea leaves, calendula flowers and pomegranate pericarp. These plants have been known for their proven biological activities and some are extensively used in the cosmetic industries.

Extraction of bioactive compounds of plants might seem very intuitive and many modern extraction techniques are available these days. Besides the choice of the technique, parameters choice needs to be finely tuned to allow a high yield recovery while reducing resource consumption and offering a sustainable approach. Therefore, chapter one is composed of a literature review. Beginning a brief classification of plants secondary metabolites and eco-extraction ethos. Followed by the description of many interesting modern extraction techniques where some are relevant to this project like ultrasound assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE) including continuous flow extraction (CFE) and subcritical water extraction (SBWE) and finally natural deep eutectic solvents (NaDESs); the principles as well as the benefits and drawbacks of each method will be presented.

Supercritical fluid extraction (SFE) is presented in detail. The history, supercritical carbon dioxide properties and their influence on the overall interactions, instrumentation, parameters influencing extraction phenomena like temperature, pressure, flow rate, modifiers, and extraction time. Finally, the interest of selective and sequential SFE applications.

The second chapter is dedicated to SFE applications on polar bioactive compounds. Flavonoids from black locust heartwood, catechins and caffeine from green tea leaves and polyphenols from pomegranates pericarp were extracted. Indeed, even though SFE applications are commonly known for non-polar compounds, in this chapter the focus will be on expanding the limits of SFE polarity. Statistical tools like design of experiments (DoE) and response surface methodology (RSM) were employed to limit the number of experiments and allowed to developpe a standardized approach for the extraction of polar compounds using SFE. In addition, comparisons between UAE and SFE were examined, and selective extraction trials were employed for the black locust and green tea.

The selective and sequential supercritical fluid extraction (S^3FE) and isolation of non-polar esterified triterpenoids and polar flavonoids and narcissin from calendula flowers is presented in the third chapter. Two experimental designs were used to optimize the extraction of both categories of compounds. Furthermore, the extraction of esterified triterpenoids was scaled-up on a pilot-scale extractor and compared to analytical scale SFE.

Finally, chapter four treated the development methodology of ultra-high performance supercritical fluid chromatography analysis of esterified triterpenoids using experimental design and Derringer functions.

On a side note, the majority of chapter two and three results are presented in forms of accepted or submitted articles. In consequence, the related bibliography for each plant composition and extractions are presented in the publication's introduction, and experimental part and developed UHPLC methods in material and methods.

Lastly, this thesis is a part of PIERIC project (*i.e.*, **P**rocédés **I**ntégrés **E**co-**R**esponsables pour des **I**ngrédients. **C**osmétiques - ARD CVL 2020) with the global aim of revisiting the main steps needed to acquire natural ingredients, starting from the plant to the final cosmetic ingredient, or even the finished product. Following sustainable approaches, to limit the volumes of (more or less toxic) solvents used and produced during the different processing steps: extraction of bioactive plant compounds, functionalization by enzymatic reactions, stabilization of the extracted compounds, pre-formulation of ingredients and formulation of the product. Various modern extraction methods were tested on common key plants. Some of these plants contain both polar and non-polar compounds, others possess primarily polar molecules. While the focus in this thesis project was SFE optimization, other extraction methods were evaluated in the framework of the project (CFE, UAE and NaDESs) for the same plants.

Introduction Générale (FR)

La relation entre les humains et les plantes est tissée depuis le début de l'existence. À l'origine, elle a commencé comme une source d'énergie nutritionnelle. Ensuite, est venue la découverte des propriétés médicinales et cosmétiques de la flore naturelle offrant des améliorations pour la santé, des avantages pour le soin de la peau et des remèdes contre les maladies. Ces avantages sont étroitement liés aux composés phytochimiques produits par les plantes et définis comme des métabolites secondaires ou spécialisés. Ces molécules existent sous forme d'une multitude de structures et peuvent faire partie de différentes catégories, ce qui leurs confère de nombreuses activités biologiques et physiologiques nécessaires à la vie quotidienne des consommateurs.

Ainsi, les civilisations anciennes ont développé des techniques d'extraction pour produire des médicaments, des élixirs de beauté et des parfums. Ainsi, les méthodes d'extraction que nous considérons maintenant comme conventionnelles étaient autrefois révolutionnaires.

Pour extraire les composés bioactifs, après la récolte des plantes, de nombreuses opérations unitaires peuvent être nécessaires : le séchage, le broyage, et la mise en contact de la plante avec un solvant d'extraction, puis la filtration, la concentration, l'évaporation et le séchage de l'extrait. Toutes ces étapes sont nécessaires pour transférer les molécules de l'intérieur de la biomasse vers un solvant liquide. Un extrait est généré et prêt à être formulé dans des produits cosmétiques, alimentaires ou pharmaceutiques.

Dans le cas des techniques d'extraction conventionnelles, certains inconvénients sont assez connus. Par exemple, de grandes quantités de solvants potentiellement toxiques pour l'environnement ou la santé humaine sont utilisées, ce qui nécessite une évaporation longue et couteuse. De plus, des temps d'extraction prolongés avec plusieurs cycles peuvent être nécessaires, augmentant globalement les coûts énergétiques et économiques de fabrication.

Au cours des cinquante dernières années, le sujet de l'impact environnementale est devenu une priorité dans différents secteurs industriels. Par conséquent, une approche durable et innovante peut permettre d'associer le progrès technologique aux ressources naturelles. Cette association permet de moduler les propriétés physico-

chimiques et de réduire les inconvénients discutés précédemment tout en offrant dans certains cas des rendements plus élevés.

Le besoin de techniques et de technologies d'extraction végétale durables respectant à la fois les humains et leur environnement a été le moteur de la recherche durant cette thèse allant de l'extraction des plantes, à l'analyse jusqu'à la concentration et à la pré-formulation des extraits.

Les objectifs de la thèse sont les suivants :

• Remplacer les méthodes d'extraction conventionnelles (de type macération) et optimiser la récupération des composés naturels bioactifs hydrophiles et hydrophobes en utilisant l'extraction par le CO_2 supercritique (SFE).

• Développer une approche SFE standardisée et sélective pour différents composés de polarités variées en utilisant des méthodes chimiométriques comme le plan d'expérience.

• Optimiser des méthodes analytiques adaptées à la quantification des composés spécifiques connus dans la littérature pour leurs propriétés biologiques.

Les techniques de séparation et d'indentification analytique utilisées dans le cadre de ce projet, ciblent des composés non-volatil polaires par chromatographie liquide à ultra-haute performance ou UHPLC et non-polaires par chromatographie en phase supercritique ou SFC.

• Mettre en œuvre des procédures d'extraction intégrées afin de simplifier et réduire les étapes unitaires en accédant directement à un extrait final ou semi final. Cela peut permettre, dans le cadre général de ce projet, de réduire les coûts énergiques, économiques et de production des extraits ou des ingrédients préformulés.

Au cours de ce projet, de nombreuses plantes connues dans la littérature pour leurs activités bénéfiques sur la santé humaine générale et plus spécifiquement la peau ont été étudiées : le bois de cœur de robinier ou faux-acacia (*Robinia pseudoacacia* L.), les feuilles de thé vert (*Camellia sinensis*), les fleurs de souci (*Calendula officinalis* L.) et le péricarpe de grenade (*Punica granatum L.).* Ces plantes et organes sont connues pour leurs activités biologiques et certaines sont déjà largement utilisées dans l'industrie cosmétique.

L'extraction de ces composés bioactifs peut sembler facile, surtout avec la multitude de techniques d'extraction, traditionnelles ou modernes, disponibles de nos jours.

Outre le choix de la technique, les paramètres doivent être finement réglés pour permettre une récupération des composés cibles avec un rendement élevé tout en réduisant la consommation des ressources par une approche durable.

C'est à partir de ce contexte que le premier chapitre présente la littérature en commençant par une brève classification des métabolites spécialisés des plantes et les principes d'éco-extraction.

Ensuite, la description de nombreuses techniques d'extraction modernes, dont certaines pertinentes pour ce projet, est présentée comme l'extraction assistée par ultrasons (UAE), l'extraction assistée par micro-ondes (MAE), l'extraction liquide sous pression (PLE), également l'extraction en flux continu (CFE), l'extraction par l'eau sub-critique (SBWE) et enfin les solvants naturels eutectiques profonds (NaDESs). Les principes ainsi que les avantages et inconvénients de chaque méthode sont discutés en englobant les points pertinents dans le cadre du sujet.

Par la suite, l'extraction par fluide supercritique (SFE) est détaillée. L'historique de la technique, les propriétés du dioxyde de carbone supercritique (SC-CO_2) et leurs influences sur les interactions soluté-solvant seront des éléments expliqués tout comme l'instrumentation au travers des paramètres influençant les phénomènes d'extraction comme la température, la pression, le débit, les co-solvants et le temps d'extraction. Enfin, l'intérêt des applications sélectives et séquentielles de la SFE est amené pour finaliser ce premier chapitre.

Le deuxième chapitre est consacré aux applications de la SFE sur les composés bioactifs polaires. On parlera des extraits tels que les flavonoïdes du bois de cœur de robinier faux-acacia, les catéchines et la caféine des feuilles de thé vert, ainsi que les polyphénols comme l'acide ellagique et les punicalagines du péricarpe de fruit de grenade.

En effet, même si les applications de la SFE sont connues pour les composés non polaires, dans ce chapitre, le point le plus important sera de tester les limites de la polarité de la SFE pour l'extraction de ces molécules hydrophiles. Des outils statistiques tels que le plan d'expériences (DoE) et la méthodologie de surface de réponse (RSM) ont été utilisés pour limiter le nombre d'expériences et ont permis de développer une approche standardisée de l'extraction des composés polaires à l'aide de la SFE. De plus, des comparaisons entre l'extraction assistée par ultrasons (UAE) et la SFE ont été examinées et des essais d'extraction sélective polaires ont été réalisés pour le bois de cœur de robinier faux-acacia et le thé vert.

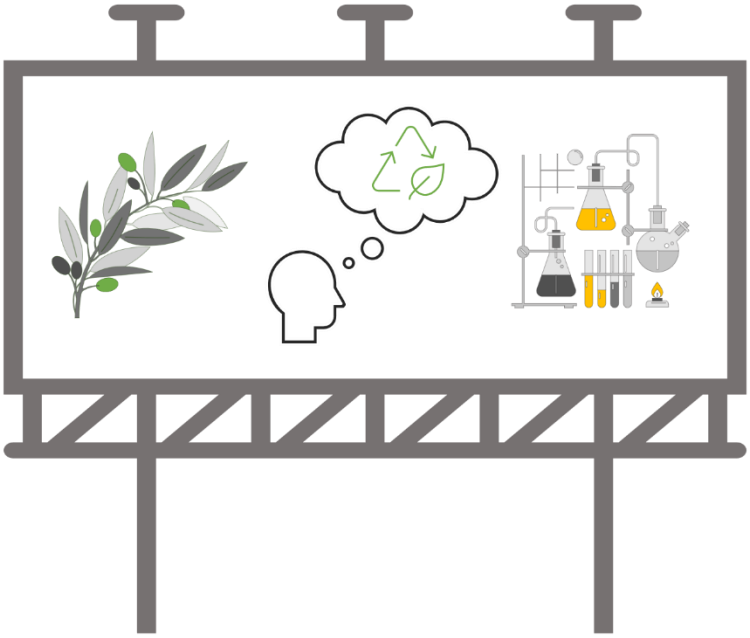
Le troisième chapitre présente l'extraction sélective et séquentielle par fluide supercritique (S^3FE) avec la séparation des composés non-polaires comme les triterpénoïdes estérifiés des composés polaires comme les flavonoïdes (narcissine) à partir des fleurs de souci. Deux modèles expérimentaux ont été conçus pour optimiser l'extraction des deux polarités. De plus, l'extraction des triterpénoïdes estérifiés a été transférée à une plus grande échelle sur un extracteur pilote et comparée à l'extraction à l'échelle analytique.

Finalement, le quatrième chapitre traite de la méthodologie de développement et de l'optimisation des méthodes analytiques en chromatographie par phase supercritique à ultra-haute performance pour l'analyse des triterpénoïdes estérifiés extraits des fleurs de souci. Ces molécules présentent une diversité de structure complexe dans la fraction non polaire des fleurs de souci c'est pourquoi un plan d'expérience et les fonctions de Derringer ont été utilisés pour ce travail.

Il faut noter que la majorité des résultats des chapitres deux et trois sont présentés sous forme d'articles acceptés. Par conséquent, la bibliographie, associée à la composition des plantes et leurs extractions, est présentée dans l'introduction de la publication ainsi que dans la partie expérimentale et la partie du développement des méthodes en chromatographie liquide à ultra-haute performance (UHPLC).

Cette thèse fait partie du projet PIERIC (Procédés Intégrés Éco-Responsables pour des Ingrédients Cosmétiques - ARD CVL Cosmétosciences) ayant pour objectif global de revisiter les principales étapes nécessaires à l'acquisition d'ingrédients naturels, depuis la plante jusqu'à l'ingrédient cosmétique final, voire le produit fini. Suivant des approches durables, le projet vise à limiter les volumes de solvants (plus ou moins toxiques) utilisés et produits lors des différentes étapes de traitement : extraction de composés bioactifs de plantes, fonctionnalisation par des réactions enzymatiques, stabilisation des composés extraits, pré-formulation des ingrédients et formulation du produit. Diverses méthodes modernes d'extraction ont été testées sur des plantes clés communes. Certaines de ces plantes contiennent à la fois des composés polaires et non polaires, tandis que d'autres possèdent principalement des molécules polaires. Bien que l'objectif de ce projet de thèse ait été mis sur l'optimisation de la SFE, d'autres méthodes d'extraction ont été évaluées par les laboratoires partenaires dans le cadre du projet (CFE, UAE et NaDESs) pour les mêmes plantes.

Chapter I: Literature review on eco-extraction of bioactive compounds from plants



1. Bioactive compounds from plants: definition and classification

Plants produce a multitude of compounds. Some can be defined as primary metabolites like carbohydrates, lipides, and proteins. These compounds are key in metabolic routes intended towards plant development and growth. Others are classified as secondary metabolites, which are not required for everyday functioning of the plant but nonetheless hold essential functions for the protection of the plant from external threats like animals, insects or free radicals generated during photosynthesis. Some secondary metabolites can be described as bioactive compounds if they stimulate a toxicological or a pharmacological effect in human beings or animals [2,3].

In contrast to primary metabolites, the absence of secondary metabolites does not cause immediate death. Instead, it affects an organism's capacity to survive, reproduce, or general health in the long term, or it might have no effect. These are frequently limited to a small number of species inside of a phylogenetic group [4]. Bioactive plant compounds can be categorized according to various elements. For example, based on their chemical structure (containing sugar, ring structure), composition (containing a nitrogen or sulfur group), their activity or according to their solubility and polarity. In some cases, certain structurally or chemically related compounds can have different clinical results, complicating their classification. Therefore, it is advisable to distinguish them based on chemical class and biochemical pathway.

The main bioactive families are the following (**Figure 1**):

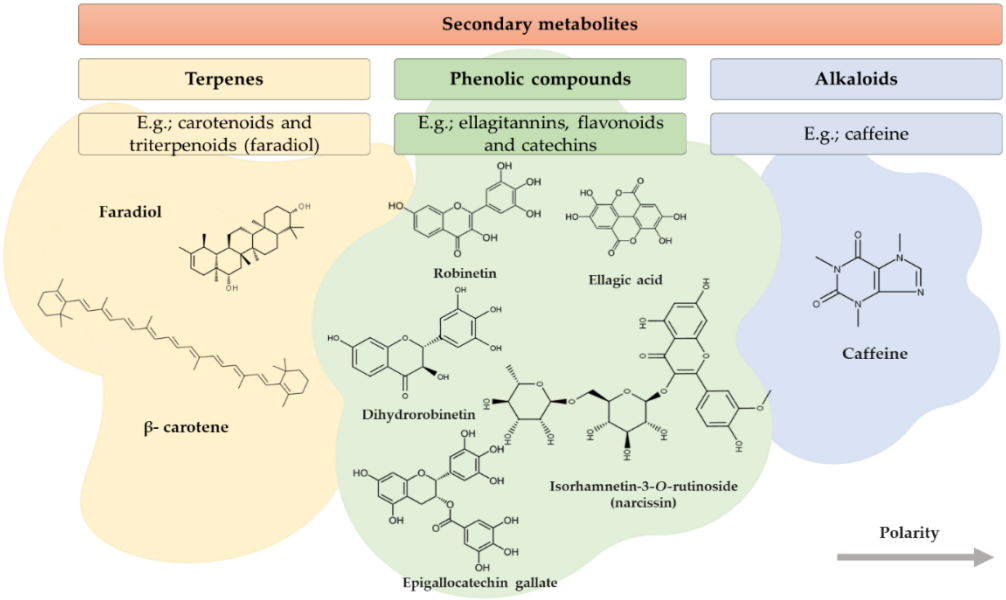


Figure 1: Simplified classification of plants secondary metabolites and some of the compounds of interest investigated in this thesis.

1.1. Terpenes (Terpenoids and Steroids)

More than 30,000 terpenes have been identified until today, they all share one common characteristic possessing a repeating five-carbon isoprene units. From two isoprene units (monoterpenes) like eucalyptol to eight units like carotenoids. Terpenes are usually thought to have both ecological and physiological functions in plants: insecticide, pollinator, hormonal, and allelopathic. They are produced by two different metabolic pathways: the mevalonate and the non-mevalonate pathway [5].

1.2. Alkaloids

Almost 12000 compounds have been identified to this day, alkaloids family is categorized as naturally occurring organic secondary compounds with a minimal of one nitrogen atom. They are involved in plant germination and defense against predators, including herbivores and microorganisms. As for human applications, alkaloids act primarily on the nervous system, offering stimulating, relaxing, or anesthetic properties. Well known alkaloids include morphine, cocaine nicotine, and caffeine [4,5].

1.3. Phenolic compounds

These compounds are biosynthesized through the shikimate, pentose phosphate and phenylpropanoid pathways [6,7]. The distribution of phenolics, considered as the most prominent secondary metabolites in plants, can be seen throughout the entire metabolic process. These compounds, also known as polyphenols, are made up of a wide range of sub-categories including simple flavonoids, phenolic acids, complex flavonoids, and colored anthocyanins [8]. These compounds have several functions in plants, including those of antioxidants, structural polymers (lignin), attractants (flavonoids), UV screens (flavonoids), signal molecules (salicylic acid and flavonoids), and defensive response chemicals (tannins and phytoalexins) [7].

The development of food additives, functional foods, pharmaceuticals, nutraceuticals, and cosmetic ingredients is based on these bioactive compounds with a wide range of functions and structural variations.

2. Eco-extraction of plants: definition and principles

Natural product extraction has probably been practiced since humans became aware of energy. Different civilizations, including the Mesopotamians, Egyptians, Chinese, Arabs, Indians, Greeks, and Romans, developed numerous inventive extraction techniques. Plant extracts have been used by all these civilizations for cosmetic and therapeutic as well as nutritive purposes [9–11]. There isn't a production process used today in the biofuel, food, pharmaceutical, cosmetic, or fragrance sectors that doesn't require extraction processes. [11].

There are numerous methods for extracting bioactive components from plants. Most of the conventional extraction techniques call for the use of significant amounts of solvent and prolonged durations. In addition, the solvents used when dealing with lipophilic or non-polar natural compounds are commonly toxic. Over the past fifty years, novel, and modern methods, also referred to as eco-extraction methods, have emerged to avoid these issues. All these methods follow some or all of the six eco-extraction principles described by Farid Chemat in 2011 (**Figure 2**)[11]:

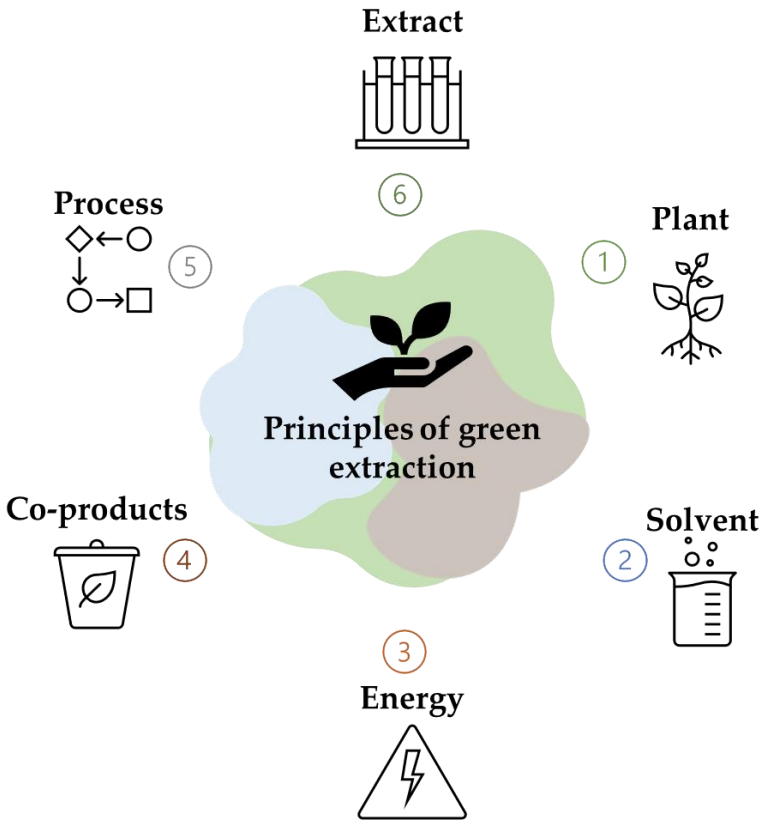


Figure 4: Illustration of the eco-extraction principles.

***Principle 1**: "Innovation by selection of varieties and use of renewable plant resources".*

After the determination of a natural compound's benefits, the next step is obtaining it in large quantities for human or animal application. However, in some cases this leads to the overutilization of plant species that may result in its extinction if not controlled. For this reason, new innovative methods should be developed to recover bioactive compounds in a regulated environment, like using natural precursors from renewable sources for semi-synthesis.

***Principle 2**: "Use of alternative solvents and principally water or agro-solvents".*

The use of agro- or bio-sourced solvents plays a significant role in an ecological and economical context to replace petrochemical solvent and volatile organic solvents. Today, for the extraction of natural compounds using agro-solvents like bio-sourced ethanol, glycerol, or oil in addition to naturally occurring resources like water and supercritical carbon dioxide is more common.

***Principle 3**: "Reduce energy consumption by energy recovery and using innovative technologies".*

Energy reduction can be achieved with either the recovery of the energy liberated during the process or using assisted extraction techniques that increase the recovered yield in shorter time.

***Principle 4**: "Production of co-products instead of waste to include the bio- and agro-refining industry".*

During an industrial process, besides the extracts many different products can be produced, corresponding to waste that needs to be eliminated, or by-products considered as residuals of the process that can be employed for other purposes. The production of by-products instead of waste can be beneficial on many aspects. It allows the reduction of the energy needed to eliminate waste and to repurpose it for other production processes giving it an economic value. This is highly related to the use of agro-solvents, where the waste can be re-utilized in other sectors or composted instead of incineration.

***Principle 5:** "Reduce unit operations and favor safe, robust and controlled processes".*

The extraction process is a multi-step operation, the reduction of these steps by using integrated process is very beneficial to the reduction of time, costs, and energy. The elimination of drying, evaporation or filtration is sought in current industries.

Principle 6: "Aim for a non-denatured and biodegradable extract without contaminants".

Depending on the plant or its origin or the extraction process, the final extracts can be contaminated with toxic solvent residues, pesticides, or metals. Whereas the natural state of an extracts does not guarantee its nontoxicity to man, animal, or environment. Controlled processes, aiming to meet regulations and market requirements, is a must for harmless extracts.

3. Modern extraction techniques: principles, benefits, and drawbacks

As previously noted, plant extraction has long been a part of our daily life. Conventional techniques for extracting natural compounds from plants include Soxhlet extraction, infusion, percolation, maceration, and hydro-distillation. These long-established techniques require the use of large quantities of organic solvents or water, which implies a subsequent and thorough evaporation step, long extraction duration and high temperatures, leading to the degradation of thermolabile compounds. Due to their numerous limitations and negative effects on the environment, conventional extractions are now avoided in sectors seeking sustainability.

This created the need to develop more modern extraction methods that follow the eco-extraction principles and at the same time minimize processing time and costs, improve extraction efficiency and selectivity, and ensure the safety of the final extract by consuming less solvent and using safer, greener alternatives in place of conventional ones. In this section various green and modern extraction techniques and in some cases relative to the thesis subject will be addressed, including the principles, the advantages, and drawbacks.

3.1. Ultrasound assisted extraction (UAE)

In contrast to audible noises, ultrasound is a mechanical wave that must propagate via an elastic material. In frequency range for UAE (comprised between 20 kHz and 2 MHz), ultrasound can cause physical and/or chemical changes in the medium, which can assist or accelerate chemical reactions. The cavitation phenomenon results from physical processes that produce, grow, and implode small bubbles of gases dissolved in the liquid, and is responsible for most of the effects of ultrasound in a liquid medium [12]. Ultrasound is primarily defined by its power (measured in W), frequency (measured in Hz), and wavelength (measured in cm), from which the ultrasonic intensity (I) is determined (measured in W/cm^2). The molecules that constitute a liquid are held together by attractive forces, and as an ultrasound wave travels through the liquid, it causes a longitudinal displacement of those molecules. This longitudinal displacement acts as a piston on the surface, it's the result of a series of compression and rarefaction phases (**Figure 3**) [13].

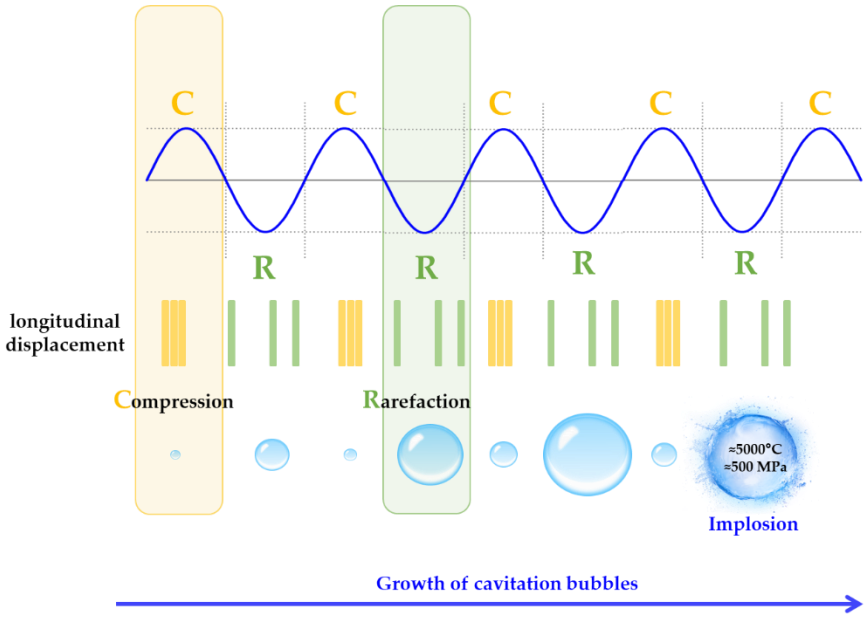


Figure 7: Illustration of compression and rarefaction cycles in UAE and the growth of cavitation bubbles reaching their implosion. Recreated from [13] .

Figure 8: Implosion of cavitation bubble and the release of plant metabolites. Recreated from [21].**Figure 9:** Illustration of compression and rarefaction cycles in UAE and the growth of cavitation bubbles reaching their implosion. Recreated from [13] .

At high power ultrasound surpasses the liquid's attractions forces, and cavitation bubbles are created. These bubbles are formed from dissolved gases are what cause the voids to be created in the medium [14–16]. A temporary hot spot is produced when the size of these bubbles reaches a critical point and collapses during a compression cycle [17,18]. The local pressure and temperature at the collapse point of cavitation bubble could reach 5000°C and 5000 bar, causing local hotspots that can drastically accelerate the extraction by destroying the cell walls and releasing compounds in the extraction solvent [18–20] (**Figure 4**) [21].

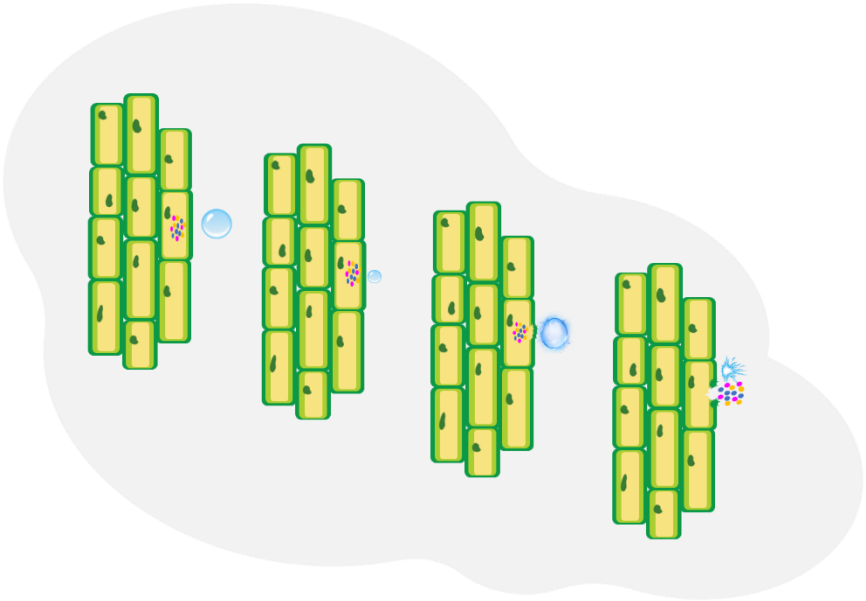


Figure 10: Implosion of cavitation bubble and the release of plant metabolites. Recreated from [21].

Figure 11: Ultrasonic bath (left) versus an ultrasonic probe (right).**Figure 12:** Implosion of cavitation bubble and the release of plant metabolites. Recreated from [21].

Two devices can be used to generate ultrasounds: the ultrasonic bath and probe (**Figure 5**). The ultrasonic bath is used to disperse solids in a solvent, it's also used to degas solutions or clean materials, as it is easier to handle and more economical than the ultrasonic probe. The disadvantage of this device is its lower intensity, which is

reduced by the bath water. Many elements can be considered to diminish the attenuation effect of using US bath, like using of flat bottom conical vessel with minimal thickness. A probe is more powerful in smaller volumes, as it transmits intensity over a smaller surface area. Additionally, it is submerged directly in the extraction solvent mixed with solid matrix, limiting the medium attenuation of the intensity. The extracts temperature must be kept under control in these circumstances because it can increase at an accelerated pace [22–26].

Figure 13: Ultrasonic bath (left) versus an ultrasonic probe (right).

Figure 14: Dipolar molecules behavior: (a) without electric field, (b) under continuous electric field, and (c) under high frequency electric field. Recreated from [29].**Figure 15**: Ultrasonic bath (left) versus an ultrasonic probe (right).

Ultrasonic power and resultant intensity have an impact on extraction in addition to the factors directly connected to the device (such as the frequency, wavelength, and amplitude of the wave). The procedure can be affected by the reactor's design as well as the probe's form. Since the extraction takes place in a solvent medium, factors like solvent type can have an impact on both the extraction yield, composition and selectivity in addition temperature and extraction time should be taken into account [24].

UAE is considered as an efficient, simple, and relatively low-cost extraction method. In addition to laboratory-scale apparatus, large-scale industrial ultrasonic devices have been successfully employed in a variety of fields. It can also be used in

combination with other conventional extraction techniques, therefore reducing long extraction durations. However, some drawbacks are the difficulty of renewing the extraction solvent during the process, the lack of a dynamic mode as well as the post extraction filtration and evaporation [27].

3.2. Microwave-assisted extraction (MAE)

This technique employs microwaves, defined as non-ionizing electromagnetic waves (generally around 2.45 GHz) to heat a medium. The basis for the dielectric heating mechanism in MAE is the existence of dipoles, which are polar molecules with opposing positive and negative ends (permanent or induced). The dipoles in a solvent are randomly orientated by the thermal agitation of the medium in the absence of an electric field. When an electric field is present continuously, molecules tend to point in that direction. A global induced dipole moment manifests after all the molecules are aligned. Dipole rotation occurs when an alternating electric field of frequency causes the dipoles to rotate in one direction for half of an alternation, becoming disoriented when the field is cancelled. Thermal energy is released when the electric field ends because the agitation causes the molecules to return to an unorganized state during the relaxation period. Therefore, heating happens because of electromagnetic waves dissipating in the irradiated medium. The complicated permittivity of the medium and the local time-averaged electric-field strength determine how much power is lost in the medium [28–30]. (**Figure 6**) [29].

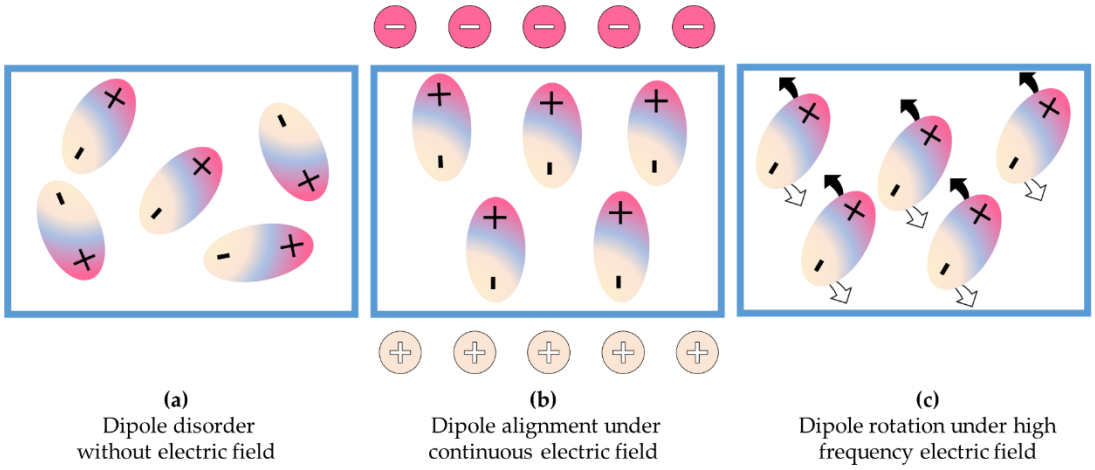


Figure 16: Dipolar molecules behavior: (a) without electric field, (b) under continuous electric field, and (c) under high frequency electric field. Recreated from [29].

Figure 17: Phase diagram of a binary system with eutectic point [41].**Figure 18:** Dipolar molecules behavior: (a) without electric field, (b) under continuous electric field, and (c) under high frequency electric field. Recreated from [29].

Solvent with high dielectric constant like water (78.4), can absorb the microwave energy and cause heating of the water, this combined with the heating of the intrinsic liquid found in the plant cell (in the case of fresh biomass), lead to their implosion and release of the secondary metabolites found inside the cell walls, thus increasing of extraction efficiency. Other solvents with lower dielectric constant like hexane (1.88) or heptane (1.92) are less affected by microwaves, and therefore will heat less compared to solvents with a higher dielectric constant.

Additionally, selective extraction can be reached with the use a solvent mixtures like water and alcohol for the extraction of polar and moderately polar compounds[31–36], and only alcohol, acetone, or chloroform for the extraction of moderately polar or non-polar compounds [37,38]. Other factors that contribute to MAE of plants optimization are solvent to plant ratio, temperatures, extraction time, number of cycles and power. In conclusion, MAE is considered as an energy efficient extraction technique since it can increase yield recovery of metabolites of interest and solvent consumption. The use of microwaves can have some limitations, for example highly non-polar compounds are less compatible with this technique, and extensive heating could damage and degrade thermolabile compounds, in addition to the requirement filtration and evaporation steps [29].

3.3. Natural deep eutectic solvents (NaDESs)

Deep eutectic solvents (DESs) have been around for almost 20 years [39], their components interact through hydrogen bonding as opposed to ionic bonds in traditional ionic liquids [40]. DESs are composed of two elements that are commonly solids, the first is called a hydrogen bond acceptor (HBA) and the second hydrogen bond donor (HBD), the interaction between both results in a reduction of the entropy lowering their corresponding melting point and creating low volatility liquid [39,40] (**Figure 7**) [41].

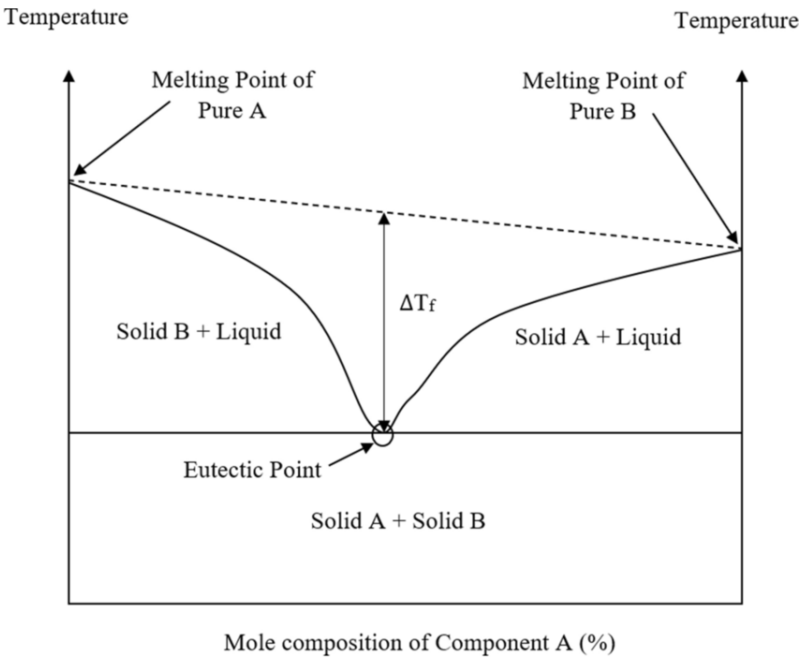


Figure 19: Phase diagram of a binary system with eutectic point [41].

Figure 20: Chemical structure of commonly used HBAs and HBDs for the preparation of NaDESs [44].**Figure 21:** Phase diagram of a binary system with eutectic point [41].

When DESs are constituted of natural elements like sugars, organic or amino acids and

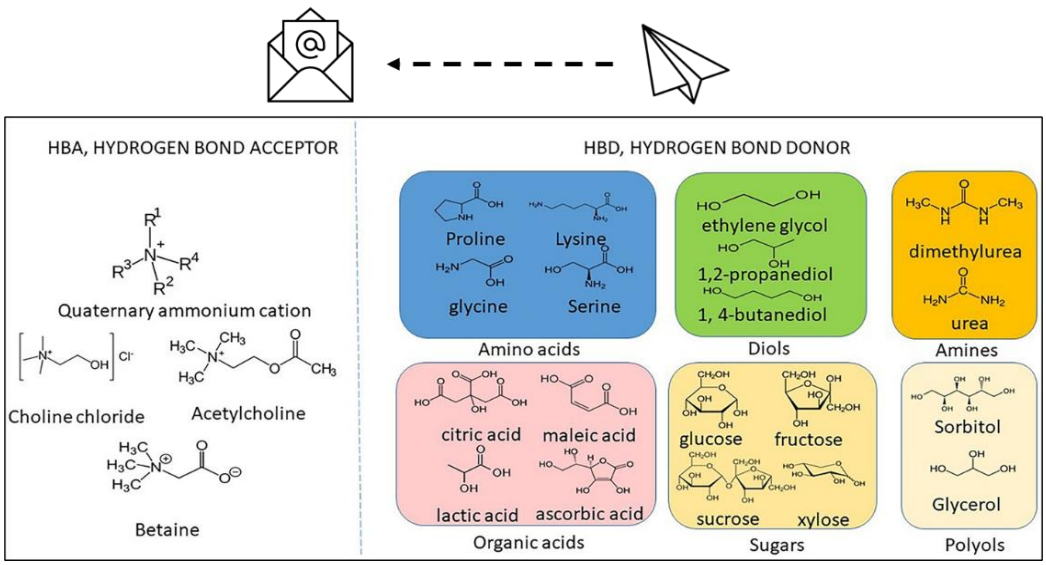


Figure 22: Chemical structure of commonly used HBAs and HBDs for the preparation of NaDESs [44].

choline derivatives, they are generally named natural deep eutectic solvents or NaDESs [42]. The most common HBA and HBD used for polar compounds extraction are presented in **Figure 8** [40,43,44].

In recent years, the use of NaDESs as alternative solvents for the extraction of natural substances has increased dramatically, this is due to the possibility of a selective extraction of both polar [45–51] and non-polar compounds [52–56]. In addition, their components are commonly inexpensive, they are easy to prepare, and their use can be combined with techniques such as UAE, MAE and PLE [57].

However, NaDESs viscosity can be considered as a limitation and result in a decrease in the extraction yield, as a results of slower penetration of the solvent into the matrix and slower diffusion rates compared to classical solvents, in addition to difficulties of filtration and handling of the extracts. However, these solvents are generally diluted in water to limit their viscosity. Coupling NaDESs with other extraction techniques has been reported. Assisted extraction processes like UAE and MAE result in an increased temperature, decreasing the viscosity of the NaDESs, thereby facilitating its penetration into the plant matrix. Other physio-chemical properties such as volatility can be problematic, given that it remains in the final extract. Since NaDESs are non-volatile solvents, it's necessary to conduct additional research on the extract stability in solvent and use NaDES-extract mixtures directly in formulations.

3.4. Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction (PLE) was introduced for the first time in 1995 as accelerated solvent extraction (ASE®) by Dionex. This process utilizes liquid solvents under pressure and high temperature, both of which affect the extraction efficacy. It is

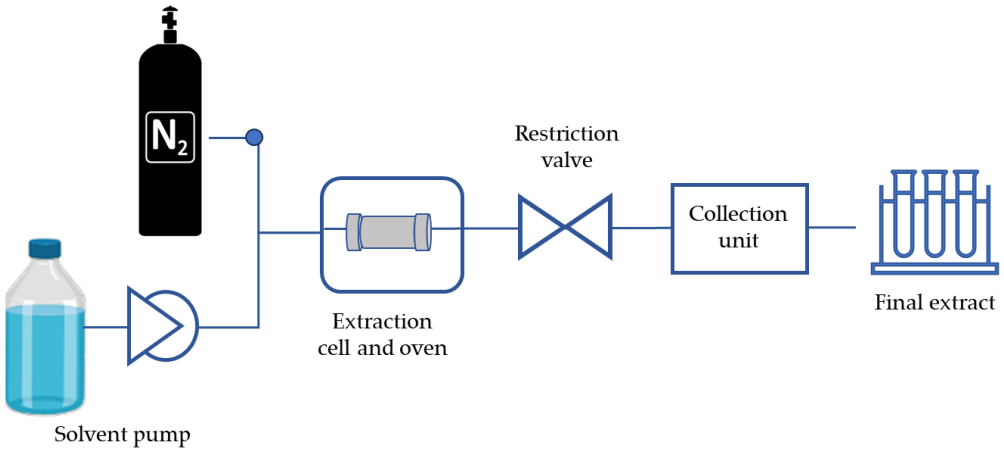


Figure 25: Schematic of a PLE instrument.

Figure 26: Change in dielectric constant of water as a function of temperature [62].**Figure 27:** Schematic of a PLE instrument.

partially derived from supercritical fluid extraction, however in PLE the liquid is maintained at its liquid and in some cases subcritical state [58,59]. Two main extraction modes are used in PLE, the first one is the classical static mode (**Figure 9**), it consists of the use of one or several static cycles, with the replacement of solvent between each cycle. The second system is a dynamic or flow mode, also called a continuous flow extraction (CFE). The flow rate is set at the beginning of the extraction and fresh solvent is continuously in contact with the matrix during the entire process [60].

Temperature is one of the most significant factors that can influence PLE performance, particularly in terms of the kinetics and velocity of the mass transfer from the plant matrix to the extraction solvent. This is partially due to high temperatures and pressure changing the physiochemical properties of the solvent. For instance, the dielectric constant of water decreases under these circumstances, resulting in polarities similar to those of organic solvents (**Figure 10**) [61,62].

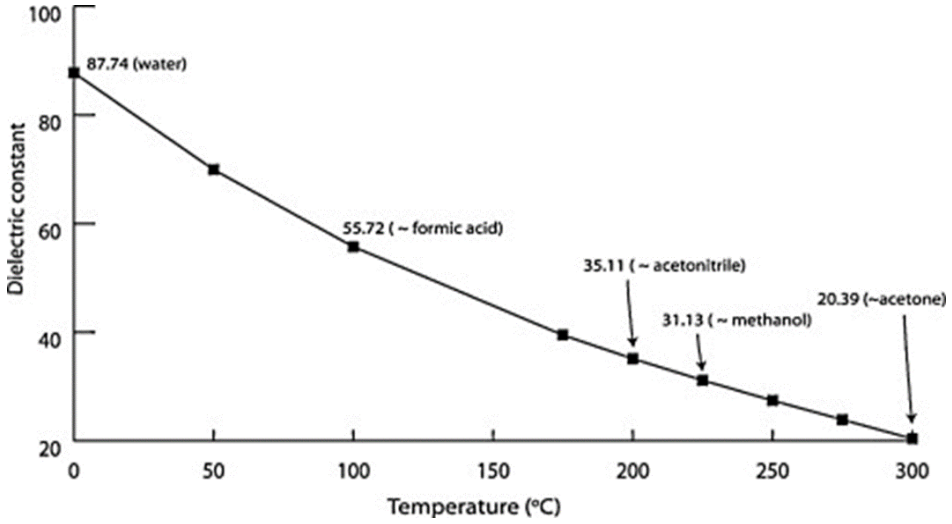


Figure 28: Change in dielectric constant of water as a function of temperature [62].

In addition, the use of high temperatures has been reported to increase the diffusion rate of the solvent by a factor of 2 to 10 with the increase of temperature from 50°C to 150°C and to decrease the viscosity of isopropanol by 9-folds [24,63], thus increasing the solvent penetration through the plant matrix. However, the use of such high temperatures is not always beneficial when working with natural compounds, this might lead to their degradation or trigger secondary reactions changing the quality of

the final product, this is why in general when working with natural matrixes the temperature range used is comprised between 40-200°C [59,63].

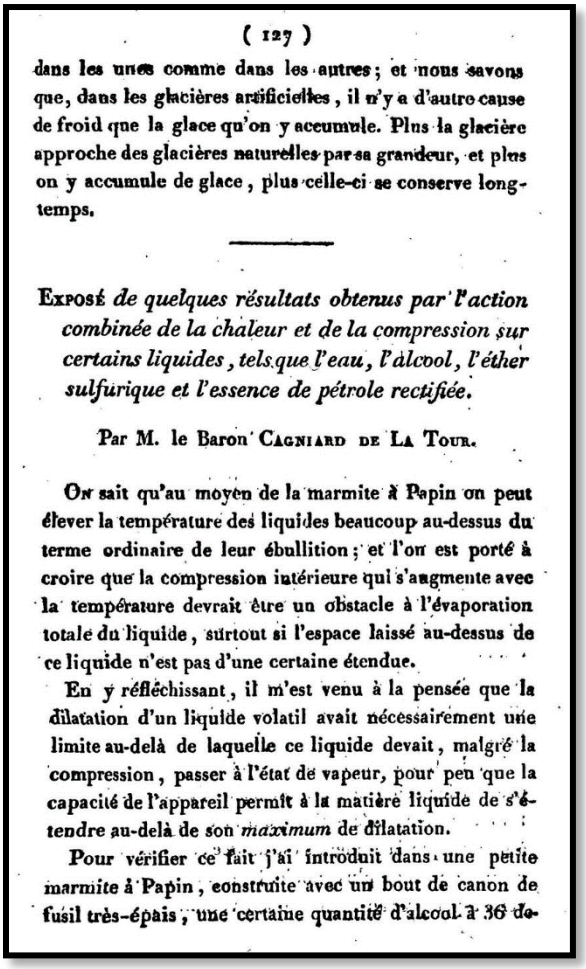
As for the pressure, generally it is kept at minimal rate to avoid the evaporation of the solvents at high temperature, this factor is temperature and solvent dependent. For example, 15 MPa of pressure is needed to prevent the evaporation of water at 200°C; however, at 300°C, the required pressure increase to 85 MPa [59,64].

Furthermore, PLE can be used with a wide range of solvents. Nonetheless, in green and sustainable approaches only non-toxic solvents can be selected. Water is the most used solvent for the extraction of polar compounds, it can be used alone or in mixtures of organic solvents to modify its polarity and therefore its selectivity for less polar compounds.

Subcritical water extraction (SBWE) or pressurized hot water extraction (PHWE) is related to PLE, it refers to the utilization of only water as an extraction solvent. Under a high temperature and pressure, a significant decrease of the dielectric constant of water is reached and getting closer to organic solvents. Additionally, the water's surface tension and viscosity drop as the temperature rises and the diffusion rates improve. SBWE have many applications on plant bioactive compounds [65–76].

Other variables are very important for a proper extraction optimization using PLE, like matrix particle size, extraction time, sample pretreatment, solvent to matrix ratio and extraction mode (dynamic or static) [58,59].

In conclusion, PLE is method that can offer higher efficiency compared to other extraction techniques [77,78]. It can be applied to all types of plants and solid matrixes, allowing the recovery of a wide range of different polarity compounds selectively, from polyphenols [79–83] to fatty acids [84,85]. In addition, due to its dynamic extraction mode and multistep sequences, operations like filtration and evaporation between each step are reduced compared to UAE and MAE. One of this method limitation in some cases is the static mode, complete extraction might not be achieved because of the limited volume of solvent used, this can be remediated with the use of several extraction cycles; resulting in final extracts that often require following concentration and clean-up prior to analysis [59].



(127)

dans les unes comme dans les autres ; et nous savons que, dans les glacières artificielles, il n'y a d'autre cause de froid que la glace qu'on y accumule. Plus la glacière approche des glacières naturelles par sa grandeur, et plus on y accumule de glace, plus celle-ci se conserve long-temps.

Exposé *de quelques résultats obtenus par l'action combinée de la chaleur et de la compression sur certains liquides, tels que l'eau, l'alcool, l'éther sulfurique et l'essence de pétrole rectifiée.*

Par M. le Baron Cagniard de La Tour.

On sait qu'au moyen de la marmite à Papin on peut élever la température des liquides beaucoup au-dessus du terme ordinaire de leur ébullition ; et l'on est porté à croire que la compression intérieure qui s'augmente avec la température devrait être un obstacle à l'évaporation totale du liquide, surtout si l'espace laissé au-dessus de ce liquide n'est pas d'une certaine étendue.

En y réfléchissant, il m'est venu à la pensée que la dilatation d'un liquide volatil avait nécessairement une limite au-delà de laquelle ce liquide devait, malgré la compression, passer à l'état de vapeur, pour peu que la capacité de l'appareil permît à la matière liquide de s'étendre au-delà de son *maximum* de dilatation.

Pour vérifier ce fait j'ai introduit dans une petite marmite à Papin, construite avec un bout de canon de fusil très-épais, une certaine quantité d'alcool à 36 de-

"... cet état particulier exige toujours une temperature trés-élevée, presque indépendante de la capacité du tube."

"This particular condition requires a very high temperature, almost independent of the tube capacity."

-Cagniard de la Tour

4. Supercritical fluid extraction (SFE) application to plant material

4.1. A historical glimpse

Charles Cagniard de la Tour initially described in 1822 the notion of critical point under the term "*état particulier*". Up until this point, pure elements were only known to exist in three states (gaseous, solid, or liquid) closely connected to their thermodynamic parameters (pressure, temperature, and volume). Nearly fifty years later, the use of supercritical extraction (SFE) was introduced. However, it was after the second half of the 20th century that the use of SFE was considered as an alternative to established extraction techniques at that time [86].

4.2. Supercritical carbon dioxide (SC-CO_2)

All pure gases and liquids have a defined critical point of temperature and pressure. Beyond this point, one homogeneous state is attained, and the properties of gas and liquid converge creating a supercritical fluid, which can be created from a large variety of compounds (**Figure 11**).

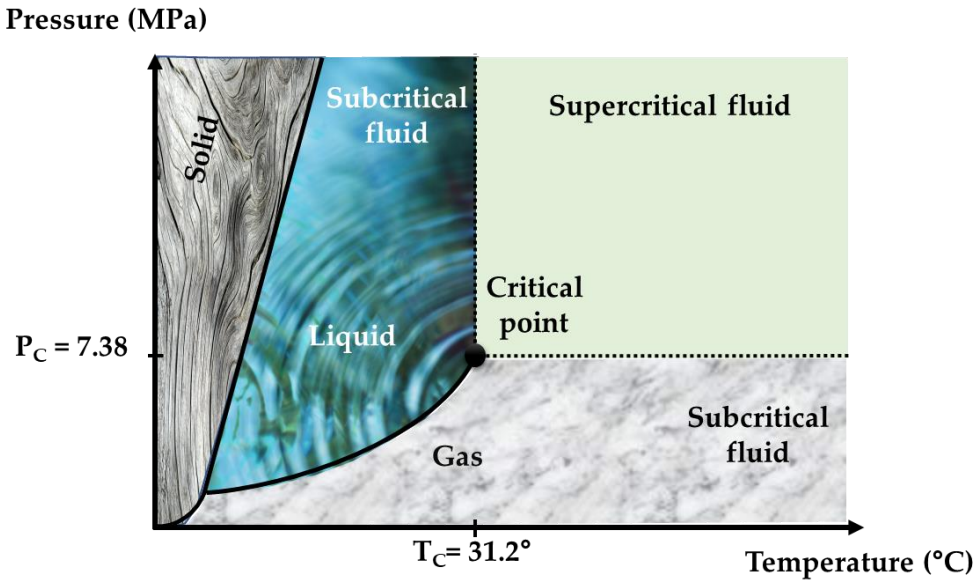


Figure 31: illustration of pure carbon dioxide pressure temperature phase diagram.

Figure 32: Schematic of a SFE instrument for solid-liquid extraction. Option (1) after BPR direct collection of the extracts. Option (2) fractionation using separators in a cascade of different pressures and temperatures.**Figure 33:** illustration of pure carbon dioxide pressure temperature phase diagram.

Nevertheless, because of its many benefits, SC-CO_2 is the most employed gas for SFE and SFC purposes. Many of these advantages for extraction are its low polarity, allowing the extraction of non-polar compounds that before were considered only accessible with hazardous organic solvent, showing to be a solvent that rivals or outperforms the strength of alkanes and ketones [87].

Due to the two opposing dipoles on the C=O bonds, CO_2 is a non-protic solvent with a low dielectric constant, no dipole moment, and a significant quadrupolar moment [87,88].

In view of its chemical structure, CO_2 critical point is easily accessible (Tc= 31°C and Pc=7.3 MPa) and makes it a great candidate for the extraction of natural compounds, decreasing the risk of degradation of the thermolabile compounds due to high temperatures. In addition, thanks to its widespread availability as a byproduct of numerous sectors and the possibility of acquiring it in sufficient purity, SC-CO_2 is particularly cost-effective, specifically when used on an industrial scale [88]. SC-CO_2 use in SFE is very advantageous since it produces concentrated extracts, reducing the need for prolonged evaporation due to its spontaneous return to its original gaseous

state at atmospheric pressure during the collection of the extracts, without leaving any residues. Following the popular saying by Paracelsus: "*the dose makes the poison*", SC-CO_2 is considered non-toxic and non-flammable, making its use very advantageous in an environmentally aware setting and for industries concerned with their carbon footprint.

4.3. Instrumentation

SFE involves the use SC-CO_2 for the extraction or isolation of compounds from a liquid or a solid matrix. This process involves two steps, first the extraction of the compounds of interest from the matrix and second the depressurization of CO_2 at atmospheric pressure, which leads to its expansion and separation from the extract. Basic SFE instrumentation is composed of several key elements for solid/liquid extraction (**Figure 12**).

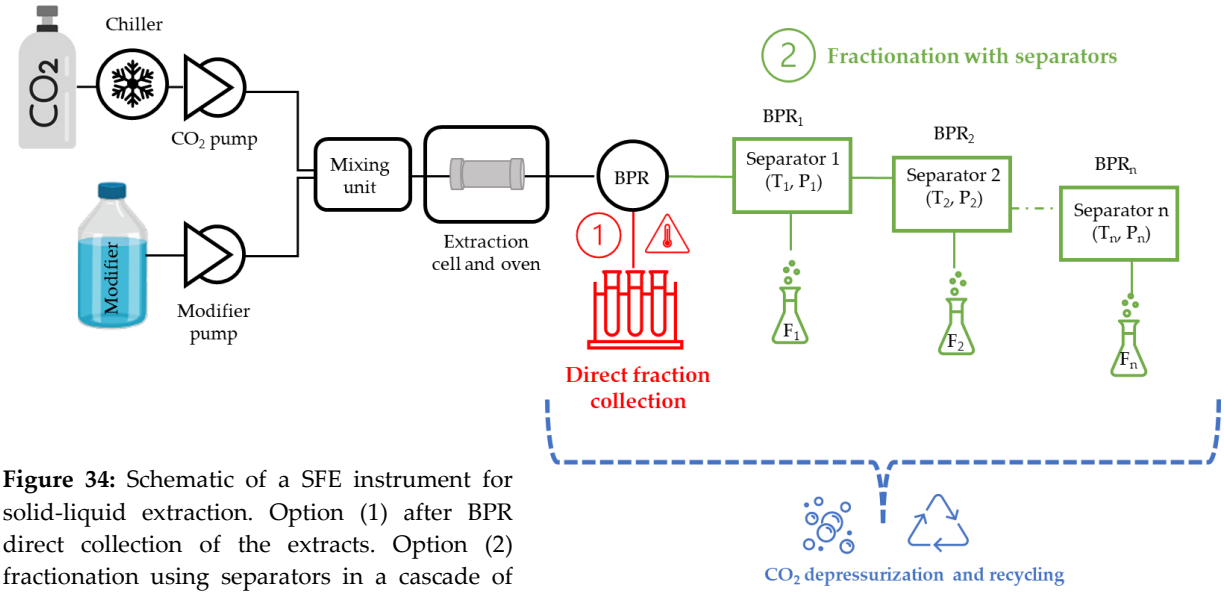


Figure 34: Schematic of a SFE instrument for solid-liquid extraction. Option (1) after BPR direct collection of the extracts. Option (2) fractionation using separators in a cascade of different pressures and temperatures.

Figure 35: Graphical representation of factors influencing SFE.**Figure 36:** Schematic of a SFE instrument for solid-liquid extraction. Option (1) after BPR direct collection of the extracts. Option (2) fractionation using separators in a cascade of different pressures and temperatures.

Solvents including the CO_2 tank and an organic solvent that can be used as modifier. A chiller which is normally maintained at a temperature of 4 to 6° C liquify CO_2 when pressurized increasing its density and pumping accuracy. The solvents are then pumped using a pumping module for both CO_2 and modifier allowing the mixing of both and delivery into the extraction oven where vessel and the solid matrix are placed, heating the solvent to reach a supercritical or subcritical state. Depending on the extraction mode the solvent can flow through the vessel in dynamic mode or accumulate in static mode. A back pressure regulator (BPR) is placed after the oven and offers a restriction of the pumped solvent in the system until it reaches the desired pressure, and when reached maintaining it at a constant value. A heat exchanger should be placed after the BPR in the absence of separators to avoid any problems of uncontrolled CO_2 solidification due to the endothermic reaction of SC-CO_2 depressurization. Followed by a collecting unit to recuperate the extracts, in some cases separators (or fractionation vessels) are employed with controlled heat and pressure, this permits a gradual fractionation respecting the compounds volatility and polarity.

In the case of a liquid-liquid SFE, instead on an extraction vessel an extraction column is used in countercurrent mode (CC-SFE) [24,89], where both the liquid matrix and the pressurized extraction solvent are pumped in opposing directions, the first introduced from the top and the latter from the bottom, in that case the compounds are isolated according to the separation factor between the compounds and the solvent [90].

4.4. Parameters influencing the SFE of plant natural compounds.

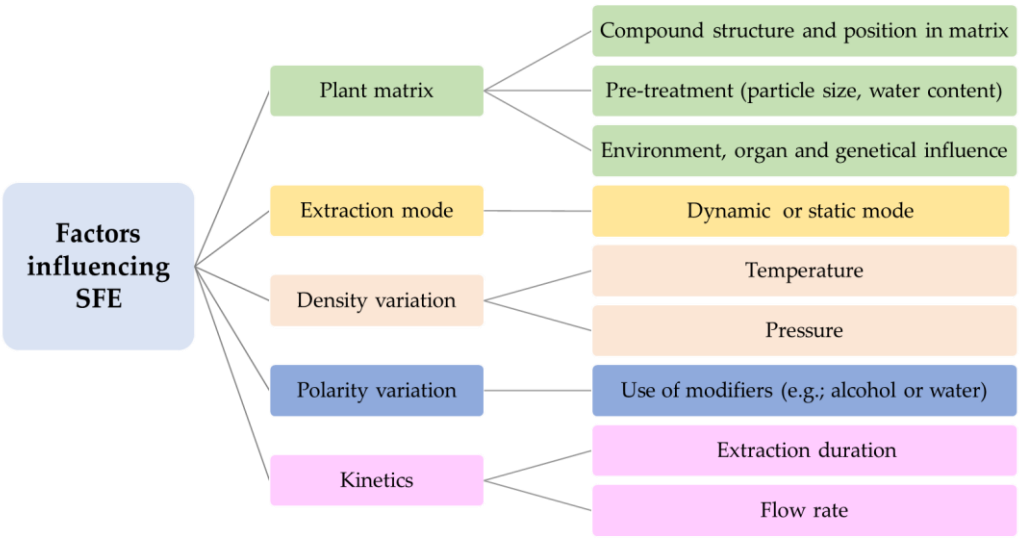


Figure 37: Graphical representation of factors influencing SFE.

4.4.1. Extraction temperature and pressure

One of the most interesting uses of CO_2 for SFE extraction is its low critical temperature given that the application of low temperatures for the extraction of bioactive compounds plays an important role for preserving the activity of the molecules of interest. Two phenomena in the SFE can be imposed by an increase in temperature at constant pressure, the first is associated with changes in CO_2 density. The density of SC-CO_2 is inversely related to temperature. An increase in temperature causes a decrease in SC-CO_2 density and as a result its solvent power. At the same time, substances transfer to the extraction solvent is facilitated due to the vapor pressure increase in the case of volatile compounds [91]. In addition, the mass transfer velocity can be impacted by temperature changes, particularly when the total viscosity of the extraction solvent is reduced. In some situations, this can result in rapid solute transfers and higher yields (temperature increase).

Furthermore, regarding the pressure it is also closely related to the fluid's density that affects the solutes solubility. An increase of the SC-CO_2 pressure is positively associated to the density of the fluid and in some cases can increase the extraction yield particularly when working with pure CO_2. However, the influence of pressure is related to bioactive molecules structure, for instance in the case of volatile compounds from essential oils, the use of higher pressure is preferred [92]. On the other hand, for the extraction of non-volatile and polar compounds like flavonoids, the use of high

pressure was beneficial to a certain value (20 MPa), and beyond this range an increase of the yield was observed [91,93].

Finally, it is important to know that temperature and pressure are modulated by a phenomenon known as retrograde behavior impacting compounds solubility. Depending on the pressure range, the temperature increase may have different effects. For instance, around the critical pressure value, the effect of fluid density is dominant; as a result, a slight temperature increase can have a significant impact on the fluid's density and reduce the extraction yield. However, when higher pressures are applied the influence of temperature increase is more closely related to the vapor pressure (volatile compounds), improving the solubility of the target substances [94–96].

4.4.2. Extraction kinetics and flow rate

Fundamental knowledge surrounding kinetic data like mass transfer coefficients is needed to optimize SFE. Extraction kinetics is represented by cumulative yield curve (peak area, mass, or concentration) versus the extraction time, the curve obtained during the extraction kinetics may depend on several parameters. Kinetics are highly dependent on the flow rate used and the plant matrix itself, therefore the use of constant flow rate for the same plant mass and the same instrument makes the comparison possible. The three fundamental phases of an extraction kinetics, commonly known as the overall extraction curve (OEC), are regulated by various mass transfer mechanisms (**Figure 14**) [91]:

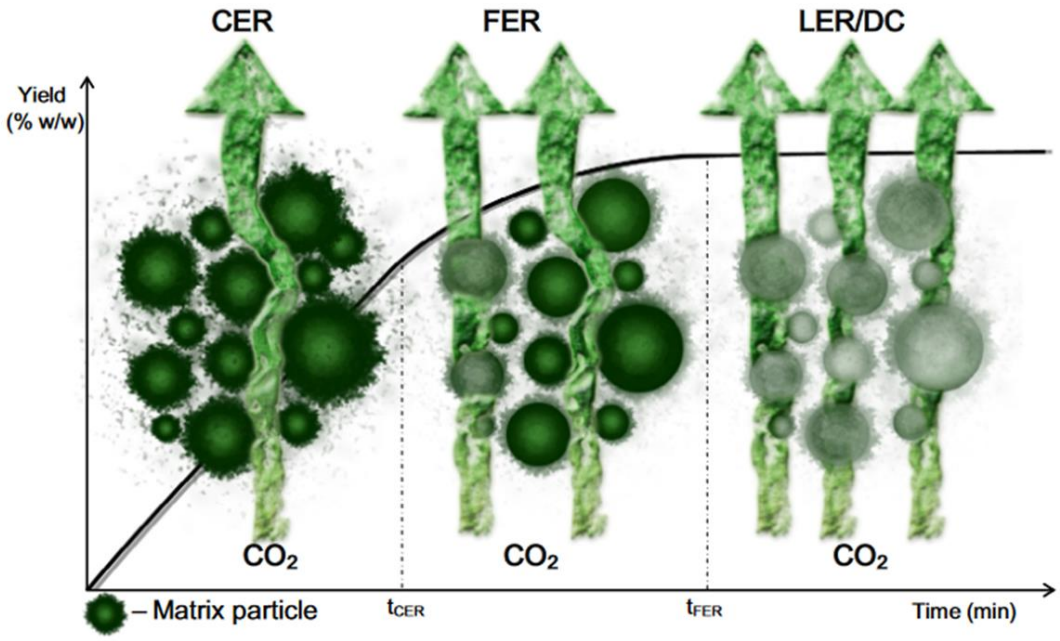


Figure 40: Graphical representation of the three periods of extraction kinetics and their correlation to extraction curve [91].

Figure 41: Summarized description of some of the unit operations required for solvent plant extraction, from the plant to the final product.**Figure 42:** Graphical representation of the three periods of extraction kinetics and their correlation to extraction curve [91].

1. **Constant extraction rate phase (CER):** During this phase, convection mechanisms dominate mass transfer, and the exterior surface of the plant matrix is covered in easily obtainable solute.
2. **Falling extraction rate phase (FER):** Convection and diffusion mechanisms are working together at this stage, and the external surface area of the plants begins to collapse, particularly the cell walls and surface oil layer.
3. **Low extraction rate phase (LER):** the external surface of the plant has been extensively weakened at this stage, and the intracellular diffusion mechanism of the extraction fluid is prominent.

The flow rate must be determined based on two factors. The first is that it must permit a quick extraction with a high yield, and the second it must allow enough contact time between the solutes and the fluid. Therefore, to reduce the overall energy and resources cost, while simultaneously allowing a high extraction yield and a quick extraction, the flow rate must be carefully selected [97].

4.4.3. Modifiers or co-solvents

There have been reports of the use of other supercritical fluids for extraction, such as propane based SFE [98]. However, SC-CO_2 use is the most common in the literature. The low polarity of CO_2 is considered as a limitation when employed in SFE. In fact, the early application of SC-CO_2 was restricted to the extraction of hydrophobic compounds (except for decaffeination) because of its low dielectric constant. However, its miscibility with a variety of organic solvents and in small amounts, water can remedy this issue. When a polar organic solvent is added to a supercritical fluid, its polarity may be greatly increased, allowing access to more polar molecules or facilitate higher molecular weight non-polar compounds extraction [97,99]. In some situations, this can result in significantly higher yields compared to other conventional approaches. Two mechanisms are thought to be at play when modifiers are added; the first is that the interaction solutes-modifier is enhanced therefore increasing the yield of more polar compounds [100–103]. The second is that the addition of organic liquid may induce the plant matrix swelling, thus facilitating the interaction of the extractables with the extraction solvent [24,97].

Many modifier choices are possible like methanol, isopropanol, and hexane. However, contrary to CO_2 that returns to its gas state after extraction, the solvents stay liquid after fraction collection, concentrating the final extract in the modifier. In a green extraction approach, toxic solvents residues are not acceptable, and ethanol is broadly used as modifier [104–108]. Water is frequently used as a modifier, but the amount must be carefully determined because of some reported drawbacks, including the possibility for compound hydrolysis and ionization, the co-extraction of saponins that may cause foam formation, and finally in the case of using separators, the risk of forming ice clogs due to the Joule-Thomson effect. Water is seldom used alone, and normally it's combined with alcohol forming a CO_2/H_2O/alcohol ternary system. Around the pressure range of 4-16 MPa and temperature of 25-100°C the existence of two phases have been reported in the literature, one CO_2-rich and the second H_2O-rich [24,97,109].

Modifier percentages traditionally ranged from 5-15%, but to increase the solubility of hydrophilic compounds, higher percentages are applied. It's important to note that modified CO_2 has a different critical point than pure CO_2, and therefore the extraction phase at higher modifier amounts is frequently regarded as being in a sub-critical state. Additionally, greater modifier percentages (15-30%) could reduce the impact of operative factors like pressure and temperature on the final density. The CO_2-modifier

is denser than pure CO_2 due to the addition of liquids, which reduces the impact of temperature and pressure on the density of CO_2. In this situation, the effect of temperature is purely dependent on mass transfer mechanism [110].

4.4.4. The plant material

Appropriate plant material conditions are elemental to achieve high recovery of bioactive compounds. Many factors can influence the accessibility of solutes during extraction, for example the location of the compounds in the plant matrix, in some cases the compounds can be found on the cell surface and therefore are more accessible by solvent, but generally they are in the intracellular space. Therefore, to facilitate their extraction pre-treatment steps like milling are required to increase the surface area of the plants with the solvent. After grinding the particle size must be carefully chosen, very small particles can be compacted causing channeling inside the extraction bed. The use of dried plant material when possible is more common. Fresh materials with higher moisture levels can be used, in some cases this can decrease the yield as the water present in the fresh plants competes with the solutes-extraction solvent interaction. And in other cases, the presence of water is recommended since it can facilitate the extraction of compounds by swelling the vegetable cells facilitating the solutes-solvent interaction [97].

A web of interconnected facets concerning the plant can additionally influence the extraction outcome like the age of the plants, the organs, the cultivation and harvesting practices, the genetics, climatic conditions, nutrient content in the soil and storage conditions. When evaluating plants for solid-liquid extraction and not just SFE, all these factors must be considered.

4.5. Selective and sequential extraction applications

The potential for selective and sequential extraction is one of the most appealing applications of SFE. Selectivity is an important factor, it could allow the enrichment of the final extracts of fraction with targeted compounds that may have various biological effects, it is made possible thanks to the fine-tuning of the SFE's operational conditions.

The low polarity of CO_2 facilitates the selective recovery of hydrophobic substances, especially lipids such as oils, fatty acids, and triglycerides [111–120]. Furthermore, the addition of polar modifier like ethanol or ethanol water mixtures make hydrophilic

substances like polyphenols accessible [121–127]. Thanks to SC-CO_2 density tunning by the variation of pressure and temperature, lipophilic substances can be recovered according to their molecular weight. Essential oils and other non-polar compounds with lower molecular weight can be extracted using high temperatures and low pressures of pure CO_2, and heavier non-polar compounds like lipids can be recovered using low temperatures and high pressures [128,129]. Avoiding the extraction of undesirable compounds is another advantage of selectivity; unwanted compounds can make the final extract unusable in certain industries. For example, chlorophylls are instable and preferably avoided in the cosmetic sector due to their instability provoking color changes in final emulsions, with a selective SFE process the bioactive molecules found in rosemary (carnosic and rosmarinic acids) were extracted separately from chlorophylls [108].

Regarding sequential SFE applications, it is possible to recover volatile, lipophilic, and hydrophilic chemicals in a single extraction sequence using same plant material through multiple steps dynamic extraction process [108,130]. Indeed, due to the use of just one extraction approach, operations like filtration and evaporation between each step are avoided, which has numerous advantages in terms of reduction of compounds loss risk, time, and therefore overall costs especially on an industrial level.

5. Integrated processes and reduction of unit operations

The extraction of plants requires the implementation of various unit operations from the plant to the final product (**Figure 15**). In solvent extraction the pre-treatment of plants is essential to ensure high extraction yields. Drying ensures the quality of the plant and in some cases avoid humidity related risks, the grinding increases contact surface area, other pretreatment to increase the availability of the compounds in the media can be employed, for example the use of ultrasound waves or enzymes.

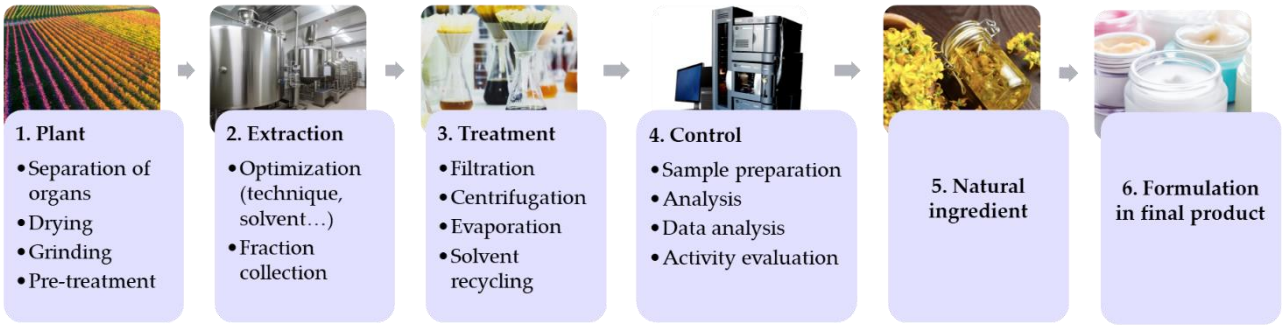


Figure 43: Summarized description of some of the unit operations required for solvent plant extraction, from the plant to the final product.

After pre-treatment steps comes the extraction procedure where the solvent is in contact with the plant followed by extract collection and separation of the plant mass from the solvent where the compounds were released. In some cases, additional steps might be required like centrifugation, solvent evaporation, and recycling, using extensive amounts of energy. After quality control checks, the extracts are now considered as a natural ingredient ready to be included in final product formulations. On an industrial scale the use of integrated process results in the reduction of unit operations and energy consumption and therefore manufacturing cost of the final products, making it more attractive for large scale industrial production.

The use of safe alternative solvents that are non-toxic or even edible for extraction is essential to eliminate the evaporation of the final extract. Extraction of lipophilic compounds like carotenoids using plant-based oils e.g., sunflower, soy, olive, sweet almond, or colza have been reported in the literature for with UAE, PLE and MAE [131–136]. As for polar compounds, the use of cosmetic solvents like glycerol and propanediol [137,138]. The same goes for NaDESs extracts as their use in formulations can be beneficial for the bioavailability of bioactive compounds in the human body compared to classical solvents [139] with the bypass of the evaporation step, direct incorporation in formulation is possible.

However, in the case of NaDESs an extensive screening of composition and influence of the solvent on the final formulation should be conducted prior to incorporation in the final formula. Since the use of pre-formulated extract can influence the final product stability or properties, and research and development process can be lengthy due to endless possibilities of NaDESs composition. As for the use of vegetable oils, in some cases this might influence the control step as it is more difficult to detect

compounds with complex matrix like oils, making the control of extract yield laborious or less reliable. In addition, oils do not evaporate therefore the final extracts cannot be concentrated to increase the final dose, the use of higher initial plant mass can help with the increase of the final concentration, this is possible when working with food waste where the plant matrix is inexpensive. However, when using rare plants other options are preferred.

Complete elimination of the solvent is a possibility. In the case of solvent free MAE, plant material is placed inside a MW reactor without the addition of any solvent or water. Plant cells containing bioactive components are compromised by heating the internal water in the plant material. The plant's internal water as well as any other substances that may be present will be released and moved from the inside to the outside of the plant, and a final pressure applied on the plant mass will allow the recovery of the extract that require minimal evaporation [30].

In the case of pure CO_2-SFE, after the extraction process CO_2 is removed and recycled, and the isolated extract is clear of all solvent remnants. The perception that supercritical fluid extraction is a more expensive technique than conventional methods has always been detrimental. However, if the entire process flow is considered rather than just the operation, we can see that this technology only necessitates a small number of individual procedures, reducing the energy required to recycle the solvent [30].

The manufacturing cost, energy consumption and carbon footprint should be based on the full extraction procedure reaching the final product rather than just focusing on the extraction itself. When each unit operation is considered, some inexpensive extraction techniques can turn very expensive and vice versa especially in sustainable economic model. Therefore, integrated processes that allow to attain high selectivity and extract purity with minimal steps are necessary.

6. References

[1] B. S. Paulsen, in *Bioact. Compd. Plants-Benefits Risks Man Anim.* **2010**.

[2] B. Aksel, in *Bioact. Compd. Plants – Benefits Risks Man Anim.* **2010**.

[3] H. A. R. Suleria, C. Barrow, *Bioactive Compounds from Plant Origin: Extraction, Applications, and Potential Health Benefits*, CRC Press **2019**.

[4] R. Tiwari, C. Rana, *IJERGS*. **2015**, *3*, 661–670.

[5] E. S. Teoh, in *Med. Orchids Asia* (Ed: E. S. Teoh), Springer International Publishing, Cham **2016**.

[6] R. Randhir, Y.-T. Lin, K. Shetty, *Process Biochem.* **2004**, *39* (5), 637–646. DOI: 10.1016/S0032-9592(03)00197-3.

[7] D. Lin, M. Xiao, J. Zhao, Z. Li, B. Xing, X. Li, M. Kong, L. Li, Q. Zhang, Y. Liu, et *al.*, *Molecules*. **2016**, *21* (10), 1374. DOI: 10.3390/molecules21101374.

[8] N. Babbar, H. S. Oberoi, S. K. Sandhu, V. K. Bhargav, *J. Food Sci. Technol.* **2014**, *51* (10), 2568–2575. DOI: 10.1007/s13197-012-0754-4.

[9] G. M. Cragg, D. J. Newman, *Pure Appl. Chem.* **2005**, *77* (1), 7–24. DOI: 10.1351/pac200577010007.

[10] D. A. Dias, S. Urban, U. Roessner, *Metabolites*. **2012**, *2* (2), 303–336. DOI: 10.3390/metabo2020303.

[11] F. Chemat, M. A. Vian, G. Cravotto, *Int. J. Mol. Sci.* **2012**, *13* (7), 8615–8627. DOI: 10.3390/ijms13078615.

[12] D. J. McClements, *Trends Food Sci. Technol.* **1995**, *6* (9), 293–299. DOI: 10.1016/S0924-2244(00)89139-6.

[13] S. Abbas, K. Hayat, E. Karangwa, M. Bashari, X. Zhang, *Food Eng. Rev.* **2013**, *5* (3), 139–157. DOI: 10.1007/s12393-013-9066-3.

[14] K. S. Suslick, *Sci. Am.* **1989**, *260* (2), 80–87.

[15] B. K. Tiwari, T. J. Mason, in *Nov. Therm. Non-Therm. Technol. Fluid Foods* (Eds: P. J. Cullen, B. K. Tiwari, V. P. Valdramidis), Academic Press, San Diego **2012**.

[16] F. Chemat, N. Rombaut, A.-G. Sicaire, A. Meullemiestre, A.-S. Fabiano-Tixier, M. Abert-Vian, *Ultrason. Sonochem.* **2017**, *34*, 540–560. DOI: 10.1016/j.ultsonch.2016.06.035.

[17] J. R. BlakePerutz, K. S. Suslick, Y. Didenko, M. M. Fang, T. Hyeon, K. J. Kolbeck, W. B. McNamara, M. M. Mdleleni, M. Wong, *Philos. Trans. R. Soc. Lond. Ser. Math. Phys. Eng. Sci.* **1999**, *357* (1751), 335–353. DOI: 10.1098/rsta.1999.0330.

[18] E. B. Flint, K. S. Suslick, *Science*. **1991**, *253* (5026), 1397–1399. DOI: 10.1126/science.253.5026.1397.

[19] W. B. McNamara, Y. T. Didenko, K. S. Suslick, *Nature*. **1999**, *401* (6755), 772–775. DOI: 10.1038/44536.

[20] K. S. Suslick, N. C. Eddingsaas, D. J. Flannigan, S. D. Hopkins, H. Xu, *Ultrason. Sonochem.* **2011**, *18* (4), 842–846. DOI: 10.1016/j.ultsonch.2010.12.012.

[21] D. Pingret Kipman, A.-S. Fabiano-Tixier, F. Chemat, *RSC Green Chem.* **2013**, 89–112. DOI: 10.1039/9781849737579-00089.

[22] J.-L. Capelo-Martinez, *Ultrasound in Chemistry: Analytical Applications*, ISBN: 978-3-527-62350-1 ed. **2009**.

[23] M. Romdhane, C. Gourdon, G. Casamatta, *Ultrasonics*. **1995**, *33* (3), 221–227. DOI: 10.1016/0041-624X(94)00023-I.

[24] J. Prado, M. Rostagno, *Natural Product Extraction: Principles and Applications*, Royal Society Of Chemistry **2022**.

[25] T. J. Mason, L. Paniwnyk, J. P. Lorimer, *Ultrason. Sonochem.* **1996**, *3* (3), S253–S260. DOI: 10.1016/S1350-4177(96)00034-X.

[26] A. Carreira-Casais, P. Otero, P. Garcia-Perez, P. Garcia-Oliveira, A. G. Pereira, M. Carpena, A. Soria-Lopez, J. Simal-Gandara, M. A. Prieto, *Int. J. Environ. Res. Public. Health*. **2021**, *18* (17), 9153. DOI: 10.3390/ijerph18179153.

[27] L.-L. Zheng, G. Wen, M.-Y. Yuan, F. Gao, *J. Chem.* **2016**, *2016*, e8768130. DOI: 10.1155/2016/8768130.

[28] M.-E. Lucchesi, Extraction Sans Solvant Assistée par Micro-ondes
Conception et Application à l'extraction des huiles essentielles, phdthesis, Université De La Réunion **2005**.

[29] E. Destandau, T. Michel, **2022**. DOI: 10.1039/9781839165894-00144.

[30] F. Chemat, A. S. Fabiano-Tixier, M. A. Vian, T. Allaf, E. Vorobiev, *TrAC Trends Anal. Chem.* **2015**, *71*, 157–168. DOI: 10.1016/j.trac.2015.02.021.

[31] M. Stramarkou, T.-V. Missirli, K. Kyriakopoulou, S. Papadaki, A. Angelis-Dimakis, M. Krokida, *Resources*. **2023**, *12* (7), 77. DOI: 10.3390/resources12070077.

[32] W. Routray, V. Orsat, *Food Bioprocess Technol.* **2012**, *5* (2), 409–424. DOI: 10.1007/s11947-011-0573-z.

[33] Z. Lou, H. Wang, S. Zhu, M. Zhang, Y. Gao, C. Ma, Z. Wang, *J. Chromatogr. A*. **2010**, *1217* (16), 2441–2446. DOI: 10.1016/j.chroma.2009.12.022.

[34] S. N. Tan, J. W. H. Yong, C. C. Teo, L. Ge, Y. W. Chan, C. S. Hew, *Talanta*. **2011**, *83* (3), 891–898. DOI: 10.1016/j.talanta.2010.10.048.

[35] C. G. Grigoras, E. Destandau, L. Fougère, C. Elfakir, *Ind. Crops Prod.* **2013**, *49*, 794–804. DOI: 10.1016/j.indcrop.2013.06.026.

[36] M. Chambaud, C. Colas, E. Destandau, *Separations*. **2023**, *10* (8), 433. DOI: 10.3390/separations10080433.

[37] A. Yustira, S. Maulina, Iriany, *AIP Conf. Proc.* **2023**, *2741* (1), 020004. DOI: 10.1063/5.0133540.

[38] S. N. M. Khazaai, P. Bhuyar, V. Strezov, N. Govindan, M. H. Ab. Rahim, G. P. Maniam, *BioEnergy Res.* **2023**. DOI: 10.1007/s12155-023-10635-1.

[39] A. P. Abbott, G. Capper, D. L. Davies, R. K. Rasheed, V. Tambyrajah, *Chem. Commun.* **2003**, *0* (1), 70–71. DOI: 10.1039/B210714G.

[40] M. Jablonský, A. Škulcová, A. Malvis, J. Šima, *J. Biotechnol.* **2018**, *282*, 46–66. DOI: 10.1016/j.jbiotec.2018.06.349.

[41] Z. Y. Lai, C. L. Yiin, S. s. M. Lock, B. Chin, N. Zauzi, S. Sar-ee, *Environ. Sci. Pollut. Res.* **2023**, 1–28. DOI: 10.1007/s11356-023-26288-8.

[42] S. P. Jeevan Kumar, G. Vijay Kumar, A. Dash, P. Scholz, R. Banerjee, *Algal Res.* **2017**, *21*, 138–147. DOI: 10.1016/j.algal.2016.11.014.

[43] D. Skarpalezos, A. Detsi, *Appl. Sci.* **2019**, *9* (19), 4169. DOI: 10.3390/app9194169.

[44] D. Li, *Front. Plant Sci.* **2022**, *13*.

[45] Y. Dai, K. H. Row, *Molecules*. **2019**, *24* (12), 2300. DOI: 10.3390/molecules24122300.

[46] Y. Dai, E. Rozema, R. Verpoorte, Y. H. Choi, *J. Chromatogr. A*. **2016**, *1434*, 50–56. DOI: 10.1016/j.chroma.2016.01.037.

[47] Z. Wei, X. Qi, T. Li, M. Luo, W. Wang, Y. Zu, Y. Fu, *Sep. Purif. Technol.* **2015**, *149*, 237–244. DOI: 10.1016/j.seppur.2015.05.015.

[48] Y. S. Wong, R. Yusoff, G. C. Ngoh, *Rev. Chem. Eng.* **2023**. DOI: 10.1515/revce-2022-0043.

[49] W. Wang, M. An, G. Zhao, Y. Wang, D. Yang, D. Zhang, L. Zhao, J. Han, G. Wu, Y. Bo, *Microchem. J.* **2023**, *193*, 108952. DOI: 10.1016/j.microc.2023.108952.

[50] C. Gómez-Urios, A. Viñas-Ospino, P. Puchades-Colera, J. Blesa, D. López-Malo, A. Frígola, M. J. Esteve, *LWT*. **2023**, *177*, 114595. DOI: 10.1016/j.lwt.2023.114595.

[51] C. G. González, N. R. Mustafa, E. G. Wilson, R. Verpoorte, Y. H. Choi, *Flavour Fragr. J.* **2018**, *33* (1), 91–96. DOI: 10.1002/ffj.3425.

[52] A. Viñas-Ospino, M. Panić, I. Radojčić- Redovniković, J. Blesa, M. J. Esteve, *Food Biosci.* **2023**, *53*, 102570. DOI: 10.1016/j.fbio.2023.102570.

[53] S. Y. Lee, Y. N. Liang, D. C. Stuckey, X. Hu, *Sep. Purif. Technol.* **2023**, *317*, 123677. DOI: 10.1016/j.seppur.2023.123677.

[54] Z. Yu, M. Xia, X. Li, R. Wang, W. Liu, R. Zheng, Z. Wang, L. Yang, Y. Shi, *Front. Chem.* **2022**, *10*.

[55] A. Viñas-Ospino, M. Panić, M. Bagović, K. Radošević, M. J. Esteve, I. Radojčić Redovniković, *Sustain. Chem. Pharm.* **2023**, *31*, 100942. DOI: 10.1016/j.scp.2022.100942.

[56] S. Eid, A. S. Darwish, T. Lemaoui, F. Banat, S. W. Hasan, I. M. AlNashef, *J. Mol. Liq.* **2023**, *382*, 121996. DOI: 10.1016/j.molliq.2023.121996.

[57] C. Cannavacciuolo, S. Pagliari, J. Frigerio, C. M. Giustra, M. Labra, L. Campone, *Foods*. **2023**, *12* (1), 56. DOI: 10.3390/foods12010056.

[58] V. Camel, *Analyst*. **2001**, *126* (7), 1182–1193. DOI: 10.1039/B008243K.

[59] A. Mustafa, C. Turner, *Anal. Chim. Acta*. **2011**, *703* (1), 8–18. DOI: 10.1016/j.aca.2011.07.018.

[60] S. S. H. Rizvi, *Separation, Extraction and Concentration Processes in the Food, Beverage and Nutraceutical Industries*, Elsevier **2010**.

[61] P. Kritzer, E. Dinjus, *Chem. Eng. J.* **2001**, *83* (3), 207–214. DOI: 10.1016/S1385-8947(00)00255-2.

[62] D. Luong, M. Sephton, J. Watson, *Anal. Chim. Acta*. **2015**, *879*. DOI: 10.1016/j.aca.2015.04.027.

[63] R. H. Perry, D. W. Green, *Perry's Chemical Engineers' Handbook*, McGraw-Hill **1984**.

[64] C. A. Meyer, American Society of Mechanical Engineers, ASME Research Committee on Properties of Steam, Eds. , *ASME Steam Tables: Thermodynamic and Transport Properties of Steam*, 6th ed ed., American Society Of Mechanical Engineers, New York **1993**.

[65] Y. Cheng, F. Xue, S. Yu, S. Du, Y. Yang, *Molecules*. **2021**, *26* (13), 4004. DOI: 10.3390/molecules26134004.

[66] B. S. Khoza, I. A. Dubery, H.-A. Byth-Illing, P. A. Steenkamp, L. Chimuka, N. E. Madala, *Food Anal. Methods*. **2016**, *9* (6), 1480–1489. DOI: 10.1007/s12161-015-0302-8.

[67] P. Mašković, V. Veličković, M. Mitić, S. Đurović, Z. Zeković, M. Radojković, A. Cvetanović, J. Švarc-Gajić, J. Vujić, *Ind. Crops Prod.* **2017**, *109*, 875–881. DOI: 10.1016/j.indcrop.2017.09.063.

[68] R. Song, M. Ismail, S. Baroutian, M. Farid, *Food Bioprocess Technol.* **2018**, *11* (10), 1895–1903. DOI: 10.1007/s11947-018-2151-0.

[69] H. Zhang, S. Liu, H. Li, F. Xue, S. Han, L. Wang, Y. Cheng, X. Wang, *RSC Adv.* **2018**, *8* (40), 22652–22658. DOI: 10.1039/C8RA02653J.

[70] H.-J. Hwang, H.-J. Kim, M.-J. Ko, M.-S. Chung, *Food Sci. Biotechnol.* **2021**, *30* (2), 217–226. DOI: 10.1007/s10068-020-00862-z.

[71] M. T. Munir, H. Kheirkhah, S. Baroutian, S. Y. Quek, B. R. Young, *J. Clean. Prod.* **2018**, *183*, 487–494. DOI: 10.1016/j.jclepro.2018.02.166.

[72] K. A. Lee, K.-T. Kim, H. J. Kim, M.-S. Chung, P.-S. Chang, H. Park, H.-D. Pai, *Food Sci. Biotechnol.* **2014**, *23* (2), 615–621. DOI: 10.1007/s10068-014-0084-6.

[73] N. T. Hiep, H. T. Duong, D. T. Anh, N. Hoai Nguyen, D. Q. Thai, D. T. T. Linh, V. T. H. Anh, N. M. Khoi, *Processes*. **2020**, *8* (9), 1028. DOI: 10.3390/pr8091028.

[74] M.-J. Ko, C.-I. Cheigh, M.-S. Chung, *Food Chem.* **2014**, *143*, 147–155. DOI: 10.1016/j.foodchem.2013.07.104.

[75] A. Cvetanović, J. Švarc-Gajić, U. Gašić, Ž. Tešić, G. Zengin, Z. Zeković, S. Đurović, *J. Supercrit. Fluids*. **2017**, *120*, 32–42. DOI: 10.1016/j.supflu.2016.10.012.

[76] K. A. Lee, W. J. Kim, H. J. Kim, K.-T. Kim, H.-D. Paik, *Int. J. Food Sci. Technol.* **2013**, *48* (5), 947–953. DOI: 10.1111/ijfs.12046.

[77] B. Martín-García, S. Pimentel-Moral, A. M. Gómez-Caravaca, D. Arráez-Román, A. Segura-Carretero, *Ind. Crops Prod.* **2020**, *154*, 112741. DOI: 10.1016/j.indcrop.2020.112741.

[78] N. Verma, Himshweta, in *Green Sustain. Process Chem. Environ. Eng. Sci.* (Eds: Dr. Inamuddin, T. Altalhi), Elsevier **2023**.

[79] N. L. Huamán-Castilla, N. Gajardo-Parra, J. R. Pérez-Correa, R. I. Canales, M. Martínez-Cifuentes, G. Contreras-Contreras, M. S. Mariotti-Celis, *Antioxidants*. **2023**, *12* (7), 1446. DOI: 10.3390/antiox12071446.

[80] A. Bebek Markovinović, S. Milošević, N. Teslić, B. Pavlić, P. Putnik, I. Brčić Karačonji, K. Jurica, D. Lasić, D. Bursać Kovačević, *Plants*. **2023**, *12* (10), 2006. DOI: 10.3390/plants12102006.

[81] M. Krstić, N. Teslić, P. Bošković, D. Obradović, Z. Zeković, A. Milić, B. Pavlić, *Molecules*. **2023**, *28* (1), 369. DOI: 10.3390/molecules28010369.

[82] D. T. Raspe, C. da Silva, S. C. da Costa, *J. Supercrit. Fluids*. **2023**, *193*, 105813. DOI: 10.1016/j.supflu.2022.105813.

[83] L. Bagatini, G. P. Zandoná, J. F. Hoffmann, J. de Souza Cardoso, F. C. Teixeira, L. S. Moroni, A. Junges, A. P. Kempka, F. M. Stefanello, C. V. Rombaldi, *Sustain. Chem. Pharm.* **2023**, *35*, 101214. DOI: 10.1016/j.scp.2023.101214.

[84] M.-T. Golmakani, J. A. Mendiola, K. Rezaei, E. Ibáñez, *J. Supercrit. Fluids*. **2014**, *92*, 1–7. DOI: 10.1016/j.supflu.2014.05.001.

[85] C. S. M. Pereira, V. M. T. M. Silva, A. E. Rodrigues, *Green Chem.* **2011**, *13* (10), 2658–2671. DOI: 10.1039/C1GC15523G.

[86] B. Berche, M. Henkel, R. Kenna, *Rev. Bras. Ensino Física*. **2009**, *31* (2), 2602.1-2602.4. DOI: 10.1590/S1806-11172009000200015.

[87] E. J. Beckman, *J. Supercrit. Fluids*. **2004**, *28* (2), 121–191. DOI: 10.1016/S0896-8446(03)00029-9.

[88] E. Lesellier, C. West, *J. Chromatogr. A*. **2015**, *1382*, 2–46. DOI: 10.1016/j.chroma.2014.12.083.

[89] Q. Gros, J. Duval, C. West, E. Lesellier, *TrAC Trends Anal. Chem.* **2021**, *144*, 116433. DOI: 10.1016/j.trac.2021.116433.

[90] F. Chemat, *Eco-Extraction Du Végétal: Procédés Innovants et Solvants Alternatifs* **2011**.

[91] R. P. F. F. da Silva, T. A. P. Rocha-Santos, A. C. Duarte, *TrAC Trends Anal. Chem.* **2016**, *76*, 40–51. DOI: 10.1016/j.trac.2015.11.013.

[92] P. B. Gomes, V. G. Mata, A. E. Rodrigues, *J. Supercrit. Fluids*. **2007**, *41* (1), 50–60. DOI: 10.1016/j.supflu.2006.08.018.

[93] M. Bimakr, R. A. Rahman, F. S. Taip, A. Ganjloo, L. M. Salleh, J. Selamat, A. Hamid, I. S. M. Zaidul, *Food Bioprod. Process.* **2011**, *89* (1), 67–72. DOI: 10.1016/j.fbp.2010.03.002.

[94] X. Yang, D. Rowland, C. C. Sampson, P. E. Falloon, E. F. May, *Fuel*. **2022**, *314*, 123033. DOI: 10.1016/j.fuel.2021.123033.

[95] A. Tarafder, K. Kaczmarski, D. P. Poe, G. Guiochon, *J. Chromatogr. A*. **2012**, *1258*, 136–151. DOI: 10.1016/j.chroma.2012.08.023.

[96] M. D. Saldaña, R. S. Mohamed, M. G. Baer, P. Mazzafera, *J. Agric. Food Chem.* **1999**, *47* (9), 3804–3808. DOI: 10.1021/jf981369z.

[97] C. G. Pereira, M. A. A. Meireles, *Food Bioprocess Technol.* **2010**, *3* (3), 340–372. DOI: 10.1007/s11947-009-0263-2.

[98] D. Sparks, R. Hernandez, M. Zappi, D. Blackwell, T. Fleming, *J. Am. Oil Chem. Soc.* **2006**, *83* (10), 885. DOI: 10.1007/s11746-006-5042-x.

[99] M. de Andrade Lima, I. Kestekoglou, D. Charalampopoulos, A. Chatzifragkou, *Molecules*. **2019**, *24* (3), 466. DOI: 10.3390/molecules24030466.

[100] A. Bamberger, G. Sieder, G. Maurer, *J. Supercrit. Fluids*. **2000**, *17* (2), 97–110. DOI: 10.1016/S0896-8446(99)00054-6.

[101] S. T. Lee, T. S. Reighard, S. V. Olesik, *Fluid Phase Equilibria*. **1996**, *122* (1), 223–241. DOI: 10.1016/0378-3812(96)03038-5.

[102] J.-H. Yoon, H. Lee, B. H. Chung, *Fluid Phase Equilibria*. **1994**, *102* (2), 287–292. DOI: 10.1016/0378-3812(94)87081-0.

[103] G.-S. Shyu, N. S. M. Hanif, K. R. Hall, P. T. Eubank, *Fluid Phase Equilibria*. **1997**, *130* (1), 73–85. DOI: 10.1016/S0378-3812(96)03196-2.

[104] N. Hassim, M. Markom, M. I. Rosli, S. Harun, *Sci. Rep.* **2021**, *11* (1), 15818. DOI: 10.1038/s41598-021-95222-0.

[105] K. M. Sharif, M. M. Rahman, J. Azmir, S. H. Shamsudin, M. S. Uddin, T. K. Fahim, I. S. M. Zaidul, *J. Ind. Eng. Chem.* **2015**, *21*, 1314–1322. DOI: 10.1016/j.jiec.2014.05.047.

[106] A. P. Jarvis, E. D. Morgan, *Phytochem. Anal.* **1997**, *8* (5), 217–222. DOI: 10.1002/(SICI)1099-1565(199709/10)8:5<217::AID-PCA366>3.0.CO;2-J.

[107] Y. Kong, Y.-J. Fu, Y.-G. Zu, W. Liu, W. Wang, X. Hua, M. Yang, *Food Chem.* **2009**, *117* (1), 152–159. DOI: 10.1016/j.foodchem.2009.03.091.

[108] T. Lefebvre, E. Destandau, E. Lesellier, *J. Chromatogr. A*. **2021**, *1639*, 461709. DOI: 10.1016/j.chroma.2020.461709.

[109] M. Leticia, **2003**.

[110] I. Brondz, B. Sedunov, N. Sivaraman, *Int. J. Anal. Mass Spectrom. Chromatogr.* **2017**, *5* (2), 17–39. DOI: 10.4236/ijamsc.2017.52002.

[111] M. J. H. Akanda, M. Z. I. Sarker, S. Ferdosh, M. Y. A. Manap, N. N. N. Ab Rahman, M. O. Ab Kadir, *Molecules*. **2012**, *17* (2), 1764–1794. DOI: 10.3390/molecules17021764.

[112] N. Rubio-Rodríguez, S. M. de Diego, S. Beltrán, I. Jaime, M. T. Sanz, J. Rovira, *J. Food Eng.* **2012**, *109* (2), 238–248. DOI: 10.1016/j.jfoodeng.2011.10.011.

[113] A. Rajaei, M. Barzegar, Y. Yamini, *Eur. Food Res. Technol.* **2005**, *220* (3), 401–405. DOI: 10.1007/s00217-004-1061-8.

[114] N. Rombaut, R. Savoire, B. Thomasset, T. Bélliard, J. Castello, É. Van Hecke, J.-L. Lanoisellé, *Comptes Rendus Chim.* **2014**, *17* (3), 284–292. DOI: 10.1016/j.crci.2013.11.014.

[115] M. E. Elsorady, *Croat. J. Food Sci. Technol.* **2023**, *15* (1), 1–7. DOI: 10.17508/CJFST.2023.15.1.01.

[116] Z. Albakry, E. Karrar, I. A. Mohamed Ahmed, A. A. Ali, Q. A. Al-Maqtari, H. Zhang, G. Wu, X. Wang, *J. Food Meas. Charact.* **2023**, *17* (3), 2429–2441. DOI: 10.1007/s11694-022-01802-7.

[117] E. P. Kalogianni, D. Georgiou, A. Charisis, S. Exarhopoulos, P. Tzika, *J. Am. Oil Chem. Soc.* **2023**, *100* (4), 317–327. DOI: 10.1002/aocs.12681.

[118] L. Manna, C. A. Bugnone, M. Banchero, *J. Supercrit. Fluids*. **2015**, *104*, 204–211. DOI: 10.1016/j.supflu.2015.06.012.

[119] C.-H. Cheng, T.-B. Du, H.-C. Pi, S.-M. Jang, Y.-H. Lin, H.-T. Lee, *Bioresour. Technol.* **2011**, *102* (21), 10151–10153. DOI: 10.1016/j.biortech.2011.08.064.

[120] D. Villanueva-Bermejo, M. V. Calvo, P. Castro-Gómez, T. Fornari, F. J. Fontecha, **2019**. DOI: 10.13039/100012818.

[121] A. M. Farías-Campomanes, M. A. Rostagno, J. J. Coaquira-Quispe, M. A. A. Meireles, *Bioresour. Bioprocess.* **2015**, *2* (1), 45. DOI: 10.1186/s40643-015-0073-5.

[122] A. Aresta, P. Cotugno, N. De Vietro, F. Massari, C. Zambonin, *Anal. Lett.* **2020**, *53* (16), 2585–2595. DOI: 10.1080/00032719.2020.1749846.

[123] C. Da Porto, A. Natolino, *J. Supercrit. Fluids*. **2017**, *130*, 239–245. DOI: 10.1016/j.supflu.2017.02.013.

[124] Y. Escobedo-Flores, D. Chavez-Flores, I. Salmeron, C. Molina-Guerrero, S. Perez-Vega, *J. Cereal Sci.* **2018**, *80*, 198–204. DOI: 10.1016/j.jcs.2018.03.002.

[125] I. J. Stavrou, A. Christou, C. P. Kapnissi-Christodoulou, *Food Chem.* **2018**, *269*, 355–374. DOI: 10.1016/j.foodchem.2018.06.152.

[126] L. T. M. Phan, K. T. P. Nguyen, H. T. Vuong, D. D. Tran, T. X. P. Nguyen, M. N. Hoang, T. P. Mai, H. H. Nguyen, *J. Chem.* **2020**, *2020*, e9548401. DOI: 10.1155/2020/9548401.

[127] N. De Zordi, A. Cortesi, I. Kikic, M. Moneghini, D. Solinas, G. Innocenti, A. Portolan, G. Baratto, S. Dall'Acqua, *J. Supercrit. Fluids*. **2014**, *95*, 491–498. DOI: 10.1016/j.supflu.2014.10.006.

[128] B. Pavlić, O. Bera, N. Teslić, S. Vidović, G. Parpinello, Z. Zeković, *Ind. Crops Prod.* **2018**, *120*, 305–312. DOI: 10.1016/j.indcrop.2018.04.044.

[129] H. Mhemdi, E. Rodier, N. Kechaou, J. Fages, *J. Food Eng.* **2011**, *105* (4), 609–616. DOI: 10.1016/j.jfoodeng.2011.03.030.

[130] Q. Gros, M. Wolniaczyk, J. Duval, S. Horie, Y. Funada, Y. Hayakawa, C. West, E. Lesellier, *J. Chromatogr. A*. **2023**, *1705*, 464168. DOI: 10.1016/j.chroma.2023.464168.

[131] M. Civan, S. Kumcuoglu, *LWT*. **2019**, *113*, 108320. DOI: 10.1016/j.lwt.2019.108320.

[132] A. M. Goula, M. Ververi, A. Adamopoulou, K. Kaderides, *Ultrason. Sonochem.* **2017**, *34*, 821–830. DOI: 10.1016/j.ultsonch.2016.07.022.

[133] H. Chutia, C. L. Mahanta, *Innov. Food Sci. Emerg. Technol.* **2021**, *67*, 102547. DOI: 10.1016/j.ifset.2020.102547.

[134] A. Elik, D. K. Yanık, F. Göğüş, *LWT*. **2020**, *123*, 109100. DOI: 10.1016/j.lwt.2020.109100.

[135] Y. Li, A. S. Fabiano-Tixier, V. Tomao, G. Cravotto, F. Chemat, *Ultrason. Sonochem.* **2013**, *20* (1), 12–18. DOI: 10.1016/j.ultsonch.2012.07.005.

[136] E. Kultys, M. A. Kurek, *Molecules*. **2022**, *27* (2), 518. DOI: 10.3390/molecules27020518.

[137] B. P. Soares, A. M. Ferreira, M. Justi, L. G. G. Rodrigues, J. V. Oliveira, S. P. Pinho, J. A. P. Coutinho, *Molecules*. **2023**, *28* (4), 1607. DOI: 10.3390/molecules28041607.

[138] M. Jabłonowska, P. Ciganović, J. Jablan, E. Marguí, M. Tomczyk, M. Zovko Končić, *Ind. Crops Prod.* **2021**, *168*, 113613. DOI: 10.1016/j.indcrop.2021.113613.

[139] S. Sut, M. Faggian, V. Baldan, G. Poloniato, I. Castagliuolo, I. Grabnar, B. Perissutti, P. Brun, F. Maggi, D. Voinovich, et *al.*, *Molecules*. **2017**, *22* (11), 1921. DOI: 10.3390/molecules22111921.

Chapter II: SFE applications on natural polar compounds from plants

Part I: *Robinia pseudoacacia* L.

In the first chapter the numerous parameters affecting extraction results in SFE have been reported, between the temperature and pressure influencing the density of the SC-CO_2. The modifier percentage and composition influencing the polarity of the extraction phase and accordingly its selectivity towards compounds, related to the interactions developed. And finally, the plant itself, every matrix is different and needs various methodologies that differ depending on the aim of the study. This makes SFE optimization very intricate, creating the need for a standardized approach.

In this regard, during this PhD, plant extraction applied to various biomasses needed to be optimized, and chemometric tools were employed to streamline this procedure. The use of statistical approaches to identify the best extraction conditions for each of the related parameters can be beneficial. It generates enough experimental data to later identify the ideal values for optimal extraction conditions depending on the chemical structure of the targeted bioactive molecules. Therefore, the aim was to standardize the experimental model, followed its validation with different plant matrixes and targeting various types of polar compounds.

The chapter is divided into three parts. Each part will present a plant, known for its richness in hydrophilic compounds.

Black locust will be presented in the first part. Dihydrorobinetin (DHR) and robinetin (Rob) are the two major flavonoids found in the tree's heartwood. The two compounds offer different applications in the cosmetic industry , DHR is known for its antioxidant and antibacterial activities. As for Rob it is naturally fluorescent and can be used as natural pigment or colorant in final products, therefore an efficient extraction of these compounds can be interesting. The employment of a Box-Behnken design (BBD) can offer a simplified approach for SFE optimization. Due to the polarity of the molecules a modifier with a composition of EtOH:H_2O 80:20 *v:v* had to be used to increase the polarity of the extraction phase and thus accessing the molecules. The influence of temperature and pressure can be assessed also to determine the significance on the yield increase.

Part I: Optimization of Supercritical fluid extraction of polar flavonoids from *Robinia pseudoacacia* L. heartwood

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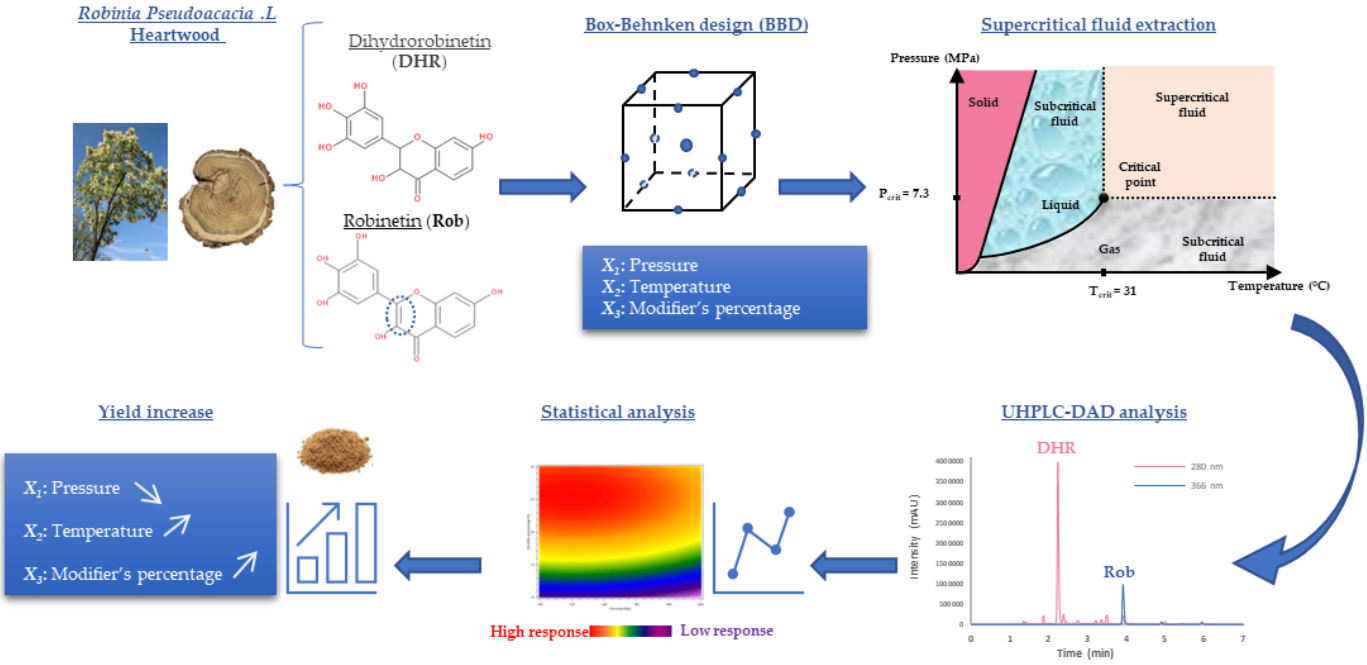
Keywords:

Eco-extraction, biomass valorization, dihydrorobinetin, robinetin, response surface methodology, black locust, modified supercritical carbon dioxide.

Graphical abstract



Abstract

The black locust is known as an invasive species. Yet it has many advantages, namely its rapid growth rate and its richness with interesting flavonoids. In this study, supercritical fluid extraction of the two main flavonoids dihydrorobinetin and robinetin, was optimized using a three level Box-Behnken response surface design. The conditions investigated to influence the extraction yield of these flavonoids were temperature (40°C-80°C), pressure (10 MPa-20 MPa) and modifier's percentage EtOH:H_2O 80:20 *v:v* (10%-30%). The response was quantified using an UHPLC-DAD optimized chromatographic method. The results showed the influence of the extraction conditions on the yield of the molecules of interest. The data were fitted into a second-order polynomial equation, the multiple regression analysis showed a high determination coefficient value (R^2) of 0.93. The optimal extraction conditions were 10 MPa, 80°C and extraction phase composition of CO_2:EtOH:H_2O 80:16:4 *v:v:v* for 30 min. These conditions allowed for the recovery of 49.2 mg of flavonoids for 1 g of dried wood powder.

1. Introduction

Black locust (*Robinia pseudoacacia* L.), also called false Acacia, was one of the first trees that crossed the Atlantic Ocean to travel from North America, its native land, to Europe at the beginning of the 17th century [1]. This species is known for its high adaptability and the capability to grow, even in areas that are different from its native range in terms of climatic conditions. The European range of this species is larger than its native range [2].

Currently, in Europe *Robinia pseudoacacia* L. is considered as the most widespread economically and environmentally significant foreign species [3]. This had led for it to be included on the European list of invasive species [4]. However, it presents many advantages; it is planted in Europe, Asia, and the United States to control erosions and for its soil-improving properties. Not to mention the flowers which are valued by beekeepers to produce honey [5].

Besides, this speed of growth associated with its durability has made the species interesting on numerous levels. Since the wood is naturally rot-resistant, it has a wide range of uses, for energy purposes, for building construction, outdoor furniture and fences which require no treatments [6].

Different organs from the tree have shown an interesting flavonoids content [7,8]. This richness in flavonoids, give the plant many biological properties, it has been proven that the reproductive organs of the tree had antimicrobial and antioxidant activity [9], the flower extract that is rich in phenolics showed an inhibitory property for tyrosinase, α-amylase and α-glucosidase [10]. Tyrosinase inhibitors are interesting for cosmetic products and dermatological treatment, mainly for their use on skin pigmentation [11].

Furthermore, the heartwood presents an interesting source of natural molecules, with a major phenolic composition, reaching a total of 41 flavonoid and non-flavonoids [12]. This abundance in phenolic compounds is responsible for the durability of the wood [13]. Two major phenolic compounds extracted from the heartwood of *Robinia pseudoacacia* L, are flavonoids Dihydrorobinetin (3,3′,4′,5′,7-pentahydroxyflavanone, DHR) and Robinetin (3,3′,4′,5′,7-pentahydroxyflavone, Rob). [12, 14–17]

Since both molecules are natural phytochemicals with great potential to be developed in the pharmaceutical and cosmetic industries, for instance due to their anti-fungi activities [16], they were considered as the targeted compounds in this study. Other studies have addressed the optimization of the extraction of these molecules with more traditional techniques, for example maceration with agitation, ultrasonic and Soxhlet [17,18]. These techniques have some disadvantages, mainly extraction time, the large consumption of chemical solvents particularly on an industrial level that will later require their evaporation or waste treatment, and the use of relatively high temperatures that might be counterproductive, especially for thermally labile liable compounds that consequently can induce their degradation. [12,16,19]. Moreover, some papers reported the use of DMSO [16], not suitable for a cosmetic use of extracts, or sequential liquid/solid extractions with filtration and evaporation steps [12], which dramatically improve the sample treatment to produce purified extracts. Besides, comparative studies achieved with Soxhlet, maceration and ultrasonic extraction, using different solvents, acetone/water 9/1 [17] or ethanol/water [20] led to different conclusions about the well -suited extraction method for the total phenolic content. For that reason, new and modern extraction methods like supercritical fluid extraction (SFE), sonication, microwave extraction and pressurized fluid extraction have been introduced, to overcome the limitations of conventional methods.

The use of supercritical fluids for the extraction of natural compounds from plants is an environment-friendly alternative to traditional extraction techniques. The SC-CO_2 has the diffusion, viscosity and surface tension of gas, and the density and solvation force of liquids, making it an intermediate state between gas and liquid. Most

commonly supercritical carbon dioxide (SC-CO_2) is the most used solvent for SFE. This is mainly due to its non-toxicity, non-flammability, and cost-efficiency. Another advantage is the natural concentration of the extracts due to the decompression of CO_2. The spontaneous return to its gaseous state leads to the production of high-quality extract that seldom needs refining or post treatment [21] and the possibility to scale up the extraction method at industrial scale with well design apparatus [22–24].

Extraction optimization is the most important aspect to consider when doing SFE since there are multiple variables and combination possibilities (extraction temperature, time, nature and amount of modifier, pressure, sample amount and size, etc.), that can influence the density of the supercritical fluid and its polarity, which in turn affects the yield of the targeted compounds. To optimize the variables in an effective manner while reducing the experimental points, it is common to use experimental designs and statistical models. Therefore, commonly experimental design is employed as a first line strategy, when it comes to producing robust extraction methods [25].

In this study's framework, the focus is to explore and optimize the extraction of DHR and Rob using SFE, that is, in our knowledge, the first application attempt of SFE to these polar polyphenols. Moreover, due to the non-polar character of carbon dioxide, the extraction of polar natural compounds is challenging. To optimize the extraction of *Robinia pseudoacacia* L. heartwood, a Box-Behnken design (BBD) [26] was employed, it is a statistical experimental design for response surface methodology (RSM); it consists of k factors and at least three levels design that can be represented as an incomplete rotatable block design. It requires experiments numbers base of $N = 2k\,(k - 1) + C_0$, k represents the number of factors and C_0 is the central point numbers. The design points avoid the extreme vertices and are positioned at the middle of the subareas of the dimension k-1; this is intentional to maintain a higher order surface definition [27,28].

SC-CO_2 is commonly used for the extraction of compounds of low polarity; its use for more polar molecules has been of more interest recently and several studies addressed the extraction of polar compounds like polyphenols and anthocyanins [29,30]. The addition of a polar modifier to carbon dioxide, for instance ethanol, allows to improve the solubility of polar compounds through hydrogen bonding. The chromatographic profile of pure CO_2 extracts and modified/CO_2 ones can dramatically change due to this additional molecular interaction. Nevertheless, to our knowledge no publication of flavonoids SFE optimization of *Robinia pseudoacacia* L. using response surface methodology (RSM) and Box-Behnken design are available until now.

2. *Methodology*

2.1. Plant material

The plant materials (*Robinia pseudoacacia* L.) consisted of dried heartwood powder with a brown color, the particle size ranged between 0.4 and 1 mm. The biomass was stored at room temperature in an airtight container.

2.2. Reference extraction

A reference extract was produced following the optimal extraction conditions of [15], the solid-liquid extraction consists of using as solvent a mixture EtOH:H_2O 50:50 *v:v*, wood powder/solvent mass ratio $R_{w/s}$ of 13%, 4 h at room temperature 25°C. This extract was used for the development of the UHPLC-DAD analysis method and was used as reference extract to compare extracts produced using SFE.

2.3. Chemical and reagents

Ultra-pure water was purified by using an Elgastat UHQ II system, (Elga, Antony, France) (resistance > 18 MW). The HPLC-grade methanol and acetonitrile used as the mobile phase and sample diluent were supplied by VWR (Fontenay-sous-Bois, France). Formic acid (FA) added to the mobile phase was obtained from Sigma-Aldrich (Merck, France).

Standards used to identify the molecules Robinetin (Rob) and Dihydrorobinetin (DHR) were from Extrasynthese (Genay, France).

In this study, an UHPLC-DAD analytical method was developed using the reference extract to quantify the extraction yield of both flavonoids of *Robinia pseudoacacia* L.

2.4. UHPLC-UV analysis

A UHPLC-DAD analytical method was developed using the reference extract to quantify the extraction yield of Rob and DHR extracted from *Robinia pseudoacacia* L. All the extract analyses were preformed using a Nexera-LC40 system of Shimadzu Corporation (Kyoto, Japan); this system is equipped with a photodiode array (PDA) detector (SPD-40), a Solvent Delivery Unit (LC-40), an Auto-sampler (SIL-40), a column oven (CTO-40) and a System Controller SCL-40. All chromatograms were

recorded on LabSolutions LC-UV 5.97 SP1 version (Shimadzu Corporation, Kyoto, Japan).

A Luna C18 (2) HST (3x100 mm) with a particle size of 2.5 μm from Phenomenex (Torrance, California, United States) was used to separate the two flavonoids. The column temperature was maintained at 30°C. The mobile phase consisted of a combination of acidified water with 0.1% of formic acid (solvent A) and Methanol/Acetonitrile 50/50 v/v mixture acidified with 0.1% of formic acid (solvent B) [15].

The total time of each analysis was 11 minutes, the elution gradient employed is the following, 0 - 6 min: 25% - 50% solvent B; 6.1 - 11 min: 25% solvent B. Equilibration time between two injections was 5 minutes. The analysis flow was maintained at 0.6 mL/min, and the injection volume was 2 μL.

Detection of the two compounds was done using a PDA detector set at specific wavelengths at 280 nm (maximal absorbance of DHR), 310 nm (absorbance of both DHR and Rob) and 366 nm (maximal absorbance Rob). Quantitative analyses of both DHR and Rob were performed by injecting a Robinetin standard from Biosynth Carbosynth (Compton, United Kingdom) at different concentrations from 0.001 mg/mL to 0.8 mg/mL. The calibration curve was obtained at 366 nm (*y=1E+07x- 268816*, $R^2 = 0.9958$), it was used to estimate the concentrations from the peak areas of Rob, DHR was quantified as an equivalent to Rob, this was possible due to their similar molar absorption coefficient (ε) at their respective maximal absorbance wavelength [15].

The injection solvent composition for all chromatographic analysis consisted of a mixture of water and methanol 70:30 respectively, to avoid any deformities in the peak shape of DHR.

In all the data presented, the extraction yield was defined as the extracted mass of the flavonoids DHR, and Rob determined by the UHPLC analysis and related to the plant material mass used for the extraction (mg/g).

2.5. SFE extraction

A Waters MV-10 ASFE was used for all the extractions; a 5-mL stainless steel extraction vessel was prepared using 1 g of plant material mixed with 1 g of dispersant (diatomaceous earth). Cotton was placed at the upper and bottom parts of the cell to

filter the extract and fill the cell entirely. A constant flow of 3 mL/ min was used, and the percentage of the modifier was calculated according to this total flow.

The modifier used for all the runs of the experimental design was a mixture of ethanol and water 80:20 respectively, since flavonoids are polar molecules and are not soluble in pure SC-CO_2. It has been reported in the literature that the addition of a polar organic modifier changes the polarity of the supercritical CO_2 and enhances the solvation of polar targeted molecules [31–33]. Indeed, due to the additional dipole-dipole and hydrogen-bondin interaction, this polar modifier addition helps to improve the efficiency of the extraction especially for phenolic compounds [34].

At the end of each experimental point, different volumes of the extracts were collected, this was due to the difference of the co-solvent percentage for each experimental point. To amend this matter, determine, and compare the extraction yield using peak areas in the calibration range; all extracts were evaporated using a nitrogen gas flow, then they were dissolved using 10 mL of methanol. Then they were filtered using a syringe filter with 0.45 μm pore size.

2.6. Ultra-sound assisted extraction (USE)

The USE was performed using a Branson 3510 (Bransonic®ultrasonic) bath from Sigma-Aldrich (Merck, France), using 1 g of black locust (*Robinia pseudoacacia* L.) dried wood powder, the extraction solvent was a mixture of EtOH:H_2O 80:20 *v:v* respectively (18 mL). The plant and solvent were mixed and sonicated for 30 minutes at room temperature (22°C), then the extract was then filtered of the remaining biomass using a paper filter (90 mm diameter) and dried using a nitrogen stream. After drying, the extract was solubilized in 10 mL of MeOH and diluted with water for UHPLC analysis.

2.7. Experimental design and Statistical analysis

The software Ellistat (Annecy, Auvergne-Rhône-Alpes) was used for experimental design, data analysis and model building. The Box–Behnken design (BBD) from response surface methodology (RSM) was used to optimize the supercritical fluid extraction of DHR and Rob from black locust.

The design was chosen to establish the model and to determine the response pattern. The three independent factors used in this study were pressure (X_1), temperature (X_2)

and modifier percentage (X_3), with three levels coded (+1) for the highest, (0) middle and (-1) the lowest level (**Table 1**).

Table 1: Design matrix in the Box-Behnken model. and the flavonoid yield as response.

Experiments	Factor Coded levels			Response
	X_1	X_2	X_3	
	Pressure (MPa)	Temperature (°C)	Modifier percentage (%)	Flavonoid yield (mg/g)
1	15 (0)	60 (0)	20 (0)	39.96
2	10 (-1)	40 (-1)	20 (0)	30.65
3	20 (+1)	40 (-1)	20 (0)	22.82
4	10 (-1)	80 (+1)	20 (0)	37.70
5	15 (0)	60 (0)	20 (0)	34.73
6	10 (-1)	80 (+1)	20 (0)	39.49
7	10 (-1)	60 (0)	10 (-1)	22.51
8	20 (+1)	60 (0)	10 (-1)	5.72
9	15 (0)	60 (0)	20 (0)	41.99
10	10 (-1)	60 (0)	30 (+1)	36.72
11	20 (+1)	60 (0)	30 (+1)	36.68
12	15 (0)	40 (-1)	10 (-1)	3.71
13	15 (0)	80 (+1)	10 (-1)	15.57
14	15 (0)	40 (-1)	30 (+1)	31.87
15	15 (0)	80 (+1)	30 (+1)	44.27

Other variables such as flow rate, extraction time and plant mass were kept constant. The dependent response or output was the sum of the yield of both DHR and Rob called flavonoid yield (mg/g) (Y). The experiments were randomized to maximize the effect of the variability in the response. Three replicates at the center point of the design were conducted to evaluate the experimental repeatability.

Due to the reduced experiment number, the ***equation (1)*** used to represent the model did not include the interactions terms between the three factors. This was done to avoid overfitting the data and in consequence misrepresenting the conclusions [35].

$$Y=\beta_0+ \beta_1 X_1+ \beta_2 X_2+ \beta_3 X_{2+} \beta_{11} X_1^2+ \beta_{22} X_2^2+ \beta_{33} X_3^2 \quad \textbf{(1)}$$

Where Y = predicted response; β_0 = the regression model constant; β_1, β_2 and β_3 = coefficients of each variable; β_{11}, β_{22} and β_{33} = quadratic coefficients and X_1, X_2 and X_3 represent the variables.

Ellistat was employed as well for the regression model and the graphical analysis of the data. The analysis of the variance using ANOVA was used to determine the significance of the independent variables on the response. This was mainly done using Fisher's test (F value), significant factors were defined by a p-value < 0.05. Other statistical values like the correlation coefficient (R^2) and adjusted correlation coefficient (adj R^2), were employed to assess the fitness of the design equation. Heat-map plots were used to visualize the interaction between the variables. And the optimal extraction condition was determined by solving the regression equation that represented the model [36].

The standard deviation (SD) was estimated to validate the reproducibility of the extraction model. Data were represented as mean values ± standard deviation.

2.8. Extraction time optimization

A kinetic experiment was carried out to determine the extraction time, as a function of the extraction yield of both DHR and Rob. It was conducted at the central point conditions ($X_1=0$, $X_2=0$ *and* $X_3=0$) of the experimental design, with all other parameters fixed. The fractions were collected every 15 minutes for one hour.

From this kinetic study, the duration of 15 minutes was chosen for the 15 points of the experimental design, to avoid the exhaustion of the molecules of interest from the plant material, therefore allowing sufficient variation of the yield with each experimental point and different extraction conditions, to determine the influence of the parameters on the response.

3. Results and discussion

The SFE of DHR and Rob was optimized using a BBD design of experiment. The pressure and temperature were chosen for their ability to modify the density of the fluid (mainly composed by carbon dioxide), as for the percentage of the modifier it was mainly due to its capacity to alter the polarity of the extraction solvent. These three factors can be representative and influence the extractive capacity of the supercritical solvent in regard to the two compounds of interest.

3.1. Statistical analysis, model, and factor significance

The model's determination coefficient (R^2) is equal to 0.93 and the value of adjusted R^2=0.88. This indicates that only 7% of the total variations was not explained by the model. The value of R^2 shows a good relation between the predicted and experimental values of the response.

The relative standard deviation (RSD %) of the center point replicate (3 points) extraction was equal to 9.63%. ***Equation (2)*** demonstrates the relationship of the three factors (X_1, X_2 *and* X_3) with the flavonoids yield (Y):

$$Y\ (mg/g) = -96.66 + 0.2233X_1 + 1.466X_2 + 5.736X_3 - 0.0009349X_1^2 - 0.009719X_2^2 - 0.1115X_3^2 \quad \textbf{(2)}$$

Table 2: the summary of the analysis of variance (ANOVA) of the regression model.

Table 2: ANOVA for response surface regression model for DHR and Rob extraction yield.

Source	DF	SS	MS	F value	Prob > F	Contribution (%)
Regression model	6	2153.5	358.91	18.7600	0.0003	
X_1 (Pressure)	1	65.345	12.623	0.6598	0.4401	0.827
X_2 (Temperature)	1	287.82	87.082	4.5517	0.0654	5.71
X_3 (Modifier %)	1	1301.1	737.9	38.5694	0.0003	48.4
X_1^2	1	5.955	20.169	1.0542	0.3346	1.32
X_2^2	1	34.1	55.807	2.9170	0.1260	3.66
X_3^2	1	459.12	459.12	23.9974	0.0012	30.1
Residuals	8	153.05	19.132			10
Total	14	2306.5				

The significance of each factor was measured with the probability > F-value or p-value.

The F- values for the regression model of the *Robinia pseudoacacia* L. two flavonoids yield was obtained as 18.76 which indicates a significant response.

The p-values indicate that the regression model is very significant with a 0.0003 value.

As for the factors, only X_3 (modifier percentage) and X_3^2 showed a p-value lower than 0.05 with a contribution of 48.4% and 30.1% respectively. This indicates the high significance for the extraction yield of both flavonoids. Nonetheless, X_2 (temperature) showed a p-value of 0.0654, which is not less than 0.05 but shows a minor significance of the model response.

In addition, X_1 (pressure) the p-value is 0.4401, and a contribution of less than 2% of the model results this indicates that these factors, is not significant on the yield of DHR and Rob.

The calculated optimal points in the response surface that allow the maximization of the output (Flavonoid's yield) are as follows: pressure of 12.07 MPa, 75.9°C temperature and a modifier percentage of 25.9%.

3.2. Optimization of the extraction parameters with Box-Behnken Design

The interaction and relationship between independent and dependent variables were illustrated. **Figure 1.a** described the effect of pressure and modifier percentage on the flavonoids yield with a constant extraction flow (3 mL/min), time (15 min) and plant mass (1 g). It revealed that the modifier percentage showed a positive influence on the extraction yield. As the modifier percentage increased from 10 to 30%, the flavonoids yield increased from 14.99 to 40.49 mg/g with the pressure and temperature fixed at 15 MPa and 60°C. This is due to the polarity of DHR (Log P= 0.9) and Rob (Log P= 1.6). And even though CO_2 is the most interesting solvent for SFE, its low polarity restricts its use for non-polar or low polarity compounds, like fatty acids or oil [37]. Polar solvents like ethanol, methanol and water are used to improve the affinity of SC-CO_2 for polar compounds like polyphenols and especially flavonoids, and accordingly increasing their extraction yield [38]. This explains the effect of the increase of the modifier percentage, which is composed of mixture of ethanol and water 80:20 v: v, a relatively polar solvent on the extraction yield.

However, the pressure had an opposite effect, it showed a negative linear influence, while the pressure increased from 10 to 20 MPa, the flavonoids yield slightly decreased from 39.41 to 33.7 mg/g with the modifier percentage and temperature fixed at 20% and 60°C. It is commonly known that the increase of pressure at specific temperatures results in an increase of the solvent's density which in turn enhance the solubility of the targeted compounds in the plant matrix [39]. However, when working with

modifier from 10 to 30%, the increase in pressure does not strongly change the fluid density, but reduces the effective diffusivity, and consequently the mass transfer rate from the matrix to the solvent [40]. Therefore, a lower pressure of 10 MPa would appear to be preferential to optimize the extraction of *Robinia pseudoacacia* L. flavonoids.

Figure 1.b indicated an increase of temperature from 40 to 80°C at a pressure and modifier percentage of 15 MPa and 20%, allowed the increase of DHR and Rob yield from 29 mg/g to 41 mg/g, while it is less than the impact of modifier percentage. Nonetheless, it shows a positive relation between the temperature and the flavonoid yield. This demonstrates that even though that the P-value of the temperature is not lower than 0.05, the increase of temperature is preferential to maximize the extraction of *Robinia pseudoacacia* L. flavonoids.

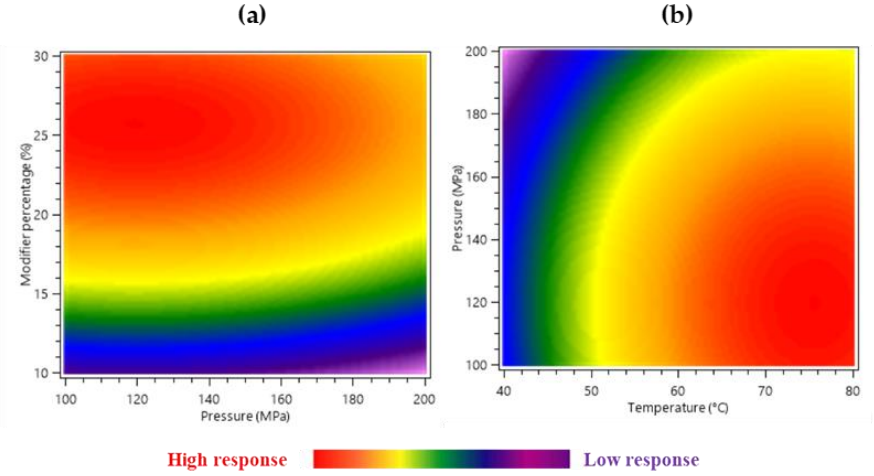


Figure 1: Response surface heatmap showing the interactive effect of pressure and co-solvent percentage on the flavonoid yield (a) and pressure and temperature (b).

The extraction temperature works on two different and opposing mechanisms during SFE. At a constant pressure, the higher the temperature the lower the solvent density, that in turns reduces the solvating power of the SC-CO_2. However, it might at the same time improve the vapor pressure of the volatile compounds, which can implicate the increase of analytes solubility and extraction yield [39,41,42]. In our case, because the studied compounds are not volatiles and because of the presence of modifier mixed to CO_2, the increase in the temperature would favor the effective diffusivity and the mass transfer [40]. This last effect seems to be predominant regarding the density change

due to temperature increase, specifically because of the low compressibility of the fluid when working with 10 to 30% of modifier. Therefore, a high temperature is favorable to the extraction of *Robinia pseudoacacia* L. flavonoids.

3.3. Extraction kinetics at optimum conditions

The regression model showed that the optimal extraction conditions of DHR and Rob are at a pressure of 12.07 MPa, 75.9°C temperature and 25.9% of modifier percentage, these extractions conditions were rounded to 10 MPa, 80°C and 30%, for further analysis.

Therefore, to determine the extraction time needed to exhaust both flavonoids content in *Robinia Pseudoacaccia* L. and to investigate the impact of temperature on the yield with a constant pressure, 10 MPa was applied for all the experiments that follow.

Four experimental points were investigated to examine the effect of the percentage of modifier and the temperature (40 and 80°C) on the speed rate of the extraction yield, with a constant backpressure of 10 MPa. This will allow the optimization of the extraction time after it was kept constant at 15 minutes for the experimental design, and therefore, obtaining the necessary time and conditions to exhaust the content of both flavonoids of *Robinia Pseudoacaccia* L.

The four points selected from the experimental design results as providing rather high yield of extraction **(Figure 2)**.

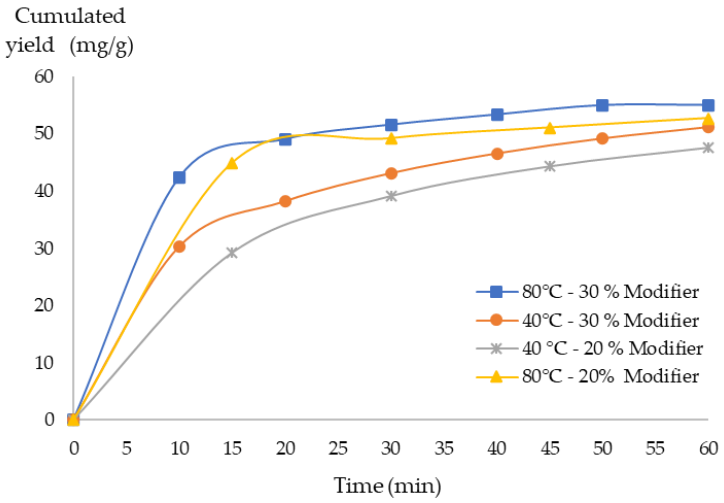


Figure 2: SFE Kinetics plot for the extraction of DHR and Rob (total flavonoid yield mg/g) from *Robinia Pseudoacaccia* L.

As indicated, a fraction was collected every fifteen minutes for the extractions performed with 20% of modifier, and every ten minutes for the extractions performed

with 30% of modifier for one hour. This strategy was employed since the extractions performed with 30% of modifier are faster than those done with 20% and had the same consumption in volume of the modifier. Therefore, 15 minutes and 10 minutes are adequate to have sufficient data points to follow the extraction kinetics.

The results of the extraction kinetics of both flavonoids were illustrated in **(Figure 2)**. Extractions made at 40°C whether it was with 20% or 30% of modifier, showed the slowest extraction kinetics of both flavonoids, and lower extraction yield with 39.20 mg/g with 20% for 30 minutes of extraction and 42.13 mg/g with 30% for the same time.

As for the extraction made at 80°C, whether it was with 20% or 30% of modifier, showed the fastest extraction kinetics of both flavonoids and higher extraction yields with 49.2 mg/g with 20% for 30 minutes of extraction and 51.6 mg/g with 30% for the same time. All the extractions kinetics showed a tendency to converge to the same point, with the increase of the extraction time above 60 min. This illustrates the exhaustion of the flavonoids of interest of the biomass.

For example, the extraction made at 30% of modifier, reached 51.17 mg/g in 60 minutes at 40°C. To reach almost the same yield (51.6 mg/g) with same percentage of modifier at 80°C only 30 minutes were necessary. This shows the impact of the temperature on the speed of the extraction, even though it showed a slight significance in the results of the design of experiments. With 15 minutes of extraction time, the kinetics showed that the combination of a temperature of 80°C effect positively not just on the yield of the extract in flavonoids but also on the velocity rate of extraction.

At 80°C with 30 minutes of extraction, the use of 30% of modifier gave a yield of 51.6 mg/g of both flavonoids, while the use of 20% of modifier gave 49.2 mg/g of flavonoids. This reveals that at high temperature the yield of flavonoids is comparable whatever the modifier percentage used. Therefore, the use of 20% is preferred since it allowed the extraction of equivalent quantity of flavonoids, additionally it permitted the usage of less quantities of modifier which is interesting on an ecological and economic level.

Consequently, the optimized conditions for SFE of DHR and Rob from *Robinia Pseudoacaccia* L. are 80°C, 20% modifier, pressure of 10 MPa for 30 minutes.

3.4. Supercritical fluid extraction vs ultrasound extraction

To compare the optimized SFE extraction with another modern extraction method, an ultrasound extraction (USE) was performed with quite similar conditions of the optimized SFE conditions, using 18 mL of EtOH:H_2O 80:20 *v:v* (an amount equivalent

for the SFE consumption of 20% of modifier for 30 minutes) during 30 min at room temperature. The flavonoid yield was compared to the yield of SFE optimized conditions.

The results illustrated with (**Figure 3***)* showed that the SFE extraction had a yield of 49.19 mg/g of both flavonoids with a 38.46 mg/g and 10.74 mg/g yield for DHR and Rob respectively. While the ultra-sound extraction led to a yield of 28.75 mg/g of both flavonoids with a 21.78 mg/g and 6.96 mg/g yield for DHR and Rob respectively.

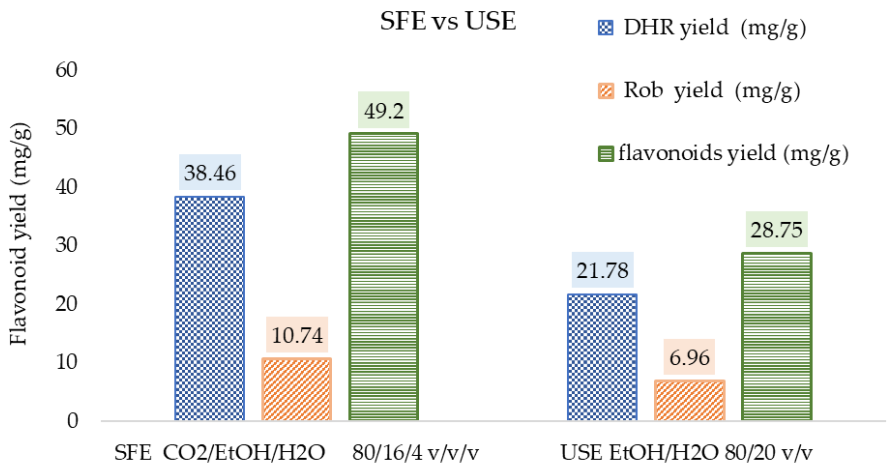


Figure 3: Comparison between SFE extraction and US extraction.

Therefore, for the same consumption of organic solvent, the optimized SFE conditions allowed the extraction of 1.7 times more of both flavonoids of *Robinia Pseudoacaccia* L. compared to standard ultrasound extraction.

3.5. Extraction selectivity

Thus far, the findings of the extraction yield were shown as a total flavonoid yield of both molecules of interest of *Robinia Pseudoacaccia* L, these extractions were made using a modifier composed of a mixture of EtOH:H_2O 80:20 *v:v*. This choice was based on previous research that showed that this composition is a compromise to extract both compounds, since DHR is more hydro-soluble, and Rob is more alcohol-soluble [14].

Therefore, in the perspective of exploring the extraction selectivity of DHR and Rob, and the possibility to extract selectively one molecule without the other with a sufficient yield, the H_2O percentage added in the modifier was investigated.

Several percentages of H_2O were used: 5, 10 and 30% (in mixture with EtOH, the total modifier content being equal to 20%) and they were compared to the previous results

with the use of 20% of EtOH:H_2O 80:20 *v:v*. Each run was operated with both 40°C and 80°C temperature.

All other extraction parameters were kept constant according to the optimization done with design of experiments and the extraction kinetics previously. The pressure was of 10 MPa, 30 minutes extraction, 20% modifier and 3 mL/min extraction flow.

The results were illustrated in **Figure 4** showed that with lower percentages of water 5 and 10%, a lower yield of flavonoids was observed, even with high temperature. This validated the choice of modifier (EtOH:H_2O 80:20 *v:v*) to optimize the extraction previously.

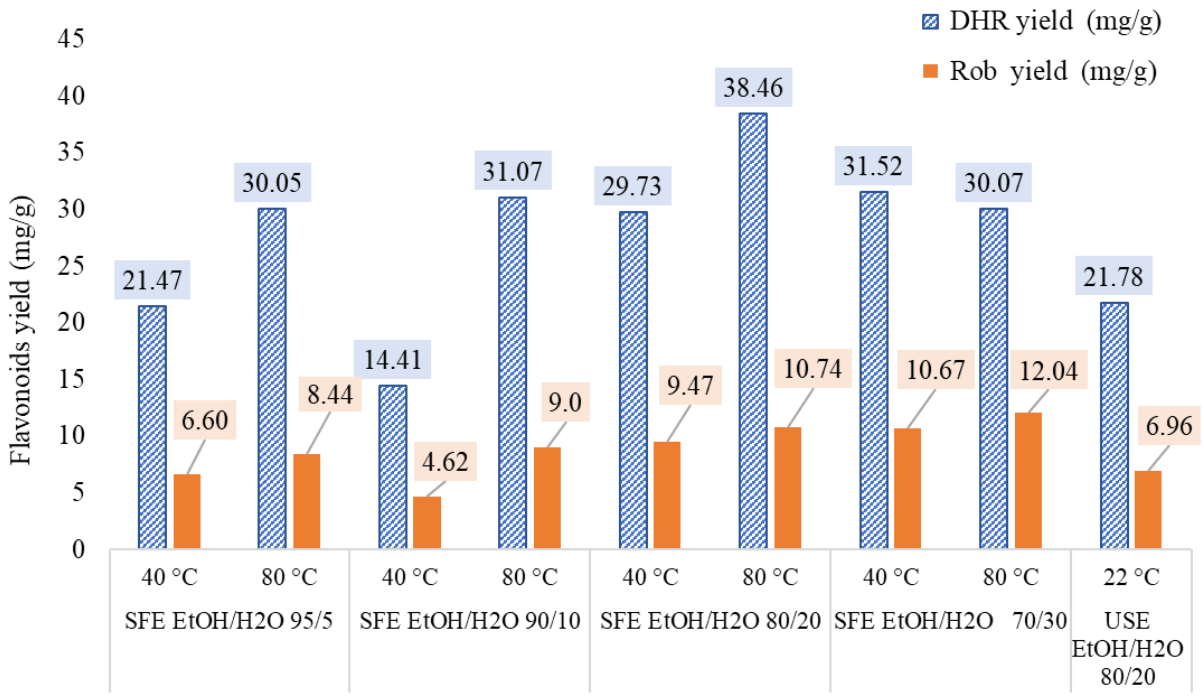


Figure 4: DHR and Rob yield for the selective extraction experiments.

They confirmed as well that the percentage of water in mixture with EtOH had little impact on the selectivity of extraction of DHR or Rob under the tested conditions. The ratio between both flavonoids was almost the same. This is not sufficient to conclude that a selective extraction is possible with the variation of the percentage of water in the modifier mixture with EtOH. Also, comparing this ratio between the extracts of SFE and USE, it is noticeable that both extraction techniques offer similar ratios, this indicates that specific SFE parameters like the combination of pressure and

temperature, that can influence the density of the SC-CO_2 did not influence the selective extraction of both compounds.

4. Conclusion

Overall, the supercritical extraction of black locust's main flavonoids was optimized by mean of design of experience; it validated the advantages of the use of SC-CO_2 mixed with polar solvents for the extraction of polar compounds. Our results show that modifier's percentage was the most influent parameter for increasing the yield of the compounds, that coupled with a higher temperature allow for a faster recovery of the flavonoids while using less solvent, the pressure showed very little influence on the yield. The possibility to consume lower amounts of organic solvents was validated by comparing the extraction yield for the same consumption of solvents between SFE and USE, indeed the SFE showed a higher yield of 49.2 mg/g compared with USE of 28.75 mg/g. Both DHR and Rob showed a similar behavior with the variation of water percentage mixed with the ethanol, which indicated a non-selective extraction of the compounds at the studied water percentages.

5. References

[1] K. Rédei, Z. Osváth-bujtás, I. Veperdi, **n.d.**

[2] A. Cierjacks, I. Kowarik, J. Joshi, S. Hempel, M. Ristow, M. von der Lippe, E. Weber, *Journal of Ecology*. **2013**, *101* (6), 1623–1640. DOI: 10.1111/1365-2745.12162.

[3] V.-N. Nicolescu, K. Rédei, W. L. Mason, T. Vor, E. Pöetzelsberger, J.-C. Bastien, R. Brus, T. Benčať, M. Đodan, B. Cvjetkovic, et *al.*, *J. For. Res.* **2020**, *31* (4), 1081–1101. DOI: 10.1007/s11676-020-01116-8.

[4] E. Weber, D. Gut, *Journal for Nature Conservation*. **2004**, *12* (3), 171–179. DOI: 10.1016/j.jnc.2004.04.002.

[5] B. Keresztesi, *Bee World*. **1977**, *58* (4), 144–150. DOI: 10.1080/0005772X.1977.11097669.

[6] C. C. Barbier, D. Merzeau, P. Pastuszka, J.-P. Charpentier, **2016**, (226), 10–19.

[7] D. Călina, N. K. Olah, E. Pătru, A. Docea, H. Popescu, M.-V. Bubulica, *Curr Health Sci J*. **2013**, *39* (4), 232–236.

[8] N. C. Veitch, P. C. Elliott, G. C. Kite, G. P. Lewis, *Phytochemistry*. **2010**, *71* (4), 479–486. DOI: 10.1016/j.phytochem.2009.10.024.

[9] I. C. Marinas, E. Oprea, E.-I. Geana, C. Chifiriuc, V. Lazar, *Journal of the Serbian Chemical Society*. **2014**, *79* (11), 1363–1378.

[10] C. Sarikurkcu, M. S. Kocak, B. Tepe, M. C. Uren, *Industrial Crops and Products*. **2015**, *78*, 110–115. DOI: 10.1016/j.indcrop.2015.10.017.

[11] Y. Cui, G. Liang, Y.-H. Hu, Y. Shi, Y.-X. Cai, H.-J. Gao, Q.-X. Chen, Q. Wang, *J. Agric. Food Chem.* **2015**, *63* (2), 716–722. DOI: 10.1021/jf505469k.

[12] M. Sanz, B. Fernández de Simón, E. Esteruelas, Á. M. Muñoz, E. Cadahía, T. Hernández, I. Estrella, E. Pinto, *J. Agric. Food Chem.* **2011**, *59* (7), 3135–3145. DOI: 10.1021/jf1042932.

[13] I. Beritognolo, E. Magel, A. Abdel-Latif, J.-P. Charpentier, C. Jay-Allemand, C. Breton, *Tree Physiology*. **2002**, *22* (5), 291–300. DOI: 10.1093/treephys/22.5.291.

[14] S. Bostyn, E. Destandau, J.-P. Charpentier, V. Serrano, J.-M. Seigneuret, C. Breton, *Industrial Crops and Products*. **2018**, *126*, 22–30. DOI: 10.1016/j.indcrop.2018.09.049.

[15] E. Destandau, J.-P. Charpentier, S. Bostyn, S. Zubrzycki, V. Serrano, J.-M. Seigneuret, C. Breton, *Separations*. **2016**, *3* (3), 23. DOI: 10.3390/separations3030023.

[16] T. Sergent, S. Kohnen, B. Jourez, C. Beauve, Y.-J. Schneider, C. Vincke, *Wood Sci Technol*. **2014**, *48* (5), 1005–1017. DOI: 10.1007/s00226-014-0656-x.

[17] V. Vek, I. Poljanšek, P. Oven, *Eur. J. Wood Prod.* **2019**, *77* (5), 891–901. DOI: 10.1007/s00107-019-01430-x.

[18] O. Dünisch, H.-G. Richter, G. Koch, *Wood Sci Technol*. **2010**, *44* (2), 301–313. DOI: 10.1007/s00226-009-0275-0.

[19] M. D. Luque de Castro, L. E. García-Ayuso, *Analytica Chimica Acta*. **1998**, *369* (1), 1–10. DOI: 10.1016/S0003-2670(98)00233-5.

[20] I. Savic Gajic, I. Savic, I. Boskov, S. Žerajić, I. Markovic, D. Gajic, *Antioxidants (Basel)*. **2019**, *8* (8), 248. DOI: 10.3390/antiox8080248.

[21] C. D. Bevan, P. S. Marshall, *Natural Product Reports*. **1994**, *11* (0), 451–466. DOI: 10.1039/NP9941100451.

[22] G. Brunner, *Journal of Food Engineering*. **2005**, *67* (1), 21–33. DOI: 10.1016/j.jfoodeng.2004.05.060.

[23] D. Pinto, M. de la L. Cádiz-Gurrea, J. Garcia, M. J. Saavedra, V. Freitas, P. Costa, B. Sarmento, C. Delerue-Matos, F. Rodrigues, *Sustainable Materials and Technologies*. **2021**, *29*, e00309. DOI: 10.1016/j.susmat.2021.e00309.

[24] M. Razgonova, T. Kalenik, V. Veselov, A. Zakharenko, A. Taghizadehghalehjoughi, V. Vuta, F. Barbuceanu, B. Izotov, A. Stratidakis, A. Tsatsakis, et *al.*, *Farmacia*. **2019**, *67*. DOI: 10.31925/farmacia.2019.1.11.

[25] F. Bianchi, M. Careri, *Current Analytical Chemistry*. **2008**, *4* (1), 55–74. DOI: 10.2174/157341108783339070.

[26] G. E. P. Box, D. W. Behnken, *Technometrics*. **1960**, *2* (4), 455–475. DOI: 10.1080/00401706.1960.10489912.

[27] B. Ait-Amir, P. Pougnet, A. El Hami, in *Embedded Mechatronic Systems 2 (Second Edition)* (Eds: A. El Hami, P. Pougnet), ISTE **2020**.

[28] J. S. Rao, B. Kumar, in *10th International Conference on Vibrations in Rotating Machinery*, Woodhead Publishing **2012**.

[29] A. T. Serra, I. J. Seabra, M. E. M. Braga, M. R. Bronze, H. C. de Sousa, C. M. M. Duarte, *The Journal of Supercritical Fluids*. **2010**, *55* (1), 184–191. DOI: 10.1016/j.supflu.2010.06.005.

[30] I. J. Seabra, M. E. M. Braga, M. T. Batista, H. C. de Sousa, *The Journal of Supercritical Fluids*. **2010**, *54* (2), 145–152. DOI: 10.1016/j.supflu.2010.05.001.

[31] M. Antunes-Ricardo, J. A. Gutiérrez-Uribe, D. Guajardo-Flores, *The Journal of Supercritical Fluids*. **2017**, *119*, 58–63. DOI: 10.1016/j.supflu.2016.09.003.

[32] E. Conde, J. Hemming, A. Smeds, B. D. Reinoso, A. Moure, S. Willför, H. Domínguez, J. C. Parajó, *The Journal of Supercritical Fluids*. **2013**, *81*, 193–199. DOI: 10.1016/j.supflu.2013.04.018.

[33] E. E. Yilmaz, E. B. Özvural, H. Vural, *The Journal of Supercritical Fluids*. **2011**, *55* (3), 924–928. DOI: 10.1016/j.supflu.2010.10.046.

[34] Y.-C. Yang, M.-C. Wei, T.-C. Huang, S.-Z. Lee, *The Journal of Supercritical Fluids*. **2013**, *81*, 55–66. DOI: 10.1016/j.supflu.2013.04.007.

[35] M. A. Babyak, *Psychosomatic Medicine*. **2004**, *66* (3), 411–421.

[36] K. M. Sharif, M. M. Rahman, J. Azmir, S. H. Shamsudin, M. S. Uddin, T. K. Fahim, I. S. M. Zaidul, *Journal of Industrial and Engineering Chemistry*. **2015**, *21*, 1314–1322. DOI: 10.1016/j.jiec.2014.05.047.

[37] A. P. Sánchez-Camargo, J. A. Mendiola, E. Ibáñez, M. Herrero, in *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, Elsevier **2014**.

[38] M. Herrero, J. A. Mendiola, A. Cifuentes, E. Ibáñez, *Journal of Chromatography A*. **2010**, *1217* (16), 2495–2511. DOI: 10.1016/j.chroma.2009.12.019.

[39] M. Cvjetko Bubalo, S. Vidović, I. Radojčić Redovniković, S. Jokić, *Food and Bioproducts Processing*. **2018**, *109*, 52–73. DOI: 10.1016/j.fbp.2018.03.001.

[40] N. R. Putra, L. N. Yian, H. M. Nasir, Z. B. Idham, M. A. C. Yunus, *IOP Conf. Ser.: Mater. Sci. Eng.* **2018**, *334*, 012057. DOI: 10.1088/1757-899X/334/1/012057.

[41] F. A. Espinosa-Pardo, V. M. Nakajima, G. A. Macedo, J. A. Macedo, J. Martínez, *Food and Bioproducts Processing*. **2017**, *101*, 1–10. DOI: 10.1016/j.fbp.2016.10.002.

[42] A. Natolino, C. Da Porto, *The Journal of Supercritical Fluids*. **2019**, *151*, 30–39. DOI: 10.1016/j.supflu.2019.05.002.

Part II :

Camellia sinensis

The finding from the first part demonstrated that the pressure was not a significant factor (in the tested range) for the extraction of polar compounds due to the high percentage of modifier mixed with CO_2, that increased the density of the extraction phase, decreasing the influence of the pressure. Therefore, in the second part, the targeted compounds of interest are catechins and caffeine from green tea leaves, these molecules have a higher polarity compared to DHR and Rob discussed in part I, and their extraction will be optimized using a modified BBD. This newly adapted model examined this time the temperature, the percentage of the modifier (EtOH or EtOH:H_2O *v:v* mixture), and the percentage of water added to the modifier. The water additive was chosen primarily to increase the polarity of the extraction phase. Nonetheless, it can be interesting to investigate the variation of its percentage on extraction selectivity.

Part II: Supercritical Fluid Extraction (SFE) of polar compounds from *Camellia sinensis* leaves: use of ethanol/water as a green polarity modifier

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green tea, caffeine, catechins, epigallocatechin gallate, water additive, natural ingredients, Box-Behnken design, polar modifiers, sequential-selective extraction.

Abstract

The use of bioactive plant extracts in cosmetic products is a common practice. Most of these extracts are obtained by maceration in organic solvents, depending on which the polarity and the structure of target molecules will vary. Polyphenols are polar compounds that often display antioxidant and/or antibacterial activities. To extract them, ethanol/water mixtures are usually selected as green solvents. This solid-liquid extraction (assisted or not) requires the use of high volumes of solvents, and many additional steps like mixing, agitation, filtration, and evaporation. Alternatively, supercritical carbon dioxide (SC-CO_2) offers many benefits for plant extraction: economical, non-toxic, and naturally concentrated extracts. However, its low polarity is not suitable to solubilize polar compounds. In this study, an experimental design was used to optimize supercritical fluid extraction (SFE) of caffeine and catechins from *Camellia sinensis*. Catechins are recognized for skin-care use (antioxidant) and caffeine is also used for its skin-care properties, and to prevent excess storage of fat in cells. The temperature, modifier content, and water additive percentage were used as independent variables. The results showed that while the temperature was an insignificant parameter, a higher percentage of water (up to 20% in ethanol) and modifier favored the extraction of the polar target molecules. Additionally, the SFE results were compared to ultrasound-assisted extraction (UAE). Finally, a sequential-selective extraction of caffeine from catechins is also presented.

1. Introduction

Tea (*Camellia sinensis*) is largely consumed around the world. The beverage obtained by the infusion of leaves in hot water (80-95°C) contains numerous polyphenols, that have several biological activities, providing beneficial effects on human health [1–5]. The major bioactive compounds are catechins, flavan-3-ol type of secondary metabolites (**Figure 1**).

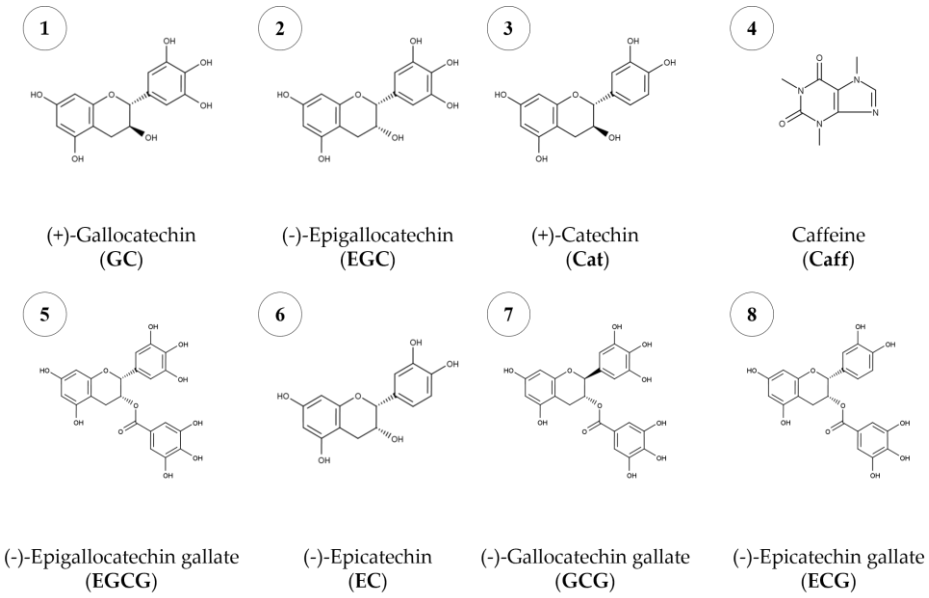

Figure 1: Chemical structure of green tea polar compounds.

The tea leaves are processed to make green tea, by roasting or steaming. Additional processes like fermentation (semi or fully) of leaves produce different variations of tea, for example oolong tea and black tea, in which the content of catechins can decrease [3,4]. The structural differences for these catechins are the hydroxyl group number on the B benzene ring (catechol), two for catechin (Cat) and catechin gallate (CG) or three for gallocatechin (GC) and gallocatechin gallate (GCG), and the presence of esterified gallic acid of the hydroxyl group in position 3 on the C ring (dihydropyran heterocycle), leading to the two gallate forms, CG and GCG of Cat and GC. Moreover, due to the presence of two asymmetric carbons, number 2 and 3 on the C ring, these four catechins can be found in the cis form, called « epi », which is less stable than the trans-form [1]. Besides, in green tea leaves, the four main flavanols are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), the two last being the most abundant [1–5].

The analysis of these compounds is generally achieved by high- or ultrahigh-performance liquid chromatography (HPLC/UHPLC), with C18-bonded stationary

phases and either water/acetonitrile [6–13], water/methanol [14–17] or water/ACN/MeOH [18] elution gradient. The retention order generally follows the same pattern, CG/ECG/Cat/EC/EGCG/GCG/ECG/CG, meaning that the free esterified forms (CG and Cat) elute before the esterified ones, GCG and CG, and for the two classes of catechins, the compounds with three hydroxyl groups on the B ring elute before the ones having two hydroxyl groups. However, when looking at "epi" and "non epi" forms, the retention order is reversed between the free and the esterified compounds, the epi forms elute after for CG and C whereas they elute before for GCG and CG. Depending on the specificity of the stationary phase, the retention order between EGCG and EC can change. Moreover, despite the satisfactory separation of standards, many coelutions could occur when working on real extracts [6,15–17].

Because the catechin content is associated to the quality of tea [19], many studies were conducted on the extracts obtained with hot water maceration, or with various modern extraction methods to study the catechin content of green and black teas, as well as how these techniques degrade these substances [1,6–16,18,20–24]. One can list the microwave-assisted extraction (MAE) [7,17,24], ultrasound-assisted extraction (UAE) in water or ethanol/ethyl acetate [12,21,23], liquid-solid extraction with varied solvents (methanol, ethanol) and/or mechanical help [11,13,18,23], ultra-high-pressure-assisted solvent extraction (UPSE) (10 to 50 MPa) [19,25], pressurized-liquid extraction (PLE) with methanol [21] or ethyl lactate/water mixtures [26], and last but not least, supercritical fluid extraction (SFE) [2–5,16,20,27–31], with CO_2-based fluids.

In addition, caffeine found in *Camellia sinensis* leaves may have an adverse effect on health [2–5]. Therefore, caffeine is often removed from the tea leaves, mainly by using SFE [2–5,16,27–30], or with solid-phase adsorption of the liquid extracts [24,32]. However, the decaffeination process can co-extract catechins from the plant, thereby reducing the positive effect of the consumed beverage. The application of SFE on green tea compounds was studied before, it showed that the highest recovery yield for both caffeine and catechins was obtained with the ethanol:CO_2 (5:95; *v:v*) composition. This was combined with a temperature of 80°C and pressure of 30 MPa [3]. However, the highest selectivity was achieved at 63°C and 23 MPa, with an extraction yield of 96% for caffeine and 40.6% for catechins [28]. In another study, the effects of particle size, temperature, modifier (EtOH) volume, and extraction duration were examined through an orthogonal array design of nine experiments. It reported that the particle size of 0.2-0.6 mm allowed the recovery of 70% of caffeine and only 6% of catechins at 80°C, 30 MPa, a CO_2 flow rate of 1.5 ml/min for 2 hours, using a total ethanol volume of 30 mL [27]. An ANOVA study of the results, based on the nine experiments, showed

that temperature, particle size, extraction time and modifier volume had a significant influence on the caffeine removal, but none of these parameters affects the concentration of EGCG, the main catechin. Besides, the smallest particle size (0.23 mm) favored the removal of caffeine, whereas the largest particle size (5.5 to 17.4 mm) reduced the extraction of EGCG [3].

The use of water as modifier degraded selectivity, yielding 70-80% recovery for the caffeine extraction and 60-70% for catechins [2,4,27]. According to a different study, the dried leaves swelled up when water was added to CO_2, enhancing the solute diffusion, and increasing the recovery of caffeine and catechins extraction. Additionally, the extraction recovery increased with the temperature, the pressure, and the water content, whereas the extraction selectivity increased with pressure and water content but decreased with the increase of temperature [5].

All these findings demonstrated that the addition of ethanol or water to SC-CO_2 is necessary to significantly enhance the solubility of polar compounds, caffeine and/or catechins [2,4,5,20,27,32]. When considering the kinetics of extraction using ethanol as a modifier, no variations in selectivity can be noted between 30 minutes and 3 hours [3,27,28]. Moreover, the extraction kinetics of catechins, caffeine and chlorophylls were very similar, regarding pressure, temperature, and modifier concentration [28].

Recently, ethyl lactate, ethyl acetate and ethanol used as modifiers were compared at 30 MPa and 70°C [32]. Ethyl lactate displayed the highest extraction recovery for caffeine, while ethyl acetate seemed better suited to achieve a higher selectivity of caffeine vs. catechins [31].

This paper describes the use of a rationalized approach to ensure the best recovery of polar compounds from green tea leaves, including caffeine and catechins and by reducing the amount of used solvent with classical maceration extraction, to provide extracts for cosmetic uses. Considering the advantages of SC-CO_2, providing concentrated extracts after the CO_2 depressurization during sample collection, and of its ecological nature, SFE with ethanol (EtOH) and EtOH:H_2O modifiers was chosen to achieve this goal. From previous studies done in our laboratory, either on the extraction of flavonoids from heartwood [33] or on the selective extraction of bioactive compounds from rosemary [34], extraction temperature, modifier percentage, and percentage of water in ethanol as additive and modifier were chosen as optimization parameters.

2. *Results and discussion*

SFE on tea leaves was optimized based on a Box-Behnken design of experiment (BBD). The percentages of modifier and water as an additive were selected because of their capacity to change the polarity of the extraction phase, while the temperature was chosen for its ability to influence the solubility of compounds in the SC-CO_2 (**Table 1**).

Table 1: Design matrix in the Box-Behnken model and responses (represented with SD values for each response) for green tea extracts (total catechins represents all the catechins including EGCG)

Exp N°	Coded levels			Results (concentration mg/g of biomass after 45 min of extraction)									Total molecules (mg/g of biomass)		
	X_1 Temperature (°C)	X_2 Modifier (%)	X_3 H_2O in modifier (%)	Caff	GC	EGC	Cat	EGCG	EC	ECG	Total catechins	Total molecules (Y mg/g)	Fraction 1 (15 min)	Fraction 2 (30min)	Fraction 3 (45 min)
1	40 (-1)	10 (-1)	10 (0)	13.04 ± 1.01	0.18 ± 0.01	0.09 ± 0.01	0.04 ± 0.00	0.12 ± 0.01	0.31 ± 0.02	0.12 ± 0.01	0.86 ± 0.06	13.90 ± 0.93	8.94 ± 1.19	3.68 ± 0.66	1.28 ± 0.20
2	80 (+1)	10 (-1)	10 (0)	13.13 ± 1.01	0.46 ± 0.02	2.46 ± 0.13	0.14 ± 0.01	4.53 ± 0.43	1.34 ± 0.08	1.88 ± 0.11	10.80 ± 0.72	23.93 ± 1.60	9.16 ± 1.22	3.42 ± 0.62	11.36 ± 1.75
3	40 (-1)	30 (+1)	10 (0)	17.24 ± 1.33	1.50 ± 0.05	14.40 ± 0.76	0.55 ± 0.03	22.75 ± 2.18	5.27 ± 0.30	6.39 ± 0.38	50.87 ± 3.40	68.11 ± 4.55	42.63 ± 5.69	15.52 ± 2.80	9.96 ± 1.53
4	80 (+1)	30 (+1)	10 (0)	19.75 ± 1.53	2.14 ± 0.08	20.35 ± 1.07	0.78 ± 0.04	32.84 ± 3.14	7.08 ± 0.40	9.79 ± 0.58	72.98 ± 4.88	92.73 ± 6.20	74.51 ± 9.94	12.14 ± 2.19	6.08 ± 0.93
5	40 (-1)	20 (0)	0 (-1)	3.59 ± 0.28	0.11 ± 0.00	0.94 ± 0.05	0.05 ± 0.00	1.17 ± 0.11	0.54 ± 0.03	0.57 ± 0.03	3.38 ± 0.23	6.97 ± 0.47	4.93 ± 0.66	1.15 ± 0.21	0.89 ± 0.14
6	80 (+1)	20 (0)	0 (-1)	9.42 ± 0.73	0.22 ± 0.01	1.55 ± 0.08	0.12 ± 0.01	2.07 ± 0.20	1.14 ± 0.07	0.99 ± 0.06	6.10 ± 0.41	15.52 ± 1.04	9.60 ± 1.28	3.29 ± 0.59	2.63 ± 0.40
7	40 (-1)	20 (0)	20 (+1)	16.78 ± 1.30	1.99 ± 0.07	19.13 ± 1.01	1.19 ± 0.05	32.07 ± 3.07	6.46 ± 0.37	9.57 ± 0.56	70.41 ± 4.70	87.19 ± 5.83	58.42 ± 7.79	23.67 ± 4.27	5.10 ± 0.78
8	80 (+1)	20 (0)	20 (+1)	13.56 ± 1.05	2.12 ± 0.08	14.56 ± 0.77	1.40 ± 0.06	20.72 ± 1.98	6.27 ± 0.36	8.23 ± 0.48	53.30 ± 3.56	66.86 ± 4.47	55.97 ± 7.47	9.20 ± 1.66	1.70 ± 0.26
9	60 (0)	10 (-1)	0 (-1)	10.45 ± 0.81	0.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	10.49 ± 0.70	8.80 ± 1.17	1.05 ± 0.19	0.64 ± 0.10
10	60 (0)	30 (+1)	0 (-1)	6.37 ± 0.49	0.34 ± 0.01	3.29 ± 0.17	0.15 ± 0.01	4.30 ± 0.41	1.34 ± 0.08	1.43 ± 0.08	10.85 ± 0.72	17.22 ± 1.15	10.44 ± 1.39	4.07 ± 0.73	2.71 ± 0.42
11	60 (0)	10 (-1)	20 (+1)	13.77 ± 1.06	1.58 ± 0.06	11.62 ± 0.61	1.06 ± 0.05	15.33 ± 1.47	4.71 ± 0.27	6.44 ± 0.38	40.74 ± 2.72	54.51 ± 3.64	12.90 ± 1.72	26.67 ± 4.81	14.94 ± 2.30
12	60 (0)	30 (+1)	20 (+1)	17.95 ± 1.39	2.00 ± 0.07	20.44 ± 1.08	1.33 ± 0.06	31.55 ± 3.02	6.88 ± 0.39	9.95 ± 0.59	72.15 ± 4.82	90.10 ± 6.02	85.10 ± 11.35	3.90 ± 0.70	1.11 ± 0.17
13	60 (0)	20 (0)	10 (0)	16.02 ± 1.24	1.66 ± 0.06	14.93 ± 0.79	0.63 ± 0.03	20.70 ± 1.98	5.68 ± 0.32	7.28 ± 0.43	50.89 ± 3.40	66.91 ± 4.47	45.64 ± 6.09	12.04 ± 2.17	9.23 ± 1.42
14	60 (0)	20 (0)	10 (0)	16.91 ± 1.31	1.76 ± 0.06	15.47 ± 0.82	0.68 ± 0.03	20.87 ± 2.00	6.49 ± 0.37	8.04 ± 0.47	53.32 ± 3.56	70.23 ± 4.69	43.18 ± 5.76	14.63 ± 2.64	12.42 ± 1.91
15	60 (0)	20 (0)	10 (0)	17.74 ± 1.37	1.75 ± 0.06	15.33 ± 0.81	0.66 ± 0.03	20.64 ± 1.98	6.29 ± 0.36	7.89 ± 0.46	52.55 ± 3.51	70.29 ± 4.70	45.53 ± 6.07	14.66 ± 2.64	10.11 ± 1.55
16	60 (0)	20 (0)	10 (0)	19.70 ± 1.52	1.83 ± 0.07	16.76 ± 0.88	0.70 ± 0.03	25.01 ± 2.39	6.58 ± 0.38	8.56 ± 0.50	59.43 ± 3.97	79.13 ± 5.29	55.41 ± 7.39	15.46 ± 2.79	8.26 ± 1.27
17	60 (0)	20 (0)	10 (0)	17.85 ± 1.38	1.72 ± 0.06	14.63 ± 0.77	0.63 ± 0.03	19.83 ± 1.90	6.12 ± 0.35	7.74 ± 0.46	50.67 ± 3.39	68.52 ± 4.58	38.77 ± 5.17	19.64 ± 3.54	10.11 ± 1.55

In our previous works, a BBD was employed to optimize the extraction of two polar flavonoids, where pressure was an optimization parameter ranging from 10 to 20 MPa [33]. It showed that pressure was an insignificant variable in that range for the extraction of such polar compounds. This was mainly due to the high density of the fluid with the addition of high percentages of modifier (up to 30%). As it showed little influence on the model's response (compounds yield), pressure was kept constant at 15 MPa during the present study and was not investigated.

2.1. Statistical analysis, model, and factor significance

The quadratic model's determination coefficient (R^2) for the total molecules was equal to 0.89, while the value of adjusted determination coefficient (adj R^2) was 0.82. This signifies that the model was unable to account for 11% of the total variance. The R^2 value demonstrates a strong correlation between the expected and experimental response values and indicates that the empirical model shows a good fit with experimental data. The relative standard deviation (RSD%) of the central point replicates (5 repetitions) for the total molecules extracted from green tea leaves was equal to 6.68%. Furthermore, the Dixon statistical test was applied and showed no outliers. This was also confirmed with the residual analysis. The correlation between the three variables (X_1, X_2, and X_3) and the total yield of the molecules of interest (Y) (**Table 1**) is presented in **Equation 1**.

$$Y\ (mg/g) = -162.8 + 3.187\ X_1 + 6.547X_2 + 6.453X_3 - 0.02537X_1^2 - 0.112X_2^2 - 0.1673X_3^2 \quad \textbf{(1)}$$

The summary of the analysis of variance (ANOVA) for the polynomial model is shown in **Table 2** and the regression values showed that the model is significant. The probability related to F-value and p-value were used to determine the significance of each factor, and their contribution percentages were studied to determine their impact on the model's response. The total yield of molecules of interest from *Camellia sinensis* regression model had an F-value of 13.55, with a related p-value of 0.0003. Both statistical terms indicate a significant model.

Table 2: ANOVA for response surface regression model for total yield of target molecules (mg/g) from green tea leaves extracts.

Source	DF	SS	MS	F value	Prob > F	Contribution (%)	Conclusion
Regression model	6	13574	2262.3	13.5449	0.0003		Very significant
X_1- Temperature (°C)	1	65.38	468.32	2.804	0.125	5	Not significant
X_2- Modifier (%)	1	3416.8	1092.2	6.5391	0.0285	12	Significant
X_3- H_2O in modifier (%)	1	7716.5	3873.1	23.1893	0.0007	42	Very significant
X_1^2	1	579.65	433.5	2.5955	0.1382	5	Not significant
X_2^2	1	616.39	528.33	3.1633	0.1057	6	Not significant
X_3^2	1	1179.1	1179.1	7.0595	0.024	13	Significant
Residuals	10	1670.2	167.02			18	
Total	16	15244					
	Degrees of freedom	Sum of squares	Mean square				

The factors X_2 (modifier percentage) and X_3 (H_2O%) showed the highest significance, with a contribution of 12% and 42% respectively. This was also validated by their p-values of 0.0385 and 0.0010 respectively. This indicates that even though the modifier percentage is significant, the contribution of water percentage to the total yield was higher. Besides, X_1 (temperature) had a low significance on the total yield, with a 5% contribution to the model and a p-value of 0.125. This suggests that the temperature variation had no substantial impact on the yield of caffeine and total catechins.

2.2. Effects of the extraction parameters assessed with BBD

Heat-maps were used as a graphical representation of the independent factors and dependent response interaction and correlated to the response of green tea compounds yield (mg/g) for 45 min of extraction time.

Figure 2.a illustrates the effect of the modifier's percentage and water content on the extract's yield. This revealed a positive impact of both the modifier's percentage and the water content on the response. When the modifier percentage increased from 10 to 30%, the total molecules yield increased from 39.15 mg/g to 80.48 mg/g, when the temperature was set at 60°C and 10% of H_2O was included in the modifier. This represented an increase of 105% of the yield. Moreover, as the H_2O% varied from 0 to 20%, the predicted total yield varied from 23.22 to 85.34 mg/g. These results confirmed

the significant effect of water as an additive, as the increase in this case was 267% compared to the 105% with the modifier's percentage.

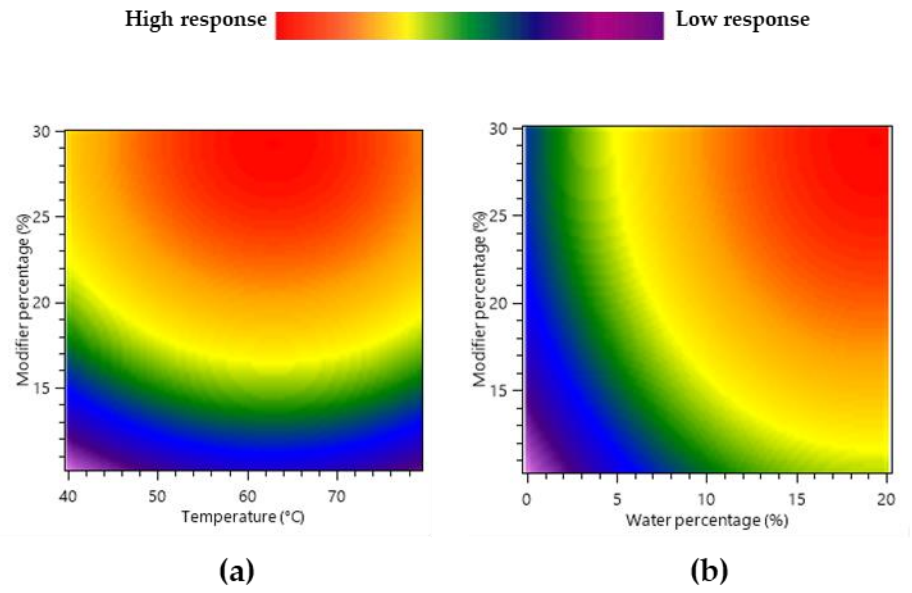

Figure 2: Response surface heat-maps showing the interactive effects of the variables on the total molecules yield (mg/g) of green tea leaves extracts.

This behavior is probably due to the high polarity of caffeine and catechins. Indeed, the low polarity of SC-CO_2 is limiting its extraction capability to non-polar or moderately polar molecules. However, the addition of ethanol and water as modifiers increased the polarity of the extraction phase, and consequently its affinity for polar compounds like the polyphenols of interest, therefore increasing the recovery yield.

Temperature is generally an essential factor in SFE optimization. It can affect the solubility of polar compounds in SC-CO_2, the diffusion rate of the compounds, and the properties of the solvent (density, viscosity). Generally, higher temperatures increase the solubility and in some cases the velocity of polar compound diffusion from the plant matrix into the extraction phase leading to higher extraction yields [35]. However, at excessively high temperatures, the polar compounds may degrade, affecting the quality of the extracted compounds. Moreover, increasing the temperature can also decrease the density of the SC-CO_2, reducing its ability to penetrate the tea matrix and solubilize the target compounds [36]. Consequently, **Figure 2.b** demonstrated that the effect of temperature on the tea compounds recovery was insignificant, in accordance with ANOVA. When working with modifier percentages from 10 to 30%, with or without water, the fluid compressibility is low,

meaning that changes in temperature cannot induce large fluid density variation or solubility changes.

2.3. Extraction optimum of caffeine and catechins

Based on **equation 1**, the computed optimum parameters, offering maximal yield of caffeine and total catechins, are as follows: temperature of 62°C, a modifier percentage of 29.3% and a water percentage in modifier of 19.3%.

These conditions are close to the extraction parameters of experiment n°12, which allowed the recovery of 90.1 mg/g of green tea compounds of interest from the biomass. The extraction kinetics (**Table 1**) of this experimental point showed that most green tea target compounds were extracted within 15 min. This indicated that, in these conditions, 15 min was sufficient, from an economical point of view, for the extraction of most of the molecules of interest. Additionally, other experimental points offered a high extraction yield of the target compounds. For instance, experiments n°4 (80°C, 30% EtOH:H_2O 90:10 *v:v*) and n° 7 (40°C, 20% EtOH:H_2O 80:20 *v:v*) had a yield of 92.73 and 87.19 mg/g respectively. These conditions could be selected in the goal to reduce both the extraction temperature (energetically favorable) and the modifier consumption. However, both experiments offered slower extraction kinetics compared to experiment n°12, where in 15 min the recovery yield was 80.3%, 67% and 94% for experiment n°4, n°7 and n°12 respectively. These recovery differences towards the extraction duration are related to different kinetics. For experiment n°4, the use of only 10% of water included in the modifier could reduce the solubility of compounds, as is also observed for experiments 13 to 17 (central point of the BBD). On the contrary, the lower temperature for experiment n°7 could mainly change the diffusion inside the matrix, as is also observed for experiment n°3.

2.4. Comparison between supercritical fluid extraction (SFE) and ultrasound assisted extraction (UAE)

The SFE was compared to UAE which is considered as an efficient and simple extraction technique. This was done in equivalent conditions: 13.5 mL solvent volume (equivalent to 30% of modifier for 15 minutes at 3 mL/min flow) and 15 min extraction time.

Three EtOH:H_2O *v:v* ratios were explored: 80:20, 50:50 and 0:100 (or 100% H_2O). The first conditions were chosen to compare the extraction yields in similar ratios of

EtOH:H_2O as for the optimized SFE conditions (EtOH:H_2O 80:20 *v:v*), and the second from conditions classically observed in the literature. The third composition (100% H_2O) was selected to investigate the effect of higher H_2O% on the yield of green tea target compounds. The results are presented in **Figure 3**. They demonstrate that both extraction methods can extract green tea compounds. However, different yields were obtained from both techniques.

Using EtOH:H_2O 80:20 *v:v*, SFE provided higher extraction yields for Caff, EGCG and total catechins with 17.95, 28.34 and 67.23 mg/g respectively compared to 10.44, 14.11 and 37.39 mg/g with UAE.

The UAE results showed that an increase of 30% of water (between EtOH:H_2O 80:20 and 50:50 *v:v*) had a positive effect on the extraction yield of the target compounds. The percentage of Caff, EGCG and total catechins increased by 46, 14.18 and 27.8% respectively.

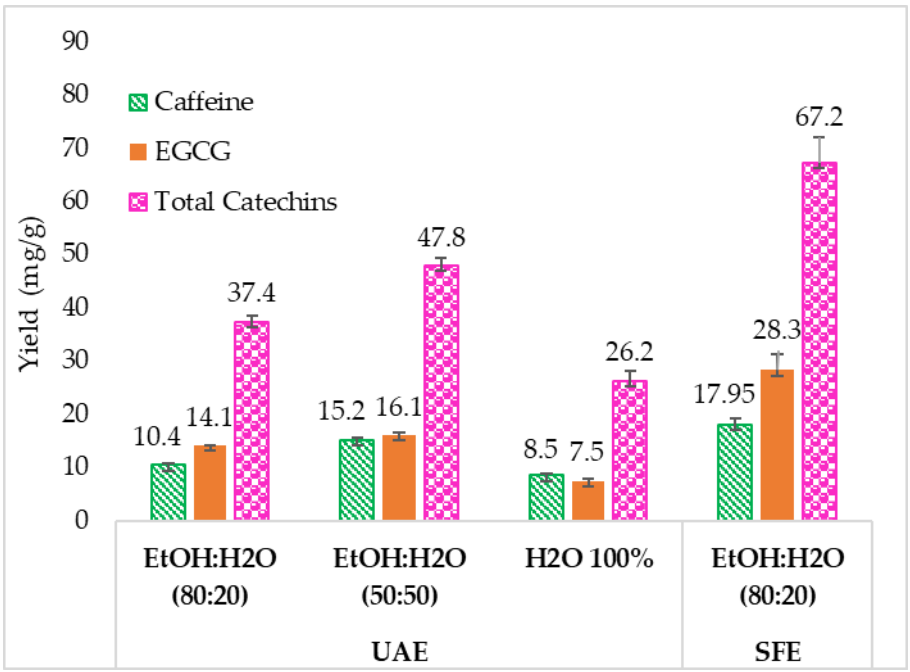

Figure 3: Comparison between supercritical fluid extraction (SFE) and Ultrasound assisted extraction (UAE) for green tea leaves compounds (total catechins represents all the catechins including EGCG).

However, the UAE with 100% water had an opposite effect on the extraction yield of green tea compounds, as a decrease of 44.07, 53.41 and 45.18% for caffeine, EGCG and total catechins extraction respectively was observed, compared to EtOH:H_2O 50:50 *v:v*.

Hence, for the same liquid solvent consumption and composition (EtOH:H_2O 80:20 *v:v*) the use of SFE increased the extraction yield by 72.11, 100.7 and 79.68% for Caff, EGCG and total catechins respectively. When comparing the two best extraction recoveries, with 50:50 EtOH:H_2O *v:v* for UAE and 80:20 EtOH:H_2O *v:v* for SFE, the higher yield of compounds was still obtained by SFE. Consequently, in the goal to produce ingredients for cosmetic products containing both caffeine and catechins, SFE can advantageously substitute UAE, leading to more concentrated extracts. Furthermore, SFE affords a lower water content (20% toward 50%). Since water requires more energy to evaporate, SFE extracts can also provide a reduction of energy consumption for the final evaporation step, and this lower water content also favors the compounds stability.

2.5. Selective extraction of caffeine

The selective extraction of caffeine from green tea or other caffeine-rich plants was previously reported in other studies [2–5]. However, for these studies the core focus was the decaffeination of the biomass while avoiding the extraction of catechins, to provide extracts containing only the bioactive catechins. Nevertheless, as previously reported in the introduction, regardless of the experimental conditions, the catechin concentration was dramatically reduced after the full extraction of caffeine. In the present study, the results of the experimental design (**Table 1**) showed that the conditions of the experiments n°1 (40°C, 10% EtOH:H_2O 90:10 *v:v*), 6 (80°C, 20% EtOH) and 9 (60°C, 10% EtOH), offered a selective extraction of caffeine.

Therefore, a selective extraction of caffeine was explored followed by an additional step to extract the remaining (minor amounts of) caffeine and the total catechins. Since we had previously selected experiment n°12 (60°C EtOH:H_2O 80:20 *v:v*) as the best suited for the overall extraction of the total compounds, this condition was chosen for the second step of the sequential extraction. Therefore, we retained experiment n°9, with a temperature of 60°C, for the extraction of caffeine in the first step, to keep a constant temperature during the sequential extraction.

Then, the sequential selective extraction kinetics was applied to 1 g of green tea biomass, with the first step using 60°C and 10% EtOH as a modifier for 45 min (left part of **Figure 4**). This first step allowed the recovery of 44.55% of the green tea leaves' extractible caffeine in 15 min; this was consistent with the amount extracted in experiment n°9. This means that the first step was able to recover almost half of the total caffeine content. The recovery of caffeine did not increase when the extraction time was extended from 15 to 45 min.

A second step was applied using the optimized SFE extraction conditions for the target

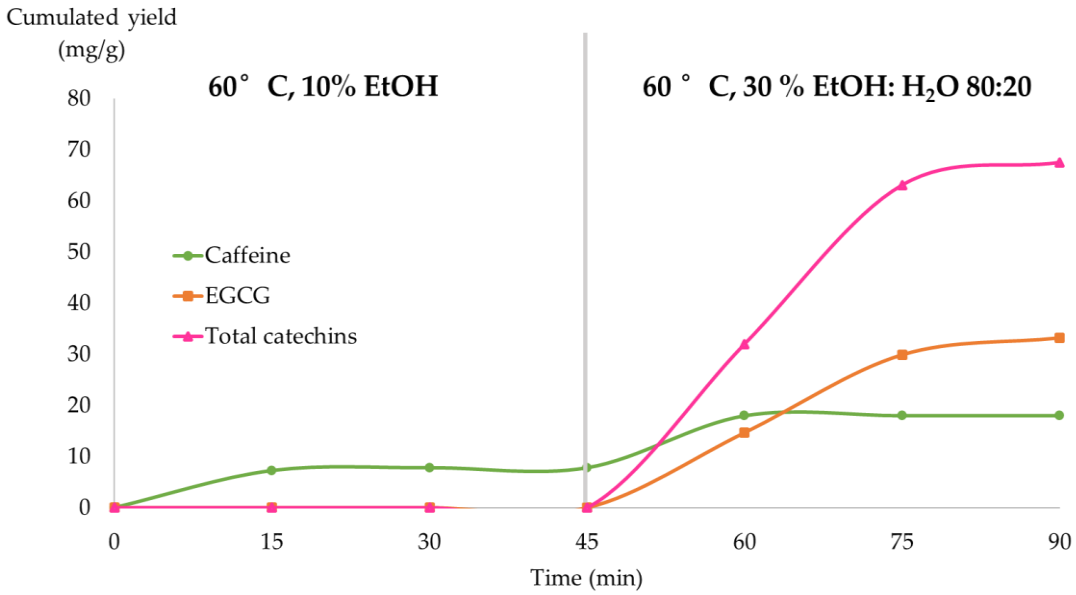

Figure 4: SFE sequential-selective extraction of caffeine from green tea leaves (total catechins represents all the catechins including EGCG).

compounds, with 60°C and 30% EtOH:H_2O 80:20 *v:v* for 45 min, corresponding to the conditions of the experiment n°12, the one displaying the highest recovery for all the compounds and catechins in the first 15 min of extraction. In the right part of **Figure 4**, we can observe that an additional amount of caffeine was extracted, leading to a final yield of 18.02 mg/g, that was approximately the same value as the one reached for the best caffeine extraction (**Table 1**). This second step also resulted in the extraction of catechins with a final yield of 67.54 mg/g. For these compounds, it was observed that the application of a first selective step to extract caffeine slowed and reduced the catechins extraction kinetics in the second step. **Figure 4** showed that in the first fraction of the second extraction step only 32.04 mg/g of catechins were extracted. In addition, the second fraction of 15 min (30 min total extraction time) allowed to recover twice as much with 63.12 mg/g cumulated yield. However, in the experimental design, with experiment n°12 only 15 min were needed to extract a major part of catechins

(**Table 1).** Furthermore, after 45 min, the total amount of extracted catechins was 67.54 mg/g, whereas it reached 72.15 mg/g for experiment n°12.

This seems to indicate, that, the first step with pure ethanol as modifier could induce a reduction in the compound accessibility into the matrix. According to other research, adding water as a modifier caused the matrix to swell, which encouraged the diffusion of the extraction solvent and consequently, the release of substances through the swollen channels of the solid matrix [2,5]. As a result, when starting with pure ethanol as a cosolvent, the effectiveness of the second step of sequential extraction is decreased since the first step's absence of water possibly prevented the matrix from swelling.

As a conclusion, from the three parameters experimental design, a sequential selective extraction was conducted, and two fractions were obtained. The first one contained only caffeine, and the second one the rest of caffeine, and around 80% of the total catechin content.

3. Materials and methods

3.1. Plant material

The green tea plant material (*Camellia sinensis* (L.) Kuntze) consisted of the dried leaves, milled into a powder of a dark green color, it was supplied by PMA 28 (Varize, France) and stored at room temperature in an airtight container.

3.2. Chemicals and reagents

CO_2 gas was supplied by Air Liquide (Fleury-les-Aubrais, France). Acetonitrile (ACN) and methanol (MeOH) were used for the mobile phase. Ethanol (EtOH) was used as extraction solvent and sample diluent. All solvents were supplied by VWR (Fontenay-sous-Bois, France). Formic acid (FA) added to the mobile phase was obtained from Sigma-Aldrich (Merck, France). Ultra-pure water (H_2O) was purified with a Milli-Q system from Sigma-Aldrich, with a resistance higher than 18 MW. Ethylenediaminetetraacetic acid (EDTA) and ascorbic acid (AA) used as stabilizing agents were supplied by Sigma-Aldrich.

The standards used to identify and/or quantify green tea target compounds (**Figure 1**) such as (-)-Epicatechin gallate (ECG), (-) Epigallocatechin (EGC) and (-)-Epicatechin

(EC) were supplied by Extrasynthese (Genay, France). (-)-Epigallocatechin gallate (EGCG); (+)-Gallocatechin (GC) and (+)-Catechin (Cat) were acquired from Sigma-Aldrich. And finally, caffeine (Caff) was supplied by Thermo-Fisher Scientific (Artenay, France).

3.3. SFE extraction

All extractions were performed using a Waters MV-10 ASFE. In the stainless-steel extraction vessel (5 mL), 1 g of plant material was combined with 1 g of diatomaceous earth powder from Sigma-Aldrich (Merck, France). Cotton was added to the top and bottom of the cell: its role is to filter the extract and to fill the cell completely. A static extraction time of 2 min was applied at the beginning of each experiment, it was then followed by continuous dynamic extraction, with a flow of 3 mL/min. The amount of modifier added was determined based on the overall extraction flow.

For each experiment in the BBD, three fractions of 15 min were collected, with a total extraction duration of 45 min. The concentrations for each fraction are shown in **Table 1**. The pressure was kept constant at 15 MPa.

Table 1 presents the list of the experiments with the responses represented by the yields (mg/g) of the major green tea compounds separately Caff and EGCG, total catechins represents all the catechins mentioned in **Figure 1** including EGCG. The total molecules yield is the sum of total catechins and the caffeine yield. This terminology is applied to all the results in this study.

3.4. Ultra-sound assisted extraction (UAE)

Using 1 g of powdered dried green tea leaves, the UAE was carried out in a Branson 3510 (Bransonic®ultrasonic) bath from Sigma-Aldrich (Merck, France). The extraction solvent used was a combination of EtOH:H_2O in various ratios, including 80:20, 50:50, and 0:100 *v:v*. The plant and 13.5 mL solvent (see section 3.4) were combined, then sonicated at room temperature for 15 min. The extracts were centrifuged at 10000 rpm for 10 min at 25°C and filtered using a 0.45 µm PTFE syringe filter from Agilent Technologies (Les Ulis, France). For UHPLC analysis, they were subsequently diluted four times with the stabilization solvent.

3.5 UHPLC-UV analysis

All extracts were analyzed with a Nexera-LC40 system of Shimadzu Corporation (Kyoto, Japan). This system was equipped with a photodiode array (PDA) detector (SPD-40), a solvent delivery unit (LC-40), an auto-sampler (SIL-40), a column oven (CTO-40) and a system controller SCL-40. All chromatograms were recorded on LabSolutions LC-UV 5.97 SP1 version (Shimadzu Corporation, Kyoto, Japan).

A Cortecs C18 (100 x 3.0 mm) column coupled to a Cortecs C18 VanGuard Cartridge (5 x 3.0 mm) both packed with 2.7 µm superficially porous particles, from Waters Corporation (Massachusetts, United States) were used for the analysis of all extracts. The column temperature was maintained at 30°C. The injection volume was 5 µL. The flow rate was maintained at 1 mL/min. Equilibration time between two injections was 5 min.

3.5.1 Elution gradient and quantification

The total time of each analysis was 18 min, the mobile phase consisted of a combination of H_2O (solvent A), MeOH (solvent B) both acidified with 0.1% of FA and ACN (solvent C). Solvent C percentage was kept constant at 3% during the whole analysis, while the percentage of solvent B varied as follows: 0-2 min: 0% B, 2-9 min: 0-30% B, 9-12 min: 87% B, 12-18 min 0% B. The separation of the target molecules and their retention time

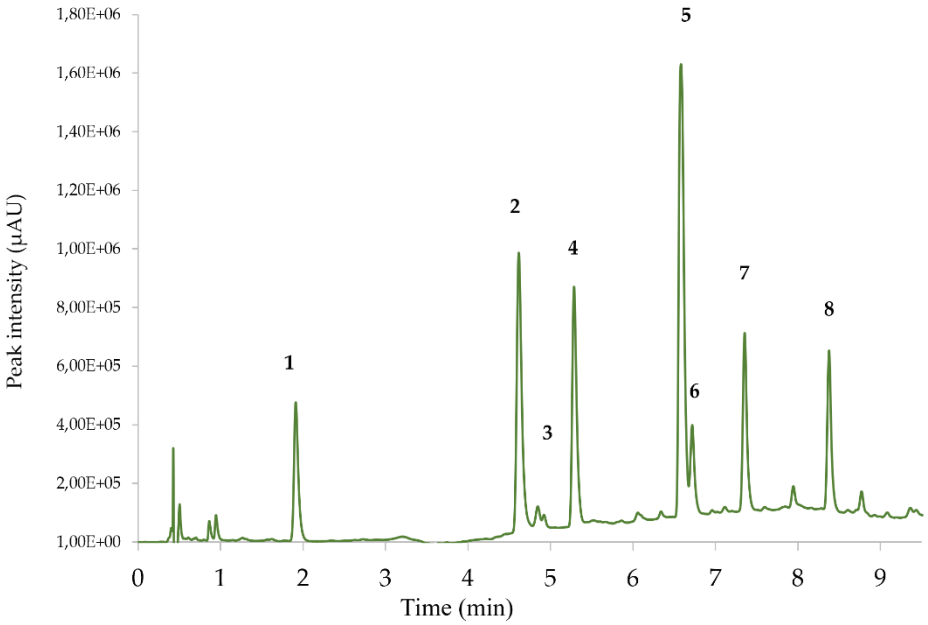

Figure 5: UHPLC separation of green tea extract (210 nm). Peak identifications: (1) GC (Tr=1.91 min), (2) EGC (Tr=4.61 min), (3) Cat (Tr=4.84 min), (4) Caff (Tr=5.29 min), (5) EGCG (Tr=6.58 min), (6) EC (Tr=6.72 min), (7) GCG (Tr=7.35 min), and (8) ECG (Tr=8.38).

(Tr min) is presented in **Figure 5**. Detection and quantification were done at 210 nm,

which allowed for the identification of all catechins, especially GCG and ECG. In addition, Gallic acid standard was eluted using this method (Tr=0.83 min), however it was not detected in SFE extracts analyzed during this study.

The identification of peaks 2, 3, 4, 5, 6 and 8 was done by the means of standards, as for peaks 1 and 7 it was estimated according to the compound's retention behavior in liquid chromatography according to the literature [6-18]. As usually found in previous papers, the retention order of catechins followed the classical pattern GC/EGC/C/EGCG/EC/GCG/ECG. As expected, the main compounds were the epi forms, EGC (peak 2) and EGCG (peak 5), and despite the high UV absorbance of many compounds at 210 nm, some coelutions of matrix peaks were observed.

Quantitative analyses of caffeine (Caff) were performed by injecting a standard at 11 different concentrations from 0.001 mg/mL to 0.5 mg/mL. The calibration curve was obtained at 210 nm ($y = 3E+07x + 378990$, $R^2 = 0.9917$), the equation was used to estimate the concentrations from the peak areas of Caff (y = concentration; x = peak area).

Quantification of EGCG and the total catechins was obtained in the same manner. It was performed by injecting a standard at 9 different concentrations from 0.001 mg/mL to 0.2 mg/mL. The calibration curves were obtained at 210 nm. The EGCG equation ($y = 4E+07x$ 188476, $R^2 = 0.9885$) was used to estimate the concentrations from the peak areas of EGCG. As for the other catechins the concentrations were quantified as an equivalent to EGCG. This approximation was possible due to their structural similarities and the similar molar absorption coefficient (ε) at 210 nm, which was studied from the spectra of standards provided by the diode-array detector.

3.6 Sample treatment

To compare and determine the extraction yields for the target compounds, all extracts were diluted in volumetric flasks of 10 mL or 20 mL with ethanol, to have a controlled dilution volume. The extracts were then filtered using a 0.45 µm PTFE syringe filter from Agilent Technologies (Les Ulis, France), and diluted 4 times in the stabilization solvent consisting of H_2O:ACN 9:1 *v:v* with EDTA and AA at a concentration of 0.25 mg/mL each. This stabilization solvent was used for several reasons: to avoid any degradation of the molecules (EDTA) [37], to homogenize and avoid any precipitation of the extracts during the analysis (AA) [38] and to adapt the injection solvent to the beginning of the gradient elution (ACN). This was also validated by a study of stability done in our laboratory, where the concentration of extracts was assessed during 12 days at 5°C with pure water or with the stabilization solvent. It showed that the

extracts diluted in water degraded by 50% and 12% for EGCG and catechins respectively. However, with the stabilization solvent the degradation after 12 days in the fridge was only 10% and 6% for EGCG and catechins respectively. Caffeine showed minimal degradation in both solvents.

The peak areas and concentrations were later normalized for all data treatment (**equation 2**) to the concentrations of the target compounds per gram of biomass used (Y).

$$Y(mg/g) = concentration\ of\ diluted\ extracts\ (mg/mL) \times dilution\ flask\ volume\ (mL) \times 4 \quad \textbf{(2)}$$

3.7 Experimental design and statistical analysis

Ellistat software (Annecy, Auvergne-Rhône-Alpes) was used for the experimental design and data analysis. A Box–Behnken design (BBD) with response surface methodology (RSM) was chosen to establish the model and to determine the response pattern. It was used to optimize the supercritical fluid extraction of target compounds from green tea leaves. The three independent factors used in this study were temperature (X_1), total modifier percentage (X_2) and water percentage added as an additive to the modifier (X_3), with three levels coded (+1) for the highest, (0) for the middle and (-1) for the lowest level.

The same software was employed for the graphical analysis of the data and the regression model. The analysis of the variance using ANOVA was used to determine the significance of the independent variables on the response. This was performed using statistical values like Fisher's test (F value), significant factors were defined by a p-value < 0.05. Correlation coefficient (R2) and adjusted correlation coefficient (adj R2) were employed to assess the fitness of the design model. To visualize the interaction between the variables, heat-map plots were utilized. Optimal extraction conditions were determined by solving the regression equation that represented the predictive model.

Flow rate and plant mass were kept constant in all SFE experiments. The dependent response or output (Y) was the sum of the yield of all target compounds (mg/g) for all three fractions. The experiments were randomized to maximize the effect of the variability in the response. Five replicates at the middle experimental conditions (X_1=0,

X_2=0 and X_3=0) of the design were conducted to evaluate the experimental repeatability. The standard deviation (SD%) was estimated to confirm the reproducibility of the extraction model, in consequence the data were represented as mean values ± standard deviation.

The polynomial **Equation 3** represents the relationship between the response and the three independent variables (X_1, X_2 and X_3).

$$Y\ (mg/g) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad \textbf{(3)}$$

Where Y = predicted response; β_0 = the regression model constant; β_1, β_2 and β_3 = coefficients of each variable; β_{11}, β_{22} and β_{33} = quadratic coefficients and X_1, X_2 and X_3 represent the variables : temperature, modifier percentage and water percentage in modifier respectively.

4. Conclusion

Overall, the supercritical extraction of polar compounds from green tea leaves (caffeine and polyphenols) was optimized by means of a design of experiments. It validated the benefits of SC-CO_2 mixed with polar solvents, mainly EtOH:H_2O for the high-yield isolation of extracts rich in polar compounds. Our results demonstrated that modifier's percentage and especially water percentage were the most influent parameters to increase the total yield of the target molecules of green tea, while the temperature had no significant effect. The optimized conditions allowed for the recovery of 90.10 mg/g of the compounds of interest.

The comparison between SFE and UAE showed that modified SC-CO_2 extraction is an effective extraction technique for the recovery of green tea leaves target compounds, that could lead to a reduction in the total solvent consumption from an industrial point of view when working with greater amount of plant. Our results show that, for the same composition of organic solvent used with both extraction methods (EtOH:H_2O 80:20 *v:v*), SFE presented an increase in the extraction yields of 72.11, 100.7 and 79.68% for Caffeine, EGCG and total catechins respectively.

A selective extraction of caffeine was also achieved, which allowed to recover 40.55% of extractible caffeine in a first step. However, a small loss of 5.37% of total catechin recovery was observed after the decaffeination step, compared to the extracted amount obtained directly from the optimal extraction conditions (experiment n°12), and what is more, an increase in the extraction duration, from 15 to 45 min for this second step.

In conclusion, SFE allowed for the recovery of high yield extracts with a reduced organic solvent consumption and a partial selective extraction of caffeine. Thus, the proposed approach can be applied not only to provide a caffeine-rich extract but additionally a catechin-rich one, both for various cosmetic and health uses.

5. References

[1] R. Ito, A. Yamamoto, S. Kodama, K. Kato, Y. Yoshimura, A. Matsunaga, H. Nakazawa, Food Chem. 2003, 83 (4), 563–568. DOI: 10.1016/S0308-8146(03)00154-7.

[2] H. S. Park, H. J. Lee, M. H. Shin, K.-W. Lee, H. Lee, Y.-S. Kim, K. O. Kim, K. H. Kim, Food Chem. 2007, 105 (3), 1011–1017. DOI: 10.1016/j.foodchem.2007.04.064.

[3] H. S. Park, H.-K. Choi, S. J. Lee, K. W. Park, S.-G. Choi, K. H. Kim, J. Supercrit. Fluids. 2007, 42 (2), 205–211. DOI: 10.1016/j.supflu.2007.03.002.

[4] K.-J. Huang, J.-J. Wu, Y.-H. Chiu, C.-Y. Lai, C.-M. J. Chang, J. Agric. Food Chem. 2007, 55 (22), 9014–9020. DOI: 10.1021/jf071161q.

[5] W.-J. Kim, J.-D. Kim, J. Kim, S.-G. Oh, Y.-W. Lee, J. Food Eng. 2008, 89 (3), 303–309. DOI: 10.1016/j.jfoodeng.2008.05.018.

[6] D. Guillarme, C. Casetta, C. Bicchi, J.-L. Veuthey, J. Chromatogr. A. 2010, 1217 (44), 6882–6890. DOI: 10.1016/j.chroma.2010.08.060.

[7] Z. Li, D. Huang, Z. Tang, C. Deng, J. Sep. Sci. 2010, 33 (8), 1079–1084. DOI: 10.1002/jssc.200900647.

[8] M. Bedner, D. L. Duewer, Anal. Chem. 2011, 83 (16), 6169–6176. DOI: 10.1021/ac200372d.

[9] M. Naldi, J. Fiori, R. Gotti, A. Périat, J.-L. Veuthey, D. Guillarme, V. Andrisano, J. Pharm. Biomed. Anal. 2014, 88, 307–314. DOI: 10.1016/j.jpba.2013.08.054.

[10] L. Xu, G. Xia, Z. Luo, S. Liu, LWT. 2019, 102, 52–57. DOI: 10.1016/j.lwt.2018.12.008.

[11] M.-K. Lee, H.-W. Kim, S.-H. Lee, Y. J. Kim, G. Asamenew, J. Choi, J.-W. Lee, H.-A. Jung, S. M. Yoo, J.-B. Kim, Eur. Food Res. Technol. 2019, 245 (5), 997–1010. DOI: 10.1007/s00217-018-3201-6.

[12] P. R. Das, Md. T. Islam, S.-H. Lee, M.-K. Lee, J.-B. Kim, J.-B. Eun, LWT. 2020, 127, 109440. DOI: 10.1016/j.lwt.2020.109440.

[13] C. T. Scoparo, L. M. de Souza, N. Dartora, G. L. Sassaki, P. A. J. Gorin, M. Iacomini, J. Chromatogr. A. 2012, 1222, 29–37. DOI: 10.1016/j.chroma.2011.11.038.

[14] P. Svoboda, H. Vlčková, L. Nováková, J. Pharm. Biomed. Anal. 2015, 114, 62–70. DOI: 10.1016/j.jpba.2015.04.026.

[15] P. Šilarová, L. Česlová, M. Meloun, Food Chem. 2017, 237, 471–480. DOI: 10.1016/j.foodchem.2017.05.133.

[16] M. Sökmen, E. Demir, S. Y. Alomar, J. Supercrit. Fluids. 2018, 133, 171–176. DOI: 10.1016/j.supflu.2017.09.027.

[17] A. A. Rahim, S. Nofrizal, B. Saad, Food Chem. 2014, 147, 262–268. DOI: 10.1016/j.foodchem.2013.09.131.

[18] K. Wei, L. Wang, J. Zhou, W. He, J. Zeng, Y. Jiang, H. Cheng, Food Chem. 2011, 125 (1), 44–48. DOI: 10.1016/j.foodchem.2010.08.029.

[19] X. Jun, Z. Shuo, L. Bingbing, Z. Rui, L. Ye, S. Deji, Z. Guofeng, Int. J. Pharm. 2010, 386 (1), 229–231. DOI: 10.1016/j.ijpharm.2009.10.035.

[20] S. M. Ghoreishi, E. Heidari, J. Supercrit. Fluids. 2012, 72, 36–45. DOI: 10.1016/j.supflu.2012.07.015.

[21] E. D. Pauli, G. B. Malta, P. M. Sanchez, I. C. Moreira, I. S. Scarminio, Anal. Chem. Res. 2014, 2, 23–29. DOI: 10.1016/j.ancr.2014.08.001.

[22] J. J. Kellogg, E. D. Wallace, T. N. Graf, N. H. Oberlies, N. B. Cech, J. Pharm. Biomed. Anal. 2017, 145, 604–610. DOI: 10.1016/j.jpba.2017.07.027.

[23] P. R. Das, J.-B. Eun, Food Chem. 2018, 253, 22–29. DOI: 10.1016/j.foodchem.2018.01.080.

[24] H. Wang, L. Chen, Y. Xu, Q. Zeng, X. Zhang, Q. Zhao, L. Ding, LWT - Food Sci. Technol. 2011, 44 (6), 1490–1495. DOI: 10.1016/j.lwt.2011.01.015.

[25] J. Xi, L. Yan, L. He, Sep. Purif. Technol. 2014, 133, 155–159. DOI: 10.1016/j.seppur.2014.06.043.

[26] D. V. Bermejo, J. A. Mendiola, E. Ibáñez, G. Reglero, T. Fornari, Food Bioprod. Process. 2015, 96, 106–112. DOI: 10.1016/j.fbp.2015.07.008.

[27] S. QingLei, H. Shu, Y. JianHui, L. JianLiang, Z. XinQiang, L. YueRong, J. Med. Plants Res. 2010, 4 (12), 1161–1168.

[28] H. S. Park, N. G. Im, K. H. Kim, LWT - Food Sci. Technol. 2012, 45 (1), 73–78. DOI: 10.1016/j.lwt.2011.07.023.

[29] B. Bahar, E. Pelvan, İ. Hasbay, C. Alasalvar, J. Supercrit. Fluids. 2013, 82, 116–121. DOI: 10.1016/j.supflu.2013.07.002.

[30] R. Joshi, G. D. K. Babu, A. Gulati, Food Res. Int. 2013, 53 (2), 693–703. DOI: 10.1016/j.foodres.2012.12.050.

[31] D. V. Bermejo, E. Ibáñez, G. Reglero, T. Fornari, J. Supercrit. Fluids. 2016, 107, 507–512. DOI: 10.1016/j.supflu.2015.07.008.

[32] D. M. Sevillano, L. A. M. van der Wielen, N. Hooshyar, M. Ottens, Food Bioprod. Process. 2014, 92 (2), 192–198. DOI: 10.1016/j.fbp.2014.02.002.

[33] S. Atwi-Ghaddar, E. Destandau, E. Lesellier, J. CO2 Util. 2023, 70, 102440. DOI: 10.1016/j.jcou.2023.102440.

[34] T. Lefebvre, E. Destandau, E. Lesellier, J. Chromatogr. A. 2021, 1639, 461709. DOI: 10.1016/j.chroma.2020.461709.

[35] O. Wrona, K. Rafińska, J. Walczak-Skierska, C. Możeński, B. Buszewski, Molecules. 2019, 24 (24), 4608. DOI: 10.3390/molecules24244608.

[36] S. Ghosh, D. Chatterjee, S. Das, P. Bhattacharjee, Ind. Crops Prod. 2013, 47, 78–85. DOI: 10.1016/j.indcrop.2013.02.030.

[37] S. Sang, M.-J. Lee, Z. Hou, C.-T. Ho, C. S. Yang, J. Agric. Food Chem. 2005, 53 (24), 9478–9484. DOI: 10.1021/jf0519055.

[38] K. O. Chu, C. C. Wang, C. Y. Chu, M. S. Rogers, K. W. Choy, C. P. Pang, J. Chromatogr. B. 2004, 810 (2), 187–195. DOI: 10.1016/j.jchromb.2004.07.031.

Part III:

Punica granatum L

In this third and final part of chapter one, the extraction of polar compounds found in pomegranate pericarp was of interest. The pericarp is a by-product of the food industry, it is rich with proven activity of polyphenols that offers positive effects on human general health. To standardize the SFE methodology of polar compounds, the same experimental model used for green tea was applied to pomegranate with different targeted compounds. This will allow us to see if the model is valid on different biomasses and can allow us to reproduce a fast and simple extraction of the compounds of interest. The selectivity of extraction was not of interest in this section; however, after the drying of the extracts their dissolution presented an issue during sample preparation, this can be translated into a concern in cosmetic handling and needed to be addressed. Therefore, a screening of cosmetic approved solvents will be presented, in the aim of bypassing a full evaporation step and recovering pre-formulated extracts ready to be incorporated in the formulations.

Part III: Integrated supercritical fluid extraction and pre-formulation process of *Punica granatum* L. pericarp polar compounds.

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Key words

Eco-extraction, food waste valorization, modified supercritical carbon dioxide, Box-Behnken design (BBD), cosmetic ingredient.

Abstract

Pomegranate (*Punica granatum* L.) is a widely used fruit in the dietary supplement industry, due to its richness in bioactive compounds. In this study, an experimental design was applied to optimize supercritical fluid extraction (SFE) of polar compounds of interest (ellagic acid and punicalagins) known for antioxidant and skin care properties from pomegranate's pericarp. The effects of temperature, modifier percentage, and water additive percentage added in the modifier were explored through a Box-Benken design, followed by a study of the extraction kinetics. The result indicated that 40°C, 20% EtOH:H_2O 80:20 *v:v* with an extraction duration of 60 min allowed for the highest recovery of the above-mentioned molecules (19.59 mg/g). Due to solubilization issues encountered by the extract, a screening of cosmetic solvents was carried out to solubilize SFE pomegranate extracts and a composition of Gly:H_2O 80:20 *v:v* was selected. Furthermore, an integrated SFE pre-formulation process of pomegranate pericarp extracts was elaborated, this allowed for the recovery of the extracts in cosmetic solvent avoiding a full evaporation. Finally, the stability of the preformulated extracts was evaluated and showed high stability for over 3 months at 5°C .

1. Introduction

Pomegranate (*Punica granatum* L.) is an indigenous fruit to a region that spans from what is now modern-day Iran to northern India. It has been grown and appreciated for its fruits in this area for thousands of years. Pomegranates have a long history across multiple cultures including the Phoenicians, Greeks, Arabs, and Romans [1,2].

Over the last ten years, research has focused on the extraction of several parts from the fruit. Preventive and attenuating effects against a variety of chronic and health/life threatening illnesses have been depicted, including cancer [3], type-2 diabetes [4] and cardiovascular diseases [5]. It's noteworthy to mention that the pomegranate's edible portion is not the only part containing all the above- mentioned nutraceutical benefits. Research conducted on the non-edible parts of the fruit and tree (such as the seeds, peel, flowers, buds, bark, and leaves) demonstrated that in some cases they can contain even higher concentrations of some nutritionally important and biologically active substances Compared to the edible fruit [6].

The pomegranate peel or pericarp is considered as a waste product after the juicing process of the fruit. Even though they are still edible, they are typically discarded during juice preparation, therefore they provide a significant difficulty in an industrial setting that is committed to environmental sustainability. This makes them a candidate for waste valorization, especially for the extraction of natural compounds that could later be used as nutraceuticals products, cosmetics ingredients or as natural preservatives for the food industry [7].

Since the peel and membrane layers of the pomegranate contain more phytochemicals than the edible meat pulp, they are a potential source of bioactive compounds [8–10]. According to studies, the peel extract have strong radical scavenging and antioxidant activity such the DPPH, superoxide ion, hydroxyl, and peroxyl radicals as well as hydrogen peroxide [11–13].

Studies have showed that pomegranate pericarp extracts (PPE) contain many polar compounds, like polyphenols such as chlorogenic, caffeic, syringic, sinapic, p-coumaric, ferulic, vanillic, ellagic, gallic and cinnamic acids [14]. Pomegranate peel cultivar from China was found to have 201.3 and 8.91 mg/g of ellagic acid and gallic acid, respectively [15]. Content in ellagic acid can go up to 50% of the total phenolic compounds depending on the varieties [16]. Additionally, flavonoids like catechin, epicatechin, quercetin, anthocyanins, and procyanidins have been detected in PPE.

Flavonoid composition changes as the fruit develops, and the concentration of flavanols and flavones varies between cultivars [17].

This composition may vary according to the freshness of the biomass, as certain studies shows the effect of treatment and packaging of the contents of some flavonoids like rutin, it was identified as the predominant flavonoids in fresh PPE but due to its high instability during prolonged cold storage (stored for four months by packing fruits inside a polyliner bag) it's content decreased by 65% [18].

High molecular weight ellagitannins are water soluble compounds that hydrolyse to produce a variety of biologically relevant by-products [19]. Punicalagins (PNGs) are a predominant pomegranate-specific hydrolysable ellagitannins that can transform to punicalin, ellagic and gallagic acid [19–21]; it was demonstrated that PNGs are responsible for more than 50% of the fruit extract antioxidant activity [22]. This bioactivity can be credited to hydrolyse process into ellagic acid (EA) both *in-vivo*, as well as their ability to cross the mitochondrial membrane *in-vitro* [23,24]. PNGs can be found naturally as two reversible α- and β-anomers [25–27] both were attributed with antibacterial activity [26,28], with β-anomer being the major form [27,29].

The extraction of polar compounds from *Punica granatum* L. pericarp have been described in literature; mostly using traditional maceration extraction and conventional solvents like methanol, ethanol, water and hydroalcoholic mixtures [30–34]. These techniques have some disadvantages, mainly extraction time that can last days in some cases, large solvent consumption, that will later require additional evaporation process. This is why in this study, the use of supercritical fluid extraction has been explored, to determine the feasibility and the extraction yield of polar compounds of interest found in PPE mainly ellagic acid (EA) and punicalagins (PNGs) with both form PNG_α and PNG_β. Besides, the extraction of natural plant compounds using supercritical fluids is an environmentally friendly approach compared to traditional extraction techniques. The supercritical carbon dioxide (SC-CO_2) is an intermediate state between a gas and a liquid; it exhibits the diffusion, viscosity, and surface tension of a gas as well as the density and solvation force of a liquid. Other benefits include the extracts' spontaneous concentration resulting from the CO_2's depressurization at atmospheric pressure, this generates extracts that require minimal refining and post extraction treatments. Moreover, the use of dimethyl sulfoxide (DMSO) to dissolve PPE was repeatedly mentioned in literature [19,30,32,35,36] in some cases to prevent the precipitation of extracts. Even though DMSO is considered as a non-toxic chemical and has applications in various fields; it's not suitable for

cosmetic use, in consequence PPE require the use of alternative solvent appropriate for cosmetic formulation.

In this study's framework, SFE of *Punica granatum* L. pericarp was optimized by the means of experimental design. Besides, a screening of stabilization solvents and a development of an integrated SFE- pre-formulation process of PPE as a cosmetic ingredient was developed.

2. *Materials and methods*

2.1.Plant material

The plant material for pomegranate (*Punica granatum* L.) consisted of the dried pericarp, milled into fine light orange yellow powder, supplied by PMA 28 (Varize, France). The biomass was stored at room temperature in an airtight container.

2.2.Chemical and reagents

CO_2 gas was supplied by Air Liquide (Fleury-les-Aubrais, France). HPLC-grade methanol (MeOH), ethanol (EtOH) and dimethyl sulfoxide (DMSO) were used for the mobile phase, extraction and sample dilution were supplied by VWR (Fontenay-sous-Bois, France). Formic acid (FA), pentanediol, glycerol (Gly), 1,3-propanediol and diatomaceous earth were obtained from Sigma-Aldrich (Merck, Semoy France). Propylene glycol (PG) and dipropylene glycol (DPG) were provided by Alban Muller (Fontenay-sur-Eure, France). Ultra-pure water was purified by using an Milli-Q® EQ 7000, from Sigma-Aldrich with a resistance > 18 MΩ.cm. Ellagic acid standard used to identify and quantify the target molecules was provided by Extrasynthese (Genay, France).

2.3.UHPLC-DAD analysis

All the extract analyses were preformed using a Nexera-LC40 system of Shimadzu Corporation (Kyoto, Japan); this system is equipped with a photodiode array (PDA) detector (SPD-40), a solvent Delivery Unit (LC-40), an auto-sampler (SIL-40), a column oven (CTO-40) and a system Controller (SCL-40). All chromatograms were recorded on LabSolutions LC-UV 5.97 SP1 version (Shimadzu Corporation, Kyoto, Japan). A Cortecs C18 (100 ×3.0 mm) column coupled to a Cortecs C18 VanGuard Cartridge (90 Å, 5 ×2.1 mm) both packed with 2.7 μm superficially porous particles, from Waters

Corporation (Milford, Massachusetts, United States) were used for the analysis of all extracts. The column temperature was maintained at 30°C. The injection volume was 5 µL and the automated sampler was kept at 10°C. The flow rate was maintained at 1 mL/min. Equilibration time between two injections was 5 min. The separation is presented in **Figure 1**.

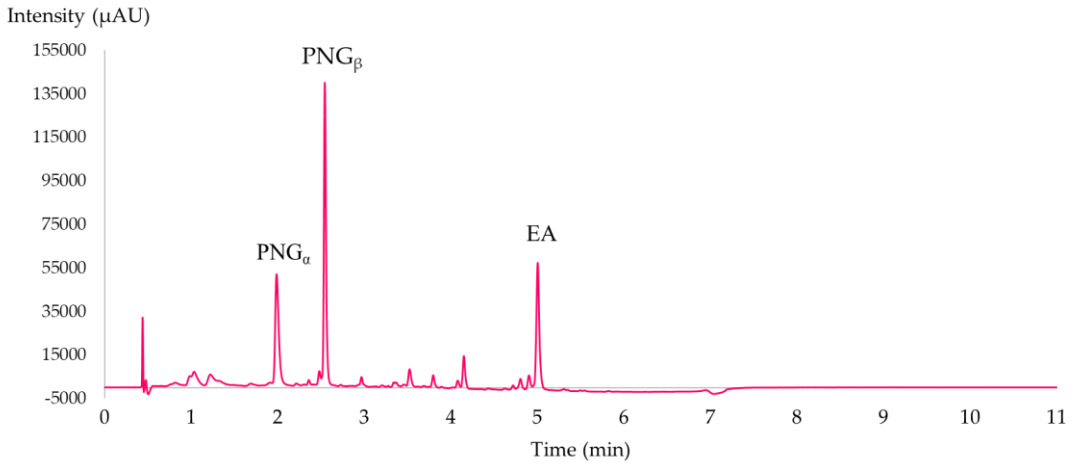

Figure 1: UHPLC-DAD analysis of polar extracts of *Punica granatum* L. pericarp diluted in DMSO:EtOH:H2O at 366 nm. PNG1 (RT= 1.83 min), PNG2 (RT= 2.45 min) and EA (RT= 4.88 min).

The total time of each analysis was 12 minutes, the mobile phase consisted of a combination of acidified water with 0.1% of FA (solvent A) and MeOH (solvent B).

The elution gradient employed is the following for solvent B (%): 0 - 0.5 min: 5%; 0.5-6 min: 5-65%; 6-6.1 min: 65-5% and 6.1-11 min: 5%. Detection of the compounds was done using a PDA detector set at 366 nm (maximal absorbance of EA) and 380 nm (maximal absorbance PNGs).

Quantitative analyses of EA and PNGs were performed by injecting an ellagic acid standard at 11 different concentrations from 0.0005 mg/mL to 0.9 mg/mL. The calibration curve was obtained at 366 nm ($y=10^7x+4916.4$, $R^2 = 0.9993$), it was used to estimate the concentrations from the peak areas of EA, PNGs (concentrations were quantified as an equivalent to EA, this was possible due to their similar molar absorption coefficient (ε) at their respective maximal absorbance wavelength). In all data presented, the extraction yield was defined as the yield of EA and PNGs (in equivalent to EA), quantified in UHPLC per one gram of plant material (mg/g of biomass). As for the PNGs, both anomers were separated and the identification was done according their PDA spectra and validated by the literature were the anomer α

is eluted before the β in reversed phase LC with the latter being the major punicalagin [19,27,29].

2.4.Supercritical fluid extraction (SFE)

A Waters MV-10 ASFE® was used for all the extractions; a 5-mL stainless steel extraction vessel was prepared using 2 g of plant powder: diatomaceous earth mixture 1:1 *w:w* . To filter the extracts and fill the extraction cell entirely, cotton (100%) was placed at the top and bottom of the extraction cell. The extraction duration for the experimental design was 30 min with a flow rate of 3 mL/min for 2 minutes at the beginning of each run, to reach the required pressure fast. At the end of the 2 min, the flow rate was reduced to 1.5 mL/min for a total of 28 min, this was done to increase the repeatability of the extraction. The percentage of the modifier was calculated according to the total extraction flow rate. After the DoE optimization, the SFE kinetics were carried out at a of 3 mL/min flow.

The use of different modifier percentages resulted in the variation of the volume of the extracts for each experimental point. The resolve this variation and compare the extraction yield of each experimental point, all extracts were evaporated using a nitrogen gas stream, then resolubilized in 4 mL with a mixture of DMSO:EtOH:H_2O 2:1:1 *v:v:v.* This solvent composition was necessary to solubilize the extracts and obtain a homogenous stable mixture that will not precipitate in time. The extracts were diluted with water 3 times, to decrease the viscosity of the injection solvent, adapt the solvent composition to the elution gradient and in consequence avoid any deformities in the PNGs peak shape.

2.5.Ultrasounds assisted extraction (UAE)

The UAE was performed using a Branson 3510 (Bransonic® ultrasonic, Semoy, France) bath purchased from Sigma-Aldrich with a power equal to 130W. 1 g of pomegranate pericarp powder was mixed with the extraction solvent in a 50 mL falcon tube.

The extraction solvents consisted of EtOH:H_2O *v:v* mixtures in 80:20 and 70:30 ratio with a volume of 36 mL. The biomass and solvent were combined using a vortex at 10000 rpm for 60 seconds, they were later sonicated at room temperature for 60 min. Finally, all extracts were centrifuged at 10000 rpm for 10 min at 25°C and filtered using a 0.45 μm polyvinylidene difluoride (PVDF) syringe filter from Agilent Technologies (Les Ulis, France) and directly analyzed without evaporation. For UHPLC analysis, they were subsequently diluted with water.

2.6.Experimental design and Statistical analysis

Ellistat software 6.4 2020/11 version (Annecy, Auvergne-Rhône-Alpes) was used for the experimental design and data analysis. A Box–Behnken design (BBD) from response surface methodology (RSM) was chosen to establish the model and to determine the response pattern (**Table 1**).

Table 1: Design matrix for the SFE design of experiment (BBD) and responses for polar compounds of interest from *Punica granatum* L. pericarp.

Experiments	Coded levels			Response (Y) : concentration in mg/g of biomass after 30 min extraction			
	X_1	X_2	X_3				
	Temperature (°C)	Modifier(%)	H_2O in modifier (%)	Ellagic acid (EA)	Punicalagine-α (PNG_α)	Punicalagine-β (PNG_β)	Total compounds
1	40 (-1)	10 (-1)	10 (0)	0.000	0.000	0.000	0.000
2	80 (+1)	10 (-1)	10 (0)	0.148	0.002	0.008	0.157
3	40 (-1)	30 (+1)	10 (0)	1.939	0.014	0.232	2.185
4	80 (+1)	30 (+1)	10 (0)	0.838	0.021	0.021	0.880
5	40 (-1)	20 (0)	0 (-1)	0.679	0.015	0.031	0.725
6	80 (+1)	20 (0)	0 (-1)	0.648	0.000	0.003	0.651
7	**40 (-1)**	**20 (0)**	**20 (+1)**	**2.104**	**3.111**	**6.690**	**11.905**
8	80 (+1)	20 (0)	20 (+1)	2.111	2.456	5.391	9.958
9	60 (0)	10 (-1)	0 (-1)	0.082	0.003	0.009	0.095
10	60 (0)	30 (+1)	0 (-1)	0.932	0.349	0.712	1.994
11	60 (0)	10 (-1)	20 (+1)	0.033	0.006	0.018	0.058
12	60 (0)	30 (+1)	20 (+1)	1.579	0.926	1.969	4.474
13	60 (0)	20 (0)	10 (0)	0.718	0.001	0.003	0.722
14	60 (0)	20 (0)	10 (0)	0.756	0.002	0.006	0.764
15	60 (0)	20 (0)	10 (0)	1.038	0.002	0.006	1.046
16	60 (0)	20 (0)	10 (0)	1.033	0.010	0.023	1.066
17	60 (0)	20 (0)	10 (0)	0.402	0.008	0.017	0.427

It was used to optimize the supercritical fluid extraction of targeted polar compounds from pomegranate pericarp. The three factors used in this study were temperature (X_1), total modifier percentage (X_2) and water percentage added as an additive to the modifier (X_3), with three levels coded (+1) for the highest, (0) middle and (-1) the lowest level.

The software was as well employed for the graphical analysis of the data and the regression model. The analysis of the variance using ANOVA was used to determine the significance of the independent variables on the response. This was performed using statistical values like Fisher's test (F value), significant factors were defined by a p-value < 0.05. Correlation coefficient (R^2) and adjusted correlation coefficient (adj R^2) were employed to assess the fitness of the design model. To visualize the effect of the variation between the factors heat-map plots were used. Optimal extraction conditions were determined by solving the regression equation representing the predictive model. To determine the presence of an outlier in the data set, Dixon statistical test was applied to the data set, and the analysis of residues between the experimental and calculated data were evaluated. The dependent response or output (Y) was the sum of the yield of EA and PNGs (mg/g). The experiments were randomized to maximize the effect of the variability in the response. Five replicates at the middle experimental conditions (X_1 = 0, X_2 = 0, and X_3 = 0) of the design were conducted to evaluate the experimental repeatability. The relative standard deviation (RSD) was estimated to confirm the reproducibility of the extraction model. The second-degree polynomial **Eq. (1)** represents the relationship between the response and the three independent variables (X_1, X_2, and X_3).

$$Y=\beta_0+ \beta_1 X_1+ \beta_2 X_2+ \beta_3 X_{2+} \beta_{11} X_1^2+ \beta_{22} X_2^2+ \beta_{33} X_3^2 \quad \textbf{(1)}$$

Where Y = predicted response; β_0 = the regression model constant; β_1, β_2 and β_3 = coefficients of each variable; β_{11}, β_{22} and β_{33} = quadratic coefficients and X_1, X_2 and X_3 represent the variables temperature, modifier percentage and water percentage in modifier respectively.

The interaction terms X_1X_2, X_1X_3 and X_2X_3 were evaluated during the analysis of the model and their addition decreased the adj R^2 suggesting overfitting therefore justifying their exclusion from the model's equation.

3. Results and discussion

3.1.SFE optimization using BBD

The SFE of pomegranate pericarp polar compounds was optimized using a BBD. The temperature (40, 60 and 80°C) was selected due to its ability to influence the solubility of compounds in the SC-CO_2, the modifier percentage (10, 20 and 30%) and water percentage added as an additive in the modifier (0, 10 and 20%) were chosen for to their capacity to increase the polarity of the extraction phase [37] (**Table 1**). The influence of pressure variation on the yield of EA and PNGS from *Punica granatum* L. was not explored in this study; and was kept at constant value of 15 MPa. This is based on our previous work treating the extraction of flavonoids from black locust heartwood using supercritical fluid extraction. Indeed, to extract polar compounds, the use of high percentages of modifier and sometimes the use of water as an additive is required in SFE; this may lead to high density of the extraction solvent. In these conditions, the pressure influence on the density of the extraction phase was minimal in the pressure range studied (10 to 20 MPa) [38]. Moreover, the influence of pressure in SFE with pure carbon dioxide have been extensively reported in the literature, it has been noted that extractions done at high pressures had a higher recovery of volatile fractions and in some cases a lower recovery of non-volatile fractions [39,40].

3.1.1. Statistical analysis, model, and factor significance

The determination coefficient (R^2) for the quadratic model was equal to 0.77 and the adjusted determination coefficient (adj R^2) was equal to 0.63. This indicated that the model was unable to describe a small part (23%) of the total response variation. The theoretical model demonstrated a satisfactory fit with the experimental data, as seen by the R^2 value > 0.7, which revealed a good correlation between expected and experimental response values. In addition, the Dixon statistical test was employed as well and no outliers were identified, this was confirmed by the analysis of residues and the distribution of the measured versus the calculated values. Five repetitions of the central level conditions were performed to evaluate the repeatability of the SFE application to the pomegranate pericarp biomass, the relative standard deviation (RSD) of the response was 32.73%, that can be related to the complexity of the matrix.

Interaction factors (X_1X_2, X_1X_3, and X_2X_3) were evaluated during the analysis of the model. They were excluded as they demonstrated signs of data overfitting where the

R^2 increased and adj R^2 decreased with the addition of each interaction term. Therefore, to avoid overfitting issues, only the quadratic terms were evaluated in this mode. The relationship between the yield of total EA and PNGs (Y mg/g) and the three variables (X_1, X_2, *and* X_3) is presented in **Eq. (2).**

$$Y\ (mg/g) = 10.14 - 0.6431 \times X_1 + 0.9461 \times X_2 - 0.2988 \times X_3 + 0.005194 \times X_1^2 - 0.02077 \times X_2^2 + 0.02927 \times X_3^2 \quad \textbf{(2)}$$

The summary of the analysis of variance (ANOVA) for the polynomial model is presented in **Table 2**. The probability related to F-value and p-value were used to determine the significance of each variable.

Table 2: ANOVA for response surface regression model for total compounds of interest (EA and PNGs) yield from *Punica granatum* L. pericarp SFE extracts

Source	DF	SS	MS	F value	p-value	Contribution (%)	Conclusion
Regression model	6	148.42	24.737	5.4351	0.0097		Very significant
X_1- Temperature (°C)	1	1.2548	19.068	4.1894	0.0679	11.3	Limit
X_2- Modifier (%)	1	10.633	22.805	5.0107	0.0491	13.6	Significant
X_3- H_2O (%)	1	65.723	8.3059	1.8249	0.2065	4.94	Not significant
X_1^2	1	19.121	18.173	3.9929	0.0736	10.8	Limit
X_2^2	1	15.613	18.165	3.9910	0.0737	10.8	Limit
X_3^2	1	36.078	36.078	7.9267	0.0183	21.5	Significant
Residuals	10	45.514	4.5514			27.1	
Total	16	193.94					
	Degree of freedom	Sum of squares	Mean square				

The p-value and F-value of the regression model was equal to 0.0097 and 5.4351 respectively, representing a significant regression. The contribution percentage of the equation terms were evaluated to assess the impact of each variable on the model. It demonstrated that within the first-degree terms X_2 had the highest contribution of 13.6%, this represented that the modifier percentage is a significant factor of the model. Furthermore, the X_1 and X_3 percentage added in the modifier had a contribution of 11.3 and 4.94% respectively, indicating a limited and a non-significant influence on the molecules of interest yield. The quadratic terms X_1^2 and X_2^2 were presented with a limited contribution of 10.8%. However, X_3^2 had the highest contribution with 21.5%, representing a significant effect on the extraction yield of EA and PNGs from *Punica granatum* L. pericarp. This result was also validated by an F-value of 7.9267 and a p-value of 0.0183. Consequently, according to the statistical analysis, the most influential factors of the statistical model on the response are the percentage of H_2O added in the modifier and the modifier total percentage.

The computed optimal conditions that allowed the highest extraction yield of both EA and PNGs was 40°C, 22.8% of EtOH:H_2O 80:20 *v:v*, the predicted extraction yield in these conditions was of 9.232 mg/g. The experiment that corresponded the most to these extraction parameters was experiment N°7 with 40°C, 20% of EtOH:H_2O 80:20 *v:v* with an experimental yield of 11.905 mg/g.

The variation between the modifier percentage (X_2) and the temperature (X_1) was presented in **Figure 2a**.

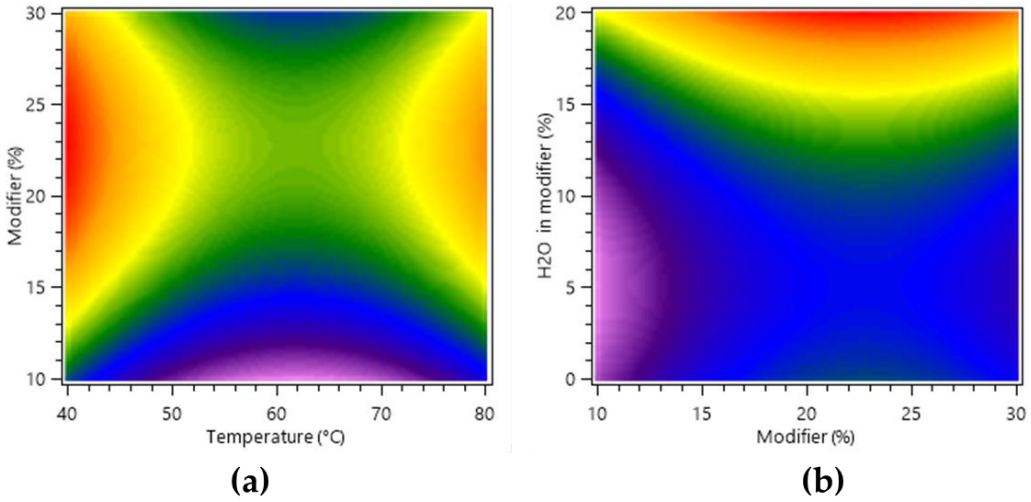

Figure 2: Response surface heat-maps showing the effects of the variation of experimental design factors on the total compounds of interest yield (mg/g) from pomegranate pericarp. (a) Interaction between temperature (X_1) and total modifier percentage (X_2) and (b) interaction between total modifier percentage (X_2) and H_2O% in modifier (X_3).

With X_3 at central level (EtOH:H_2O 90:10 *v:v*) the variation of temperature at 40, 60 and 80°C resulted in a predicted extraction yield of total compounds of 3.278, 0.805 and 2.486 mg/g. This indicated that the temperature had a minor positive effect on the response with an optimal 40°C. The influence of the modifier percentage and H_2O (%) added as an additive to the modifier on the response was described **Figure 2b**, at constant temperature (40°C). The zone with the maximal response was observed when the water percentage was 20% combined with a modifier percentage ranging from 20% to 30%. At a modifier composition of 100% EtOH (without water), increasing the modifier percentage from 10% to 30% slightly raised the yield of total compounds of interest from 0.1095 to 2.415 mg/g. However, using a modifier composition of EtOH:H_2O 80:20 *v:v* and percentages of 10%, 20%, and 30% of the modifier resulted in yields of 5.842, 9.072, and 8.148 mg/g, respectively. Consequently, a combination of 20% H_2O and a modifier percentage in the range of 20% to 30% is required to achieve

high yields of EA and PNGs from PPE. These variations are related to the solubility change of the polar compounds, and perhaps to the modifications in the matrix solvation, which are favored by both the water and the modifier percentage increase in the examined range.

3.1.2. Validation of experimental design and extraction kinetics

The results of the BBD indicated that the optimal extraction conditions were 40°C, 22.8% EtOH:H_2O 80:20 *v:v* with an extraction duration of 30 minutes and a flow rate of 1.5 mL/min. In the aim of assessing the impact of the flow rate on the yield of EA and PNGs and determine the extraction time required to exhaust the molecules of interest from *Punica granatum* L. Four extraction kinetics were performed with a fraction collection every 15 min for two hours. The modifier composition was EtOH:H_2O 80:20 *v:v* and flow rate was 3 mL/min for all extractions. Four experiments were explored in these conditions. The factors investigated were the temperature (40 and 60°C) and the modifier percentages (20 and 30%). This approach allowed to investigate finely the influence of temperature (predicted optimal 40°C) and modifier percentage (predicted optimal 22.8% EtOH:H_2O 80:20 *v:v*) on the extraction yield with a higher flow rate and longer extraction duration. The results of the extraction kinetics for total EA and PNGs yield were presented in **Figure 3**. The first 60 min of the kinetics corresponded to the constant extraction rate (CER) period, at this point the surface area of plant matrix particles is covered with easily accessible solute and mass transfer is dominant [39,41].

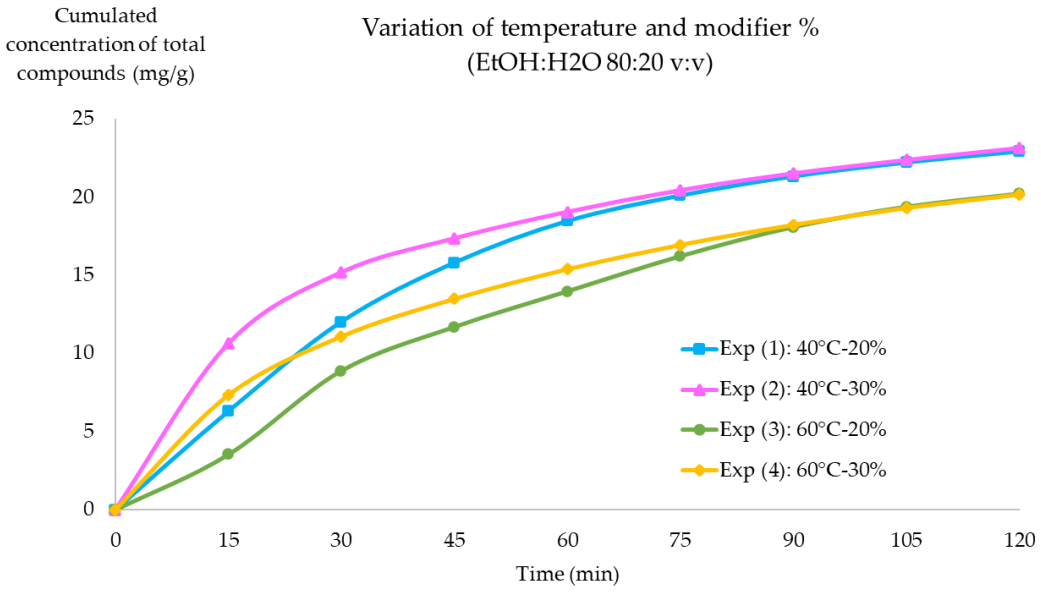

Figure 3: SFE kinetics for the total sum of compounds of interest (EA and PNGs) concentration in mg/g. Constant: 15 MPa, 1 g of plants, 3 mL/min flow rate, modifier composition EtOH:H_2O 80:20 *v:v*.

The fastest extraction rate was the exp n°2 (40°C, 30% modifier) and the slowest was exp n°3 (60°C, 20% modifier). The extraction yield for both conditions at 60 min was 19.04 and 13.96 mg/g respectively. This indicated a 26.68% difference of the yield and validated the influence of lower temperatures for the extraction of the compounds of interest from PPE. As for the exp n°1 (40°C, 20% modifier) and exp n°4 (60°C, 30% modifier) for the first 30 min of extraction both conditions had similar extraction behavior and yields difference of 7.82%. However, after this time the extraction rate of exp n°1 was faster, the difference between exp n°1 and n°4 at 60 min was of 16.68%. Furthermore, a distinct contrast between the experiments carried out at 40 and 60°C was observed, where at 120 min the yield was greater at a temperature of 40°C, independent from the modifier percentage. The extraction yield for exp n°1 and n°2 around 120 min was 22.91 and 23.12 mg respectively as for exp n°3 and n°4 it was of 20.20 and 20.16 respectively.

This validated the calculated results of the temperature on the extraction of *Punica granatum* L. pericarp compounds, where the extraction conducted at 40°C had the superior yield compared to higher temperatures. Besides, even though exp n°2 had the fastest extraction rate in the first 45 min, after 60 min exp n°1 had a similar yield with the use of lower amount of modifier. Therefore, the optimal extraction condition selected was exp n°2 with 40°C and 20% of modifier for 60 min, this extraction condition allowed for the recovery of 18.46 mg/g of compounds of interest from pomegranate pericarp. It was noted that a two-hour extraction resulted in a higher extraction yield of 22.91 mg/g. The second 60 minutes, however, produced a 24.10% yield increase while consuming twice as many resources. Therefore, 60 minutes was sufficient under these conditions to reduce energy and resource consumption, with 40°C and 20% EtOH:H_2O 80:20 *v:v.*

3.2.Comparison between Supercritical Fluid Extraction (SFE) and Ultrasound-Assisted Extraction (UAE)

The determined optimal extraction conditions in SFE were compared to UAE which is considered as a simple and efficient modern extraction technique. The extractions were conducted in comparable extraction conditions to SFE: plant mass, extraction time, solvent consumption and composition equivalent to the modifier used.

Two SFE were conducted in the previously determined optimal conditions for 1 g of plant, 60 min, at 40°C and 20% modifier composed of EtOH:H_2O 80:20 *v:v* and EtOH:H_2O 70:30 *v:v*. Indeed, since the percentage of water added to the modifier as an

additive demonstrated a significant effect of the yield of compounds of interest from pomegranate pericarp in the experimental design optimization (**Table 2**), the increase of H_2O% in the modifier from 20% to 30% was additionally investigated. Furthermore, the UAE was conducted for 60 min using 36 mL of solvent which is in equivalent conditions of time and organic solvent consumption (20% of modifier for 60 min with a total flow of 3 mL/min) with the same ratios of EtOH:H_2O 80:20 and 70:30 *v:v*.

The results are presented in **Figure 4.**

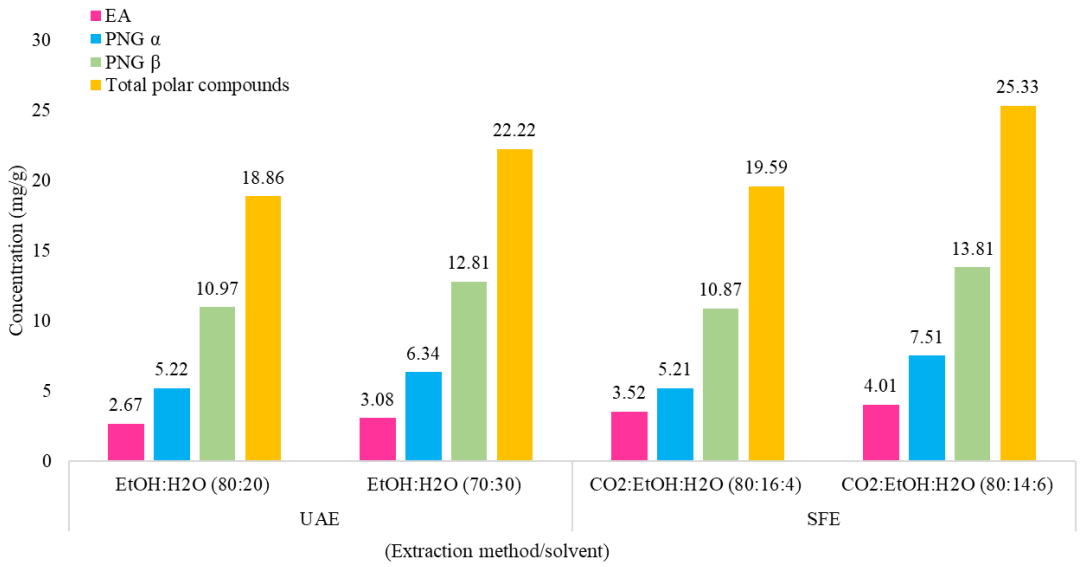

Figure 4: Comparison between supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) for polar compounds of *Punica granatum* L. pericarp.

For the experiments carried out in UAE when H_2O percentage in the extraction solvent increased for 20 to 30%, it resulted in an increase for EA, PNG_α and PNG_β of 15.35%, 21.45% and 16.77% respectively. This indicated that the use of higher H_2O percentage in the extraction solvent is beneficial for the recovery of pomegranate pericarp polar compounds. Moreover, the experiments performed with SFE exhibited the same behavior as UAE with an increase of the H_2O percentage. The SC-CO_2 extraction conducted with a modifier composition of EtOH:H_2O 70:30 *v:v* when compared to 80:20 *v:v*, allowed for a yield increase for EA, PNG_α and PNG_β of 13.92%, 44.14% and 27.05% respectively.

Furthermore, when comparing the extraction yield of both techniques, SFE allowed for an increase of total compounds by 3.87% using EtOH:H_2O 80:20 *v:v*, and 13.99% using EtOH:H_2O 70:30 *v:v* compared to UAE. This demonstrated that both techniques can extract compounds of interest from *Punica granatum* L. and similar yields could be

recovered. Moreover, even though SFE is mostly known for the extraction of non-polar compounds, it is a comparable extraction method to UAE for the polar compound's extraction. Since SFE is a dynamic on-line process, its use can diminish the need for sample or post extraction preparations (e.g., filtration, centrifugation, and evaporation)

3.3.Integrated process: SFE and pre-formulation of natural cosmetic ingredients

During SFE optimization a difficulty for the solubilization of the dried extracts before analysis in conventional polar and non-polar solvents have been observed. After the screening of many solvent compositions and ratios, DMSO:EtOH:H_2O 2:1:1 *v:v:v* allowed for a homogenous solubilization of the extracts and was retained for chromatographic analysis. However, this solvent composition is not compatible with cosmetic formulations. Thus, to bypass the complete evaporation and solubilization of the dried extract in cosmetic products, an integrated SFE pre-formulation of extracts process in cosmetic solvents was developed.

3.3.1. Dissolution solvent screening

An initial screening of cosmetic solvents was carried out, to find an adapted composition that allowed a homogenous aspect of the dissolved extracts, avoiding precipitation in time and the use of DMSO which is not compatible with cosmetic use. An important point was that the selected solvent does not influence the chromatographic analysis of the PPE to allow the possibility of monitoring the stability of the extracts and the influence of storage duration on compound concentration. Numerous solvents and compositions were selected for their use in the cosmetic industry. The initial study showed that the use of water at first for the dissolving of the dried extracts was necessary, however after a while precipitation was inevitable, this is why a stabilizing solvent was needed. However, water percentage was to be limited to 20% in the final composition, this was to reduce any microbiological growth in PPE and to increase the probability of a stable extract, especially since no preservatives were used. A composition of solvent:H_2O 80:20 v:v was assessed and for chromatographic analysis a dilution of the extracts in water was needed.

The extracts showed a homogenous and stable aspect with these cosmetic approved solvents: dipropylene glycol (DPG), propylene glycol (PG), glycerol (Gly), 1,3-propanediol and pentanediol. The results of UHPLC-DAD analysis of PPE dissolved were presented in **Figure 5.**

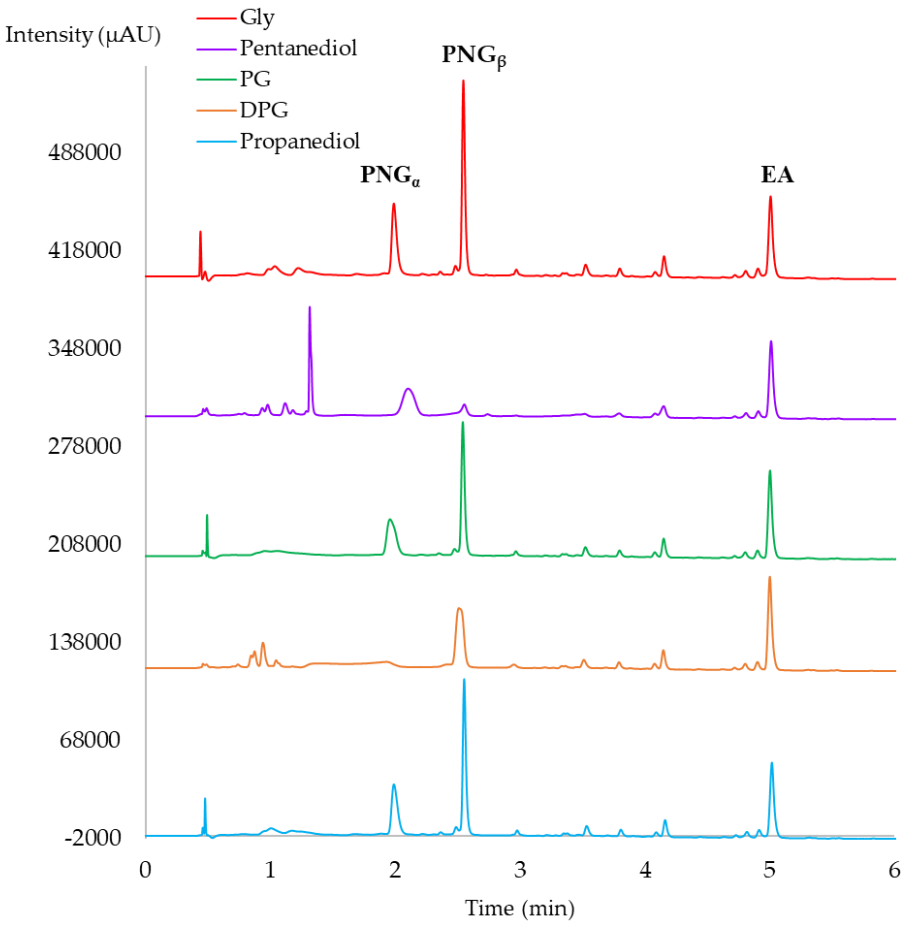

Figure 5: UHPLC-DAD analysis of pomegranate pericarp polar extracts solubilized in cosmetic solvent (composition solvent:H_2O mixture) at 366 nm. Glycerol **(Gly) red**, pentanediol **purple**, propylene glycol **(PG) green**, dipropylene glycol **(DPG) orange** and 1,3-propanediol in **blue**.

EA peak shape was not affected since it was eluted later in the gradient, as for the peak shape of both PNGs was strongly influenced when using pentanediol and DPG, moreover the use of PG influenced the symmetry of PNGα. Hence, Gly and propanediol were the only solvents that did not influence the analysis and quantification of PNGs. Lastly, Gly was selected for the stabilization of PPE, because of the slight improvement of the peak high, possibly due to enhanced solubility of the studied compounds and the lower price point when compared to propanediol.

3.3.2. *Integrated pre-formulation of pomegranate pericarp SC-CO_2 extracts*

The integrated process for SFE extraction of *Punica granatum* L. pericarp is composed of two main steps illustrated in **Figure 6**.

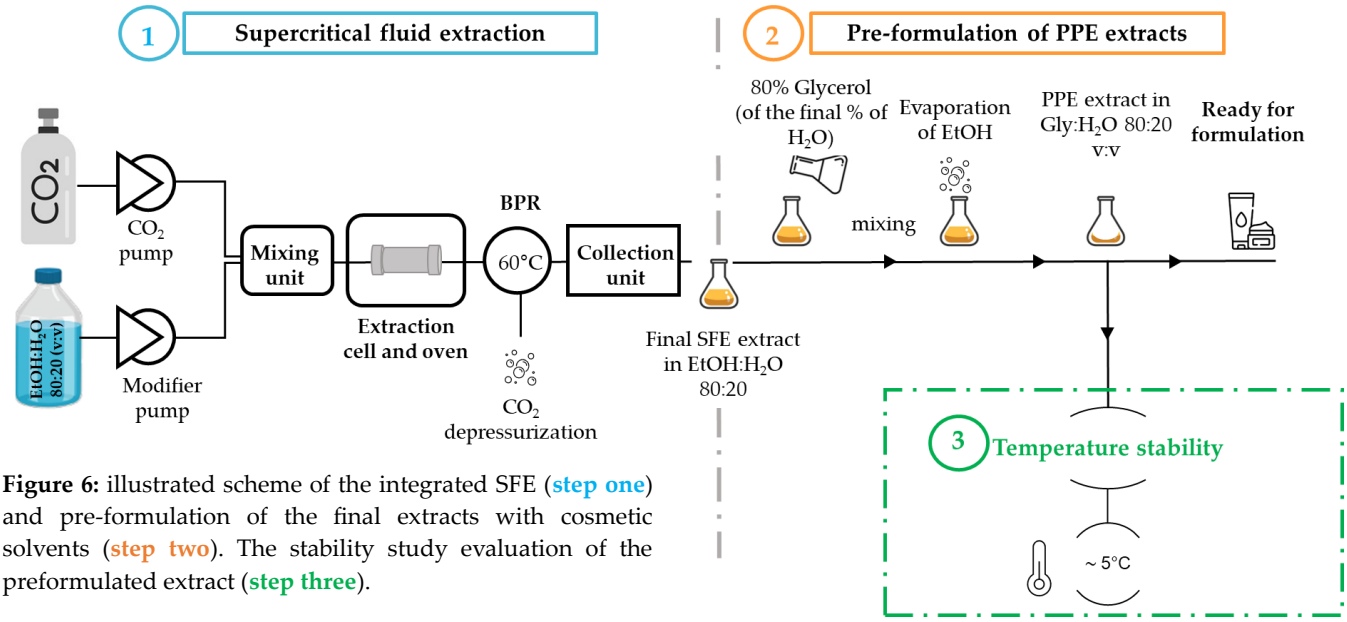

Figure 6: illustrated scheme of the integrated SFE (**step one**) and pre-formulation of the final extracts with cosmetic solvents (**step two**). The stability study evaluation of the preformulated extract (**step three**).

The first step is the supercritical fluid extraction of the plant mass using a mixture of SC-CO_2 and modifier as extraction solvent. The optimized extraction conditions were employed with a composition of EtOH:H_2O 80:20 *v:v* was employed to limit the H_2O% to 20% in the final extract. After the back pressure regulator (BPR) the CO_2 depressurizes and returns to its gaseous state and final SFE extract is collected in the modifier. The second step included the pre-formulation of the SFE extract in a Gly:H_2O 80:20 *v:v* this composition allowed the stabilization of the extracts while keeping only 20% of H_2O, minimizing the influence of water on the stability of the extracts. To achieve this composition, a volume of Gly equal to the EtOH volume is added to the final SFE extract, the mixtures of EtOH:Gly:H_2O *v:v:v* is then mixed manually to achieve a homogenous mixture. The following step included the evaporation of EtOH using nitrogen flow. The evaporation of EtOH first and then adding Gly after to the extract in water was tested, however the PPE was not fully stable in only water, this eventually led to its precipitation and was later needed centrifugation, adding a unit operation to the post-treatment process. Therefore, Gly was added directly to the SFE extract and EtOH was later evaporated. Finally, after this step the pomegranate extract is solubilized in a Gly:H_2O 80:20 *v:v* mixture, pre-formulated to be included in cosmetic emulsions of formulas.

3.3.3. *Stability evaluation of the pre-formulated extracts*

The preformulated PPE were stored at 5°C, this temperature was chosen since the extracts did not contain any preservatives. Evaluation of the stability was conducted following UHPLC-DAD quantification of compounds concentration over time. Samples were analyzed eight times in triplicate over the course of the 104 days that followed the integrated extraction and pre-formulation process. The data were presented as the concentrations of the compounds of interest in mg/g of biomass and their relative variation percentage compared to the initial values at day one, with a positive variation indicating an increase and a negative a decrease (**Table 3**). At the final 104th day the yield of EA increased by 0.27% while that of PNG$_\alpha$ and PNG$_\beta$ decreased by 0.06 and 1.97%, respectively, indicating a minimal variability of the compounds of interest. This proved that the *Punica granatum* L. pericarp SFE extracts in Gly:H_2O 80:20 *v:v* was stable when stored at a low temperature of 5°C without the use of chemical preservatives.

Table 3: Stability evaluation of PPE solubilized in Gly:H_2O 80:20 mixture, the results are presented in terms of concentration in mg/g of plant and the relative variation percentage (day 104) compared to the initial concentration values at day one of the compounds of interest.

EA (mg/g)		PNG$_\alpha$ (mg/g)		PNG$_\beta$ (mg/g)	
Day 1	Day 104	Day 1	Day 104	Day 1	Day 104
0,612	0.614 (+0.27%)	0,483	0.483 (-0.06%)	0,783	0.768 (-1.97%)

4. Conclusion

This study, the application of SFE for the extraction of polar compounds of interest from *Punica granatum* L. pericarp was presented. Using an experimental design, the extraction conditions were optimized and the following conditions of 40°C, 20% modifier of EtOH:H_2O 80:20 *v:v*, 60 min, 15 MPa, 3mL/min for 1 g of plant, allowed for the recovery of 19.59 mg/g of EA and PNGs, both considered as potent antioxidants. The comparison between a UAE maceration and SFE in equivalent conditions, particularly the extraction duration, composition and consumption of solvents demonstrated that SFE is a comparable extraction technique to UAE and that both methods can offer similar yields in the condition range explored. Additionally, to remedy the issue of precipitation and the difficulty of dissolving dried PPE, a screening

of alternative solvents was carried out and a composition of Gly:H_2O 80:20 *v:v* was selected. This allowed for the development of an integrated SFE and pre-formulation of PPE as cosmetic ingredient, while at the same time avoiding full evaporation of the extracts. Finally, stability of PPE in Gly:H_2O 80:20 *v:v* was carried out and a variation of the compounds of interest lower than 2% in three months was achieved at 5°C.

5. References

[1] M. Ozgen, C. Durgaç, S. Serçe, C. Kaya, *Food Chemistry*. **2008**, *111* (3), 703–706. DOI: 10.1016/j.foodchem.2008.04.043.

[2] P. Melgarejo, D. Núñez-Gómez, P. Legua, J. J. Martínez-Nicolás, M. S. Almansa, *Trends in Food Science & Technology*. **2020**, *102*, 232–236. DOI: 10.1016/j.tifs.2020.02.014.

[3] O. Orgil, E. Schwartz, L. Baruch, I. Matityahu, J. Mahajna, R. Amir, *LWT - Food Science and Technology*. **2014**, *58* (2), 571–577. DOI: 10.1016/j.lwt.2014.03.030.

[4] S. Banihani, S. Swedan, Z. Alguraan, *Nutrition Research*. **2013**, *33* (5), 341–348. DOI: 10.1016/j.nutres.2013.03.003.

[5] A. Al-Jarallah, F. Igdoura, Y. Zhang, C. B. Tenedero, E. J. White, M. E. MacDonald, S. A. Igdoura, B. L. Trigatti, *Atherosclerosis*. **2013**, *228* (1), 80–89. DOI: 10.1016/j.atherosclerosis.2013.02.025.

[6] P. Sestili, C. Martinelli, D. Ricci, D. Fraternale, A. Bucchini, L. Giamperi, R. Curcio, G. Piccoli, V. Stocchi, *Pharmacological Research*. **2007**, *56* (1), 18–26. DOI: 10.1016/j.phrs.2007.02.003.

[7] N. Hasnaoui, B. Wathelet, A. Jiménez-Araujo, *Food Chemistry*. **2014**, *160*, 196–203. DOI: 10.1016/j.foodchem.2014.03.089.

[8] R. Boggia, F. Turrini, C. Villa, C. Lacapra, P. Zunin, B. Parodi, *Pharmaceuticals*. **2016**, *9* (4), 63. DOI: 10.3390/ph9040063.

[9] A. Masci, A. Coccia, E. Lendaro, L. Mosca, P. Paolicelli, S. Cesa, *Food Chemistry*. **2016**, *202*, 59–69. DOI: 10.1016/j.foodchem.2016.01.106.

[10] L. Tamborlin, B. R. Sumere, M. C. de Souza, N. F. Pestana, A. C. Aguiar, M. N. Eberlin, F. M. Simabuco, M. A. Rostagno, A. D. Luchessi, *Food Science & Nutrition*. **2020**, *8* (10), 5483–5496. DOI: 10.1002/fsn3.1831.

[11] E. A. Akuru, C. I. Chukwuma, C. E. Oyeagu, O. L. Erukainure, B. Mashile, R. Setlhodi, S. S. Mashele, T. J. Makhafola, J. O. Unuofin, T. O. Abifarin, et *al.*, *Journal of Food Biochemistry*. **2022**, *46* (4), e13913. DOI: 10.1111/jfbc.13913.

[12] A. T. Kishawy, S. A. Amer, M. E. A. El-Hack, I. M. Saadeldin, A. A. Swelum, *Asian-Australas J Anim Sci*. **2019**, *32* (8), 1161–1171. DOI: 10.5713/ajas.18.0522.

[13] Y. Li, C. Guo, J. Yang, J. Wei, J. Xu, S. Cheng, *Food Chemistry*. **2006**, *96* (2), 254–260. DOI: 10.1016/j.foodchem.2005.02.033.

[14] W. Elfalleh, N. Tlili, N. Nasri, Y. Yahia, H. Hannachi, N. Chaira, M. Ying, A. Ferchichi, *Journal of Food Science*. **2011**, *76* (5), C707–C713. DOI: 10.1111/j.1750-3841.2011.02179.x.

[15] G.-Z. Ma, C.-M. Wang, L. Li, N. Ding, X.-L. Gao, *Food Sci Biotechnol*. **2015**, *24* (5), 1887–1892. DOI: 10.1007/s10068-015-0247-0.

[16] O. A. Fawole, N. P. Makunga, U. L. Opara, *BMC Complementary and Alternative Medicine*. **2012**, *12* (1), 200. DOI: 10.1186/1472-6882-12-200.

[17] X. Zhao, Z. Yuan, Y. Fang, Y. Yin, L. Feng, *Journal of Agricultural Science and Technology*. **2014**, *16*, 1649–1659.

[18] R. R. Mphahlele, O. A. Fawole, N. P. Makunga, U. Linus Opara, *Food Measure*. **2017**, *11* (4), 2233–2246. DOI: 10.1007/s11694-017-9608-0.

[19] N. Seeram, R. Lee, M. Hardy, D. Heber, *Separation and Purification Technology*. **2005**, *41* (1), 49–55. DOI: 10.1016/j.seppur.2004.04.003.

[20] M. Abid, H. Yaich, S. Cheikhrouhou, I. Khemakhem, M. Bouaziz, H. Attia, M. A. Ayadi, *J Food Sci Technol*. **2017**, *54* (9), 2890–2901. DOI: 10.1007/s13197-017-2727-0.

[21] C. Pagliarulo, V. De Vito, G. Picariello, R. Colicchio, G. Pastore, P. Salvatore, M. G. Volpe, *Food Chemistry*. **2016**, *190*, 824–831. DOI: 10.1016/j.foodchem.2015.06.028.

[22] D. Rakel, in *Clinical Men's Health* (Ed: J. J. Heidelbaugh), W.B. Saunders, Philadelphia **2008**.

[23] A. P. Kulkarni, H. S. Mahal, S. Kapoor, S. M. Aradhya, *J. Agric. Food Chem.* **2007**, *55* (4), 1491–1500. DOI: 10.1021/jf0626720.

[24] N. P. Seeram, R. Lee, D. Heber, *Clinica Chimica Acta*. **2004**, *348* (1), 63–68. DOI: 10.1016/j.cccn.2004.04.029.

[25] D. Rongai, P. Pulcini, G. Di Lernia, P. Nota, P. Preka, F. Milano, *Horticulturae*. **2019**, *5* (3), 52. DOI: 10.3390/horticulturae5030052.

[26] C. Gosset-Erard, M. Zhao, S. Lordel-Madeleine, S. Ennahar, *Food Chem*. **2021**, *352*, 129396. DOI: 10.1016/j.foodchem.2021.129396.

[27] J. Lu, K. Ding, Q. Yuan, *Chroma*. **2008**, *68* (3), 303–306. DOI: 10.1365/s10337-008-0699-y.

[28] Ž. Peršurić, L. Saftić Martinović, M. Malenica, I. Gobin, S. Pedisić, V. Dragović-Uzelac, S. Kraljević Pavelić, *Molecules*. **2020**, *25* (24), 5916. DOI: 10.3390/molecules25245916.

[29] S. U. Mertens-Talcott, P. Jilma-Stohlawetz, J. Rios, L. Hingorani, H. Derendorf, *J. Agric. Food Chem.* **2006**, *54* (23), 8956–8961. DOI: 10.1021/jf061674h.

[30] J. A. Khan, S. Hanee, **n.d.**

[31] M. Singh, K. E. Lee, R. Vinayagam, S. G. Kang, *Biotechnol Bioproc E*. **2021**, *26* (5), 728–737. DOI: 10.1007/s12257-021-0211-1.

[32] D. Dey, S. Debnath, S. Hazra, S. Ghosh, R. Ray, B. Hazra, *Food and Chemical Toxicology*. **2012**, *50* (12), 4302–4309. DOI: 10.1016/j.fct.2012.09.001.

[33] H. S. Elshafie, L. Caputo, L. De Martino, S. H. Sakr, V. De Feo, I. Camele, *Plants*. **2021**, *10* (1), 153. DOI: 10.3390/plants10010153.

[34] P. C. Anibal, I. T. A. Peixoto, M. A. Foglio, J. F. Höfling, *Braz. J. Microbiol.* **2013**, *44*, 839–848. DOI: 10.1590/S1517-83822013005000060.

[35] R. García-Villalba, J. C. Espín, K. Aaby, C. Alasalvar, M. Heinonen, G. Jacobs, S. Voorspoels, T. Koivumäki, P. A. Kroon, E. Pelvan, et *al.*, *J. Agric. Food Chem.* **2015**, *63* (29), 6555–6566. DOI: 10.1021/acs.jafc.5b02062.

[36] S. Seifabadi, G. Vaseghi, M. Ghannadian, S. Haghjooy Javanmard, *Iran J Pharm Res*. **2019**, *18* (1), 348–357.

[37] S. Atwi-Ghaddar, L. Zerwette, E. Destandau, E. Lesellier, *Molecules*. **2023**, *28* (14), 5485. DOI: 10.3390/molecules28145485.

[38] S. Atwi-Ghaddar, E. Destandau, E. Lesellier, *Journal of CO2 Utilization*. **2023**, *70*, 102440. DOI: 10.1016/j.jcou.2023.102440.

[39] R. P. F. F. da Silva, T. A. P. Rocha-Santos, A. C. Duarte, *TrAC Trends in Analytical Chemistry*. **2016**, *76*, 40–51. DOI: 10.1016/j.trac.2015.11.013.

[40] P. B. Gomes, V. G. Mata, A. E. Rodrigues, *The Journal of Supercritical Fluids*. **2007**, *41* (1), 50–60. DOI: 10.1016/j.supflu.2006.08.018.

[41] N. Mezzomo, J. Martínez, S. R. S. Ferreira, *The Journal of Supercritical Fluids*. **2009**, *51* (1), 10–16. DOI: 10.1016/j.supflu.2009.07.008.

Chapter III: Selective-Sequential Supercritical Fluid Extraction (S^3FE)

Calendula officinalis L.

In the second chapter, the application of SFE to extract polar compounds was highlighted using experimental design. Accordingly, due to the inherent physiochemical properties of SC-CO_2 (low polarity) and the possibility of dynamic and multistep extraction process, the sequential extraction of non-polar and polar compounds is highly advantageous. This offers a fast and simple extraction mainly due to the reduction of sample preparation steps and the immediate change of extraction parameters, allowing the saving of time and energy.

The recovery of lipophilic compounds needed to be optimized using suitable parameters, for instance since water incorporation should be avoided, and in the case of the targeting of both polar and non-polar compounds in the plant biomass, the non-polar compounds should be extracted first since polar compounds required the use of water additives. Consequently, in this chapter another type of experimental model was developed to extract non-polar compounds from *Calendula officinalis* L, a plant rich in a wide range molecule of different polarity. After the optimization of the extraction of non-polar compounds, what now is known as standard experimental design for polar compounds was applied (the same as for green tea and pomegranate), allowing the validation of this model on a third plant. In addition, the exploration of extraction scale-up for lipophilic compounds will be addressed in this chapter.

Exploring the Sequential-Selective Supercritical Fluid Extraction (S³FE) of flavonoids and esterified triterpenoids from *Calendula officinalis* L. flowers

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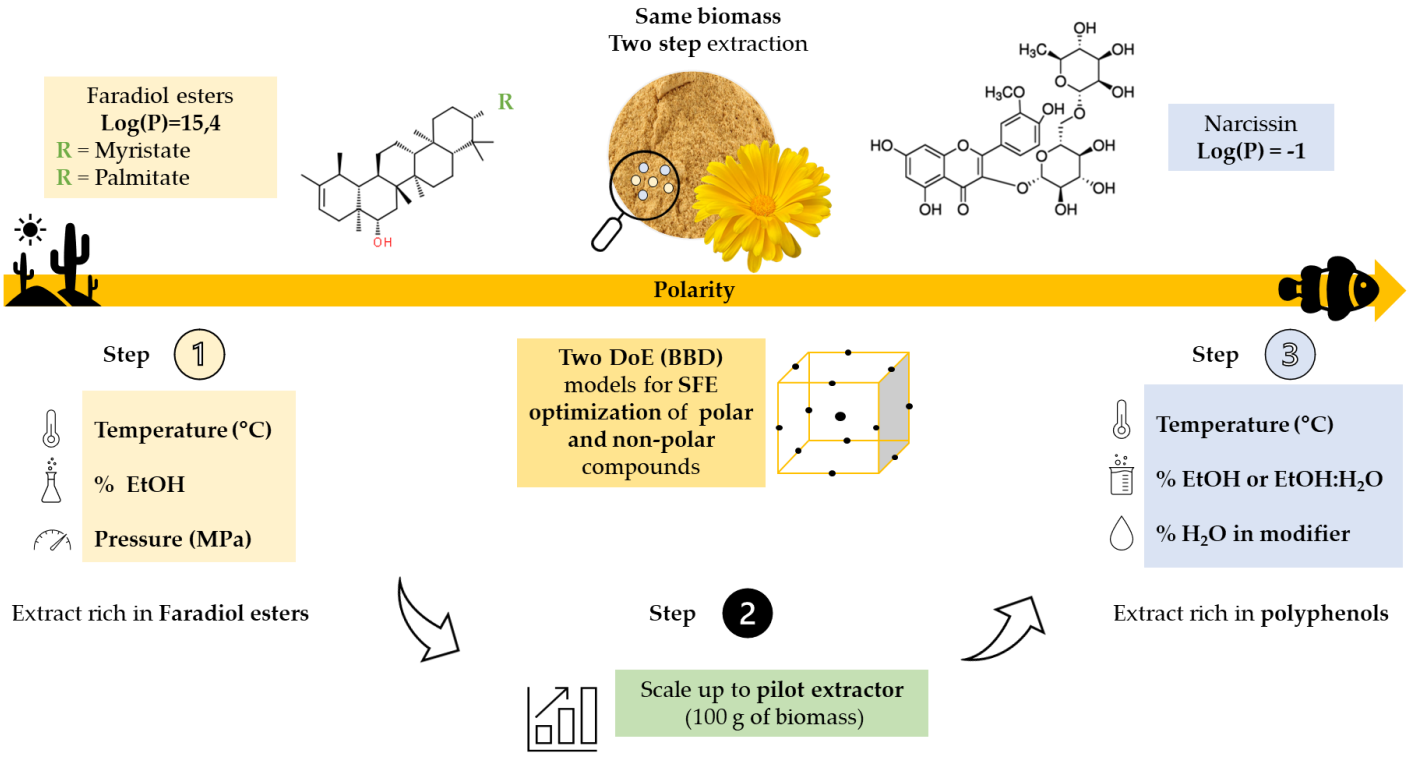
Graphical abstract



Abstract

One of the many advantages of supercritical fluid extraction (SFE) is the possibility of using it in sequential and selective approaches. This is due to the use of a dynamic extraction mode in addition to the possibility of altering the composition of the modifier during the extraction process. In this study, the optimization of *Calendula officinalis* L. extraction of non-polar and polar compounds was achieved using three-level Box-Behnken designs (BBD). For non-polar compounds the factors were pressure, temperature and EtOH percentage. As for the polar compounds the three variables were temperature, total modifier percentage and H_2O added in modifier as an additive. The recovery of selectively rich extracts in triterpendiol esters and narcissin was possible using a sequential two step SFE. The first step was performed at 80°C and 15% EtOH and the second at 40°C and 30% EtOH:H_2O 80:20 *v:v* with a total 60 min extraction. Additionally, the SFE extraction of non-polar compounds was scaled-up on a pilot-scale extractor, demonstrating similar results. Finally, the SFE results were compared to ultrasound-assisted extraction (UAE).

1. Introduction

Plant-derived extracts have long provided a reliable source to supply pharmaceutical, nutraceutical, and cosmetic products rich in bioactive compounds. After the identification of the plant and the compounds of interest, a suitable extraction procedure allowing the best final extract activity and yield should be selected.
Therefore, numerous traditional and modern extraction methods have been developed and used in several studies in this regard. Soxhlet, maceration, or hydro-distillation are among the techniques that are regarded as traditional, advantages of these methods are the simplicity of use and low cost. However, these methods have some limitations, such as the necessity for large volumes of solvent and extended extraction times that might in some cases result in a low yield recovery. In addition, selective extraction of bioactive chemicals can be achieved by using several solvents in series [1,2]. In this regard, green and modern extraction methods have emerged. Primarily, on an analytical scale that was later scaled-up on a pilot and industrial level. This allowed the use of less solvent, faster extraction times and often a higher extract purity [3–6].

One of these green extraction techniques is the use of supercritical carbon dioxide (SC-CO_2) for the extraction of plant compounds, its low polarity made possible the replacement of toxic solvents like hexane, while extracting non-polar compounds like triglycerides, carotenoids, fatty acids, and essential oils [7–10]. Moreover, the use of polar modifiers like ethanol (EtOH) and EtOH:H_2O *v:v* mixtures modulated its polarity in order to access more polar molecules like polyphenols [11–14]. This flexibility of the SC-CO_2 polarity allowed the extraction of a larger spectrum of chemicals with different polarity and consequently different bioactivities and skin care properties, while using the same plant biomass decreasing sample treatment steps.

Calendula officinalis L. (Asteraceae) also known as pot marigold is used as a traditional therapeutic plant for the treatment of various diseases [15–23], this is due to its abundance in secondary metabolites. The phytochemical composition of marigold flowers has been extensively studied for both polar and non-polar compounds.

The composition of marigold's lipophilic extract has been known since the late 1960s [24]. Active terpenoids such as ψ-taraxasteol, lupeol, erythrodiol, calenduloside, cornulacic acid acetate, faradiol, calendiladiol, maniladiol, β-amyrine, and arnidiol have been frequently emphasized for their abundance in the plant [24–29]. Most of these triterpenoids are found in an esterified form. In relation to that, a quantitative study of 10 varieties of *Calendula officinalis* L. dichloromethane extracts studied the content of triterpendiol monoesters, it identified faradiol-3-*O*-palmitate, faradiol-3-*O*-myristate, faradiol-3-*O*-laurate as major compounds. In addition to, arnidiol-3-*O*-palmitate, arnidiol-3-*O*-myristate, arnidiol-3-*O*-laurate, calenduladiol-3-*O*-palmitate, calenduladiol-3-*O*-myristate and calenduladiol-3-*O*-laurate as minor ones [25].

Marigold yellow to orange color can be attributed to its carotenoids content [30]. Pigments like neoxanthin, luteoxanthin, antheraxanthin, flavoxanthin, mutatoxanthin, lactucaxanthin, lutein, zeaxanthin, rubixanthin, lycopene, γ-carotene, α-carotene, and β-carotene have been identified in the flowers. However, the composition of carotenoids may differ depending on the variety and color of the flower [31]. The analysis of carotenoids found in the petals of six different cultivars identified 19 carotenoids, with 10 being unique to orange color cultivars. However, it was recurrent to see that luteoxanthin, flavoxanthin and in some cases lutein were the most abundant [30].

Lipid content in *Calendula officinalis* L. seed extracts have been previously examined [32–36]. Fatty acid content in the petals of the flowers like lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic acids has been reported. It was also noted that the major fatty acids were the palmitic and myristic ones [37]. In addition, it was found that the petals contain tocopherol in α, β, and γ forms [38,39].

Many extraction methods have been applied to extract lipophilic compounds from marigold flowers. Some noted studies used Soxhlet with diethyl ether [40], others UAE with dichloromethane [41]. However, the application of supercritical fluids has been more frequent along the years, this is due to the non-polar compounds' compatibility with SC-CO_2 polarity [42–49]. The extraction kinetics model of lipophilic compounds (*i.e.*, oleoresin) was studied, it determined that the increase in pressure improved the extraction yield, while the increase in the solvent flow rate decreased it. The temperature influence showed an increase in the yield only when the pressure was higher than 15 MPa [43]. In addition, major anti-inflammatory triterpendiol esters of marigold flowers (faradiol 3-*O*-laurate, palmitate and myristate) were extracted using SFE with a pressure of 50 MPa and a temperature of 50°C for 3 hours, this resulted in an extraction yield of 5% for the dried extract with recovery of 85% of total faradiol esters from the crude herb [44]. Lastly, a theoretical model of the scale-up extraction of these molecules using SC-CO_2 extraction was obtained, it determined that a good simulation of the kinetic behavior should be produced while maintaining a constant CO_2 residence duration in the various size vessels [46].

For polar compounds, flavonoids and polyphenols have been reported in marigold extracts like calendoflavoside, isoquercitrin, rutin, isorhamnetin, quercetin, narcissin (isorhamnetin-*3*-*O*-rutinoside), astragalin, hesperidin and kaempferol [27,50–52]. Finally, flavonol glycosides like isorhamnetin 3-*O*-*β*-*D*-glucopyranoside, quercetin 3-*O*-*β*-*D*-glucopyranoside and quercetin *3*-*O*-*β*-*D*-galactopyranoside [53] were identified. In addition to some coumarins like scopoletin, umbelliferone, esculetin [54]. It was demonstrated that the yield of polyphenols is associated with the antioxidant bioactivity of the extracts [55,56]. For the polar compounds of marigold, many studies were conducted using various extraction techniques. Microwave assisted extraction (MAE) was optimized using response surface methodology (RSM) with three factors: temperature, extraction time and solvent/solid ratio. The extracts were evaluated, and it was reported that the solvent (EtOH) concentration had the highest impact to the total phenolic content (TPC) and antioxidant activities [55]. Another study tested several extraction methods for polar compounds, including homogenizer-assisted extraction (HAE), maceration (MAC), Soxhlet, and ultrasound-assisted extraction (UAE). These methods all used methanol (MeOH) as extraction solvent. It stated that the flower extracts provided the highest total phenolic content. The yields were examined for the four extraction techniques and the study determined that the phytochemical profile of the extracts was different for each extraction process [52].

The sequential and selective extraction of non-polar then polar compounds from *Calendula officinalis* L. was explored before, mainly due to the different properties

found in each fraction. As the hydrophilic fraction offers an antioxidant activity and the lipophilic one offers anti-edematous and anti-inflammatory properties [41].
The sequential maceration at room temperature for two days with n-hexane, dichloromethane, acetone, ethyl acetate, methanol, and distilled water allowed the recovery of extracts with different polarity from the flowers [57]. In addition, two different extraction process SC-CO_2 extraction followed by UAE with EtOH:H_2O 50:50 *v:v* was reported as a green extraction method [42].
However, these approaches required either long extraction times, drying of the extracts for the next step and changing the solvents to fit the polarity of the compounds or using two different extraction processes. This is a valid approach for the characterization and testing the activities of the fractions, but an industrial extraction for cosmetic or pharmaceutical use requires a reduction of the process step and in certain cases, the use of only green solvents.
The sequential-selective supercritical fluid extraction (S^3FE) of different polarity compounds helped to overcome some of the drawbacks previously reported [58,59]. In this paper, this approach was applied to *Calendula officinalis* L. flowers. In the objective of limiting the extraction solvents volume and unit operations with the use of dynamic extraction mode. A rationalized approach is described in this paper. Both steps were optimized using an experimental design model adapted for the polarity of the targeted compounds.
Finally, the extraction for non-polar compounds was scaled-up from an analytical scale (1 g of plant) to a pilot sale extractor (100 g of plant). To our knowledge, this topic has never been addressed by other publications.

2. *Results and discussion*

SFE applied to *Calendula officinalis* L. flowers was optimized based on two Box-Behnken designs (BBD). The first model targeted non-polar compounds (CNPE), the temperature and pressure were chosen for their ability to change the SC-CO_2 density, and the EtOH percentage used as modifier was used for its capacity to modify the polarity of the extraction phase **(Table 1)**.

Table 1: Design matrix for the selective SFE design of experiment (BBD) of CNPE with responses for non-polar compounds of interest (faradiol esters) and narcissin (UV response 354 nm UC-DAD) expressed in peak area normalized for one gramme of biomass.

Experiment N°	Coded levels			Results (after 30 min of extraction)				
	X_1	X_2	X_3	Faradiol myristate (mAU×min)	Faradiol palmitate (mAU×min)	Both faradiol esters (Y) (mAU×min)	Narcissin (µv×sec)	Dried extract yield (mg/g)
	Temperature (°C)	Pressure (MPa)	EtOH (%)					
1	40 (-1)	10 (-1)	10 (0)	3.11×10^{8}	2.54×10^{8}	5.64×10^{8}	0.00	58.9
2	**80 (+1)**	**10 (-1)**	**10 (0)**	$\mathbf{5.46\times10^{8}}$	$\mathbf{4.65\times10^{8}}$	$\mathbf{1.01\times10^{9}}$	$\mathbf{4.08\times10^{5}}$	**88.5**
3	40 (-1)	20 (+1)	10 (0)	2.78×10^{8}	2.31×10^{8}	5.08×10^{8}	0.00	62.5
4	80 (+1)	20 (+1)	10 (0)	5.50×10^{8}	4.77×10^{8}	1.03×10^{9}	0.00	76.1
5	40 (-1)	15 (0)	5 (-1)	3.94×10^{8}	3.27×10^{8}	7.20×10^{8}	0.00	56.5
6	80 (+1)	15 (0)	5 (-1)	1.99×10^{8}	9.61×10^{7}	2.95×10^{8}	0.00	41.5
7	**40 (-1)**	**15 (0)**	**15 (+1)**	$\mathbf{5.39\times10^{8}}$	$\mathbf{4.66\times10^{8}}$	$\mathbf{1.00\times10^{9}}$	$\mathbf{3.23\times10^{5}}$	**69.7**
8	**80 (+1)**	**15 (0)**	**15 (+1)**	$\mathbf{1.39\times10^{9}}$	$\mathbf{1.26\times10^{9}}$	$\mathbf{2.66\times10^{9}}$	**0.00**	**92.3**
9	60 (0)	10 (-1)	5 (-1)	1.30×10^{8}	5.79×10^{7}	1.88×10^{8}	0.00	35.2
10	60 (0)	20 (+1)	5 (-1)	3.65×10^{8}	3.13×10^{8}	6.79×10^{8}	0.00	55.5
11	**60 (0)**	**10 (-1)**	**15 (+1)**	$\mathbf{6.81\times10^{8}}$	$\mathbf{5.73\times10^{8}}$	$\mathbf{1.25\times10^{9}}$	$\mathbf{3.04\times10^{5}}$	**85.4**
12	**60 (0)**	**20 (+1)**	**15 (+1)**	$\mathbf{6.50\times10^{8}}$	$\mathbf{5.53\times10^{8}}$	$\mathbf{1.20\times10^{9}}$	$\mathbf{3.15\times10^{5}}$	**102.6**
13	60 (0)	15 (0)	10 (0)	5.77×10^{8}	4.93×10^{8}	1.07×10^{9}	0.00	73.4
14	60 (0)	15 (0)	10 (0)	5.68×10^{8}	4.68×10^{8}	1.04×10^{9}	0.00	70.7
15	60 (0)	15 (0)	10 (0)	5.82×10^{8}	4.82×10^{8}	1.06×10^{9}	0.00	68.1
16	60 (0)	15 (0)	10 (0)	6.59×10^{8}	5.49×10^{8}	1.21×10^{9}	0.00	71.5
17	60 (0)	15 (0)	10 (0)	4.59×10^{8}	3.86×10^{8}	8.45×10^{8}	0.00	65.5

The preliminary screening of the EtOH percentage to be included in the CNPE design of experiment (DoE), determined that ethanol was necessary for the extraction of triterpendiol esters from marigold in the pressure and temperature ranges chosen for this design. In addition, the second model targeted the extraction the polar compounds (CPE). Temperature, total modifier percentage and water percentage added in the modifier were selected as the three parameters to be investigated for the extraction of marigold flavonoids, this choice was based on previous work done for the extraction of caffeine and total catechins from green tea [11]. As for the pressure, in prior research to optimize the extraction of two polar flavonoids, with pressure serving as the optimization parameter with a range of 10 to 20 MPa; it was demonstrated that in that range, pressure was an insignificant variable for the extraction of such polar compounds [60]. This was mainly due to the fluid's high density, especially with high percentages of modifiers (up to 30%). Therefore, the pressure was maintained constant at 15 MPa throughout the present investigation and was not explored because it had little impact on the model's response (compounds yield).

2.1.Optimization of calendula non-polar extracts (CNPE)

2.1.1. Statistical analysis, model, and factor significance

To achieve a selective high recovery of non-polar compounds without the co-extraction of polar ones, the optimization of triterpendiol esters extraction was carried out first**.** The total peak areas of major triterpendiol esters (faradiol myristate and faradiol palmitate) found in *Calendula officinalis* L. non-polar extracts (CNPE) were used as the response (Y).

The model's determination coefficient (R^2) was equal to 0.93, while the value of adjusted determination coefficient (adj R^2) was equal to 0.88. This signifies that the model was unable to account for 7% of the total variance. The R^2 value demonstrates a highly strong correlation between the model's expected and experimental response values and indicated that the theoretical model shows a good fit with experimental data. The relative standard deviation (RSD%) of the central point replicates (5 repetitions) for the peak area of both molecules and the dried extract yield (mg/g) were equal to 12.42% and 4.42% respectively. Furthermore, the Dixon statistical test was applied and showed no presence of outliers in the responses, this was validated by the analysis of the residuals between predicted and experimental values. The results are shown in **Table 1** and the correlation between the three variables (X_1, X_2, and X_3) and

the response (*Y*) is presented in **Equation 1**. The terms presented were chosen to obtain the highest pred R^2, therefore increasing the predictive ability of the model for the response. The quadratic terms and the interaction between the temperature and the modifier percentage were included, and the predicted R^2 was equal to 0.54.

$$Y\ (mAU \times min) = 7.976 \times 10^7 - 4.881 \times 10^7 X_1 + 3.73 \times 10^8 X_2 - 2.7664 \times 10^8 X_3 + 8.843 \times 10^4 X_1^2 - 1.21 \times 10^7 X_2^2 + 3.556 \times 10^6 X_3^2 + 5.189 \times 10^6 X_1 X_3 \quad \textbf{(1)}$$

The results of the polynomial model's analysis of variance (ANOVA) are summarized in **Table 2**, and the regression results indicated that the model is significant. Each factor's significance was assessed using the probability associated with its F-value and p-value, and the percentage of each factor's contribution to the model's response was examined. The marigold regression model's total sum of faradiol esters peaks area had a F-value of 17.57 and a corresponding p-value of 0.001. A significant model is presented by both statistical terms.

Table 2: ANOVA for response surface regression model for total peak area of faradiol esters (faradiol myristate and faradiol palmitate) in selective CNPE.

Source	DF	SS	MS	F value	p-value	Contribution (%)	Conclusion
Regression model	9	4.3485×10^{18}	6.2121×10^{17}	17.5737	0.0001		Very significant
X_1- Temperature (°C)	1	5.9963×10^{17}	9.8515×10^{16}	2.7869	0.1294	3.75	Not significant
X_2- Pressure (MPa)	1	1.9942×10^{16}	4.0094×10^{17}	11.3423	0.0083	15.3	Very significant
X_3- EtOH (%)	1	2.2417×10^{18}	3.0974×101^{7}	8.7624	0.0160	11.8	Significant
X_1^2	1	2.3401×10^{15}	5.2683×10^{15}	0.1490	0.7084	0.2	Not significant
X_2^2	1	3.7452×10^{17}	3.8531×10^{17}	10.9002	0.0092	14.7	Very significant
X_3^2	1	3.3276×10^{16}	3.3276×10^{16}	0.9414	0.3573	1.27	Not significant
$X_1\times X_3$	**1**	**1.0771×10^{18}**	**1.0771×10^{18}**	**30.4700**	**0.0004**	**41**	**Very significant**
Residuals	7	3.1814×10^{17}	3.5349×10^{16}			12.1	
Total	16	4.6666×10^{18}					
	Degree of freedom	Sum of squares	Mean square				

The factors X_2 (pressure) and X_2^2 (pressure × pressure) were significant with 15.3% and 14.7% of contribution respectively. This was also validated by their p-values of 0.0083 and 0.0092 respectively. However, the interaction term between the temperature and EtOH percentage ($X_1\times X_3$) had the highest significance with 41% of contribution and 0.0004 p-value. The separate term of temperature (X_1) was non-significant to the model's response with and 3.75% contribution and p-values of 0.1294, as for the EtOH percentage (X_3) the factor was significant with a contribution of 11.8% and p-value of 0.0160. This indicated that the response was less influenced by the distinct variations in temperature and EtOH percentage. However, the extraction yield of both faradiol myristate and faradiol palmitate was significantly impacted by the interaction between the two variables. The interaction terms of $X_1\times X_2$ and $X_2\times X_3$ were excluded from the DoE, their influence on the response was assessed during the analysis of the model. However, when they were included, these factors had a low significance and decreased the predicted R^2 to 0.33 (supplementary data).

2.1.2. Effects of the extraction parameters on CNPE assessed with Box-Behnken design

Heat-maps were used as a graphical representation of the independent factors and dependent response variations, they were correlated to the response of marigold

faradiol myristate and faradiol palmitate peak areas (mAU×min) for 30 min of extraction time (**Figure 1**).

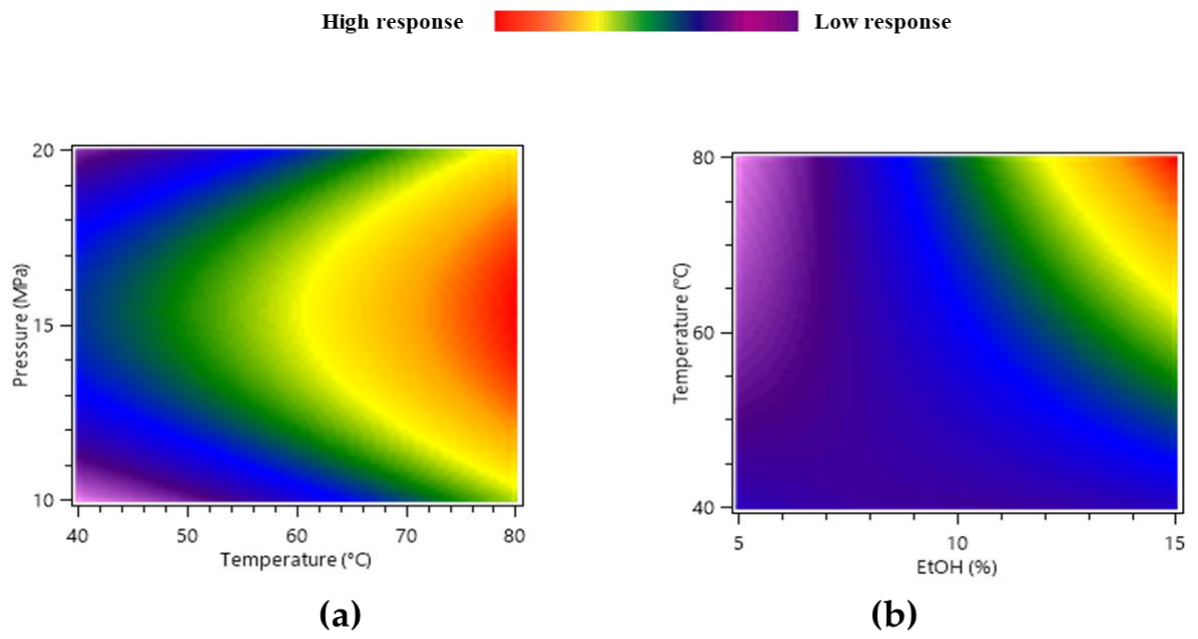


Figure 1: Response surface heat-maps showing the effect of the factors variation on the total faradiol esters (faradiol myristate and faradiol palmitate) peak area (mAU×min). (a) Interaction between pressure (X_1) and temperature (X_2), (b) interaction between temperature (X_2) and EtOH percentage (X_3).

Figure 1a represented the effect of temperature (X_1) and pressure (X_2) at 10% of EtOH on the extraction yield of faradiol esters. The heat-map demonstrated that the pressure's influence on the extraction yield is weak and independent from the temperature, and the optimal pressure is equal to 15 MPa, for the full temperature range studied. The same behavior was noticed with the pressure and EtOH percentage interaction (supplementary data).

Figure 1b illustrated the effect of temperature (X_1) and EtOH percentage (X_3) variation on the response, it showed that both factors were influential for the extraction of faradiol esters. The increase of EtOH percentage from 5 to 15% at 40°C and 15 MPa had little effect of the peak areas of non-polar targeted compounds, their calculated value increased from 8.848×10^8 to 9.056×10^8 mAU×min (2.35%). Nonetheless, the same increase of EtOH at 80°C resulted in an augmentation of peak areas from 3.945×10^8 to 2.491×10^9 mAU×min (531.43%). Besides, when comparing the results between 40°C and 80°C using 5% of EtOH, it showed that the temperature had the opposite effect where a decrease of 55.41% was noticed in the yield with the increase of the temperature. This can explain the influence of the interaction term $X_1\times X_3$ represented

in the ANOVA analysis in **Table 2**, where the influence of the high temperature is only related to the simultaneous increase of EtOH percentage. In this case, the effect of density variation linked to temperature increase is disadvantageous for yield increase at lower percentages of modifier. However, at high percentages (15% EtOH), density variation is minimal, and the variation in solubility is predominant. Hence, favoring extraction at 80°C. In conclusion the combination of temperature and ethanol percentage have a highly significative influence on the results.

2.1.3. Optimal selective extraction of faradiol esters

Based on **Equation 1**, the computed optimum parameters, offering maximal yield of faradiol myristate and palmitate, are as follows: temperature of 80°C, a pressure of 15.52 MPa and an ethanol percentage of 15%.

These conditions corresponded to the experiment n°8 (80°C, 15MPa and 15% EtOH), for which the highest yield of faradiol palmitate and myristate was found with a peak area of 2.66×10^9 mAU×min and dry extract mass of 92.3 mg/g of biomass **(Table 1)**. Other experiments that offered a close yield in set are experiment n°2, 11 and 12. Additionally, **Table 1** showed that these conditions offer a selective extraction of the non-polar compounds without co-extracting the polyphenols of marigold, the UC-DAD analysis of the experiment n°8 CNPE did not detect the extraction of polar compounds especially the targeted molecule of narcissin. In contrast, the experiments n°2 (80°C, 10MPa, and 10% EtOH), n°7 (40°C, 15MPa, and 15% EtOH), n°11 (60°C, 10MPa, and 15% EtOH), and n°12 (60°C, 20MPa, and 15% EtOH) demonstrated a co-extraction of these polar molecules. The combination of all these values validated the relevance of experiment n°8 for the selective and efficient extraction of faradiol esters. One can notice that the extracted amount of triterpenoid esters is strongly higher than the one reached by using pure CO_2 [26,61]

2.1.4. *Experimental design validation: extraction kinetics CNPE*

The experimental design presented the results for 30 minutes of extraction time, to validate these results while including the time factor in the response, extraction kinetics were investigated in several experimental conditions (**Figure 2**).

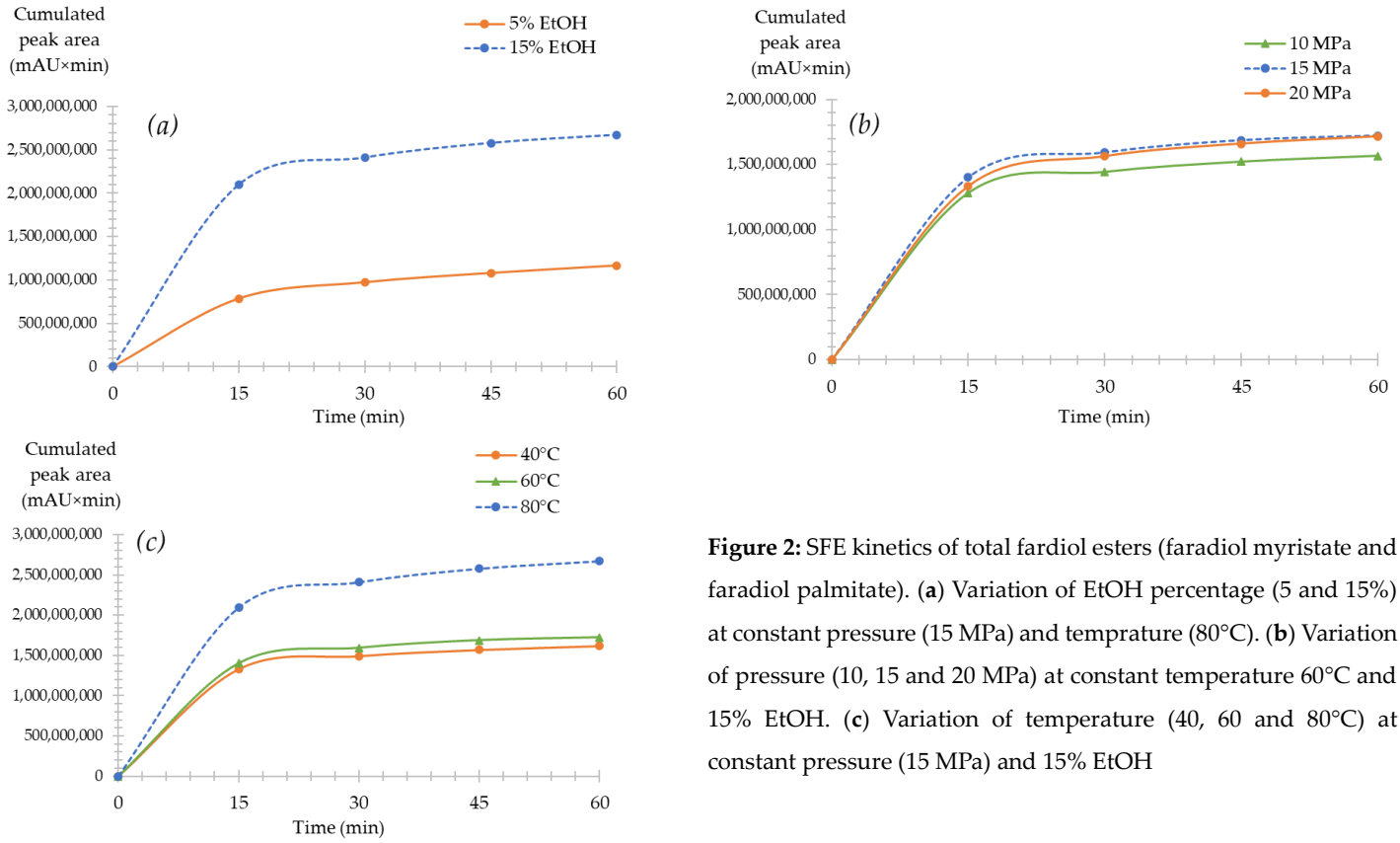


Figure 2: SFE kinetics of total fardiol esters (faradiol myristate and faradiol palmitate). (**a**) Variation of EtOH percentage (5 and 15%) at constant pressure (15 MPa) and temprature (80°C). (**b**) Variation of pressure (10, 15 and 20 MPa) at constant temperature 60°C and 15% EtOH. (**c**) Variation of temperature (40, 60 and 80°C) at constant pressure (15 MPa) and 15% EtOH

Figure 2a showed the extraction kinetics of faradiol esters for two EtOH percentages at 5 and 15%, both extractions were done at 15 MPa and 80°C. The results validated that a high EtOH percentage is needed to attain a high extraction yield of faradiol esters for calendula flowers. Moreover, when comparing both yields at 30 min, 15% EtOH had a 175% higher percentage when compared to 5% EtOH. This validated the results of the experimental design demonstrating that a high percentage of modifier at 80°C is needed to increase the yield of faradiol esters from marigold petals.

Figure 2b represented the extractions kinetics of faradiol esters at a constant temperature of 60°C (median level), EtOH of 15% and three different pressure values: 10, 15 and 20 MPa. The extraction kinetics at the three pressure points had minimal variations for all extractions with 15 MPa having the highest yield. This demonstrated that pressure modifications had a relatively slight effect on the yield of non-polar compounds extracted from marigold flowers at this modifier percentage.

In addition, the influence of temperature was demonstrated in **Figure 2c**. With a constant pressure and EtOH percentage of 15 MPa and 15%, the extraction kinetics of three different temperatures were investigated: 40, 60 and 80°C. The results from experimental design **Figure 1a** were validated as with an 80°C temperature the highest extraction yield was obtained. Besides, at 40 and 60°C the yield kinetics had a similar extraction yield during the total extraction time. At 30 min a yield increase of 79% was noted with the increase of temperature from 40 to 80°C.

The kinetics behavior was similar with **Figure 2b** (at 60°C), as within the first 30 min of extraction, 90% of faradiol esters were extracted compared to the total yield obtained at 60 min considered as 100%.

Finally, for all extraction kinetics comparable behavior of the yield's percentage variation was observed. After the first 30 min almost 90% of yield is obtained and for the second 30 min only 10% is extracted. This might be explained by a matrix effect of the plant mass, where at the initial 30 minutes the highest obtainable extraction yield within the conditions applied is reached, and any additional extraction time applied increases the extraction yield of only 10%. In most cases, the increase of extraction time in SFE allows for convergence of yield for non-optimal to the yield of optimal conditions; meaning that theoretically the highest extraction yield is reached faster with optimal conditions. Nonetheless, for less optimal conditions the same extraction yield can be reached but it will require longer extraction times and higher energy consumption [62]. However, for *Calendula officinalis* L. in conditions range used in this study, the use of longer extraction duration for non-optimal conditions, did not allow to reach the same recovery yield compared to the one obtained for optimal ones (for instance **Figure 2a** from 5 to 15% of modifier, **Figure 2b** or from 60 to 80°C). Consequently, the optimal conditions should be applied, and only 30 min is necessary to obtain almost 90% of the optimal extraction yield.

2.1.5. Pilot-scale supercritical extraction of CNPE

A pilot-scale extraction (PE) of 100 g of biomass was conducted to compare and validate the extraction results with the analytical-scale extractor (AE) of 1 g biomass. The extraction conditions chosen for the investigation of the scale-up were 60°C, 15% EtOH and 15 MPa. The temperature of 60°C was chosen to favor a lower energy consumption, required for the cosmetic industry, and to facilitate the extraction cell handling. A comparison between both extractors is presented in **Figure 3**.

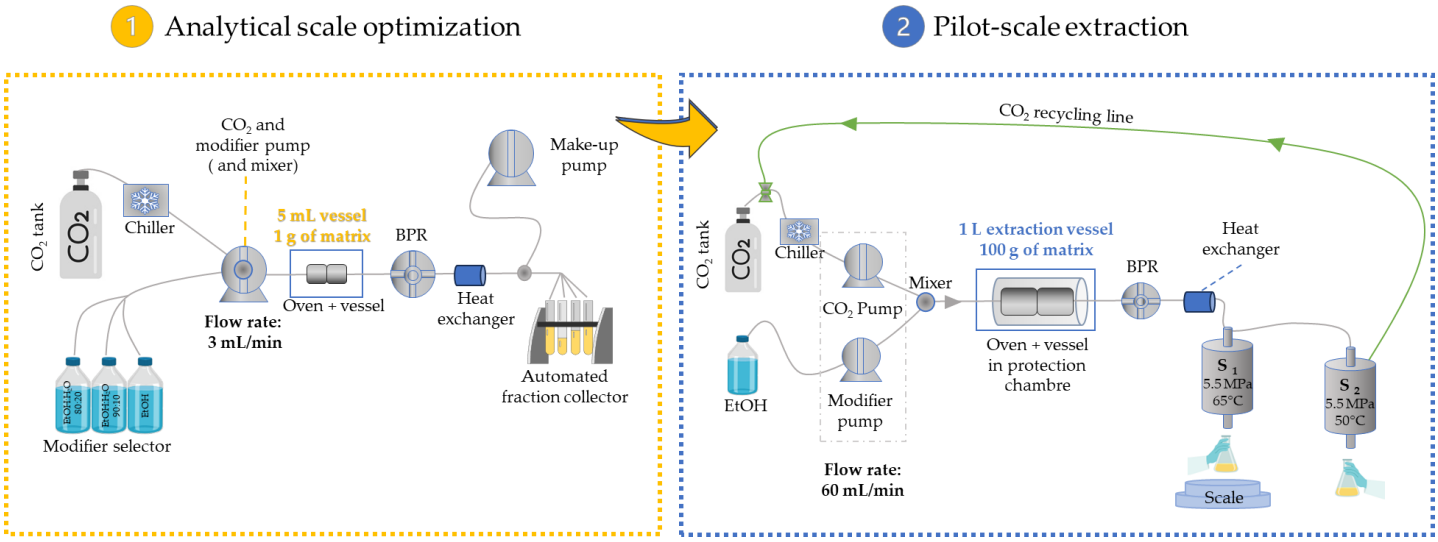


Figure 3: Schematic representation of the analytical scale extractor (AE) and pilot-scale extractor used in this study.

The results in **Figure 4** represented the cumulated fardiol myristate and faradiol palmitate yields normalized for 1 g of plant mass for both pilot and analytical scale extractors. When comparing the extraction kinetics of both AE and PE, the results in **Figure 4.a** showed that the analytical scale extractor offered a higher and faster extraction yield when compared to PE with 768% of difference at around 15 min time.

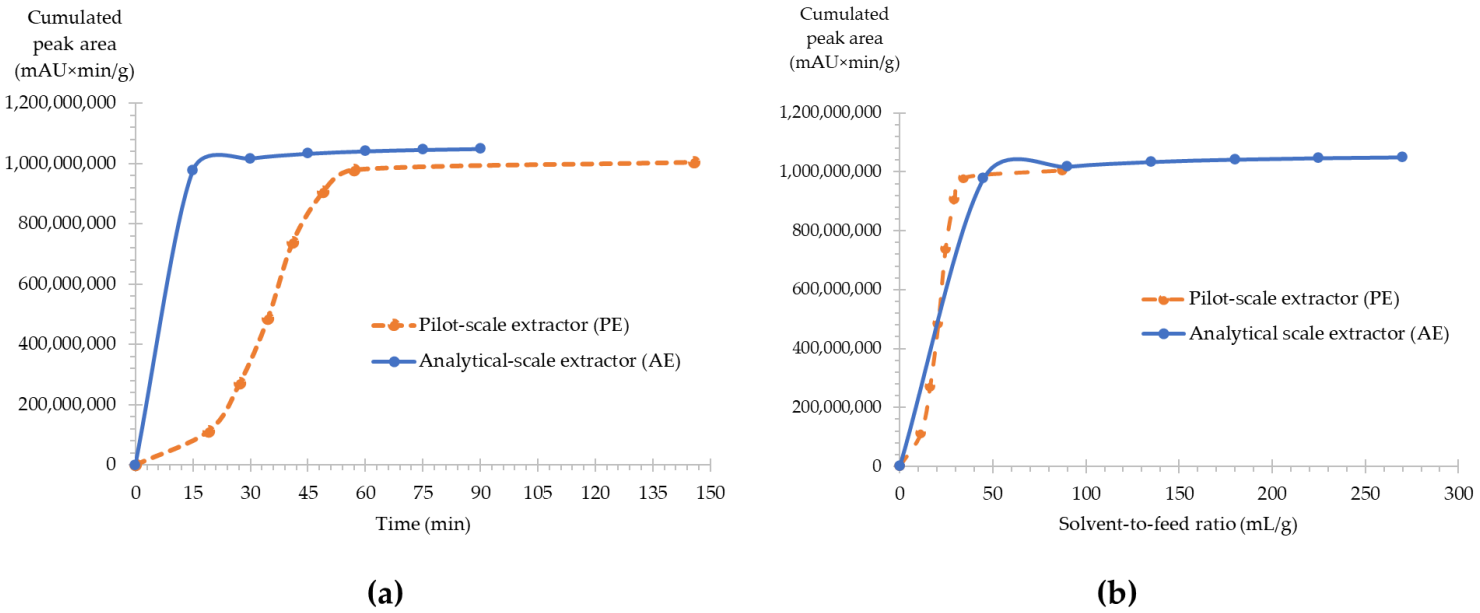


Figure 4: Comparison of the peak areas (mAU×min/g) of major faradiol myristate and faradiol palmitate, between analytical-scale extraction (AE) and pilot-scale extraction (PE) for CNPE. (a) Extraction kinetics of the total yield of faradiol (b) variation of the total yield in function to solvent-to-feed-ratio.

Though, the yield of PE slowly progressed to reach only 6.42% difference with AE at 60 min.
However, the kinetics representation is not ideal when working with different extraction flow rates thus the comparison between both extractors was also conducted in function to solvent-to-feed ratio F (mL/g). F was calculated following **Equation.2:**

$$F\ (mL/g) = [Flow\ rate\ (mL/min) \times Extraction\ time\ (min)]/Plant\ mass\ (g) \quad \textbf{(2)}$$

Indeed, when comparing AE and PE in function to solvent to flow ratio, it allows a higher understanding of the scale-up procedure. The results presented in **Figure 4.b** showed that both extractors had very similar results when flow rate, time and plant mass are considered.
Finally, PE fractions were analyzed to determine if polar compounds, specifically narcissin, were extracted. Since none of these compounds were detected, it demonstrated that scale-up offered an effective and selective extraction of non-polar compounds.

2.1.6. Comparison between supercritical fluid extraction (SFE) and ultrasound assisted extraction (UAE) for CNPE

SFE was also compared with UAE for the extraction of non-polar compounds. UAE is regarded as an efficient and simple extraction technique. This comparison was conducted in equivalent conditions of organic solvent consumption: 13.5 mL of solvent volume equivalent to 15% of modifier for 30 min at 3 mL/min flow. Only using modifier equivalent volume and not the whole CO_2-EtOH volume was chosen to reduce the consumption of organic solvents. In the case of using CO_2-EtOH volume consumed for 30 min at 3 mL/min, 90 mL of organic solvent would be used, very high volume for only 1 g of plants and that later needs to be evaporated.
Based on previous literature, three traditional extraction solvents were explored: heptane (hept), dichloromethane (DCM) and acetone (Ace). The results are presented in **Figure 5**.

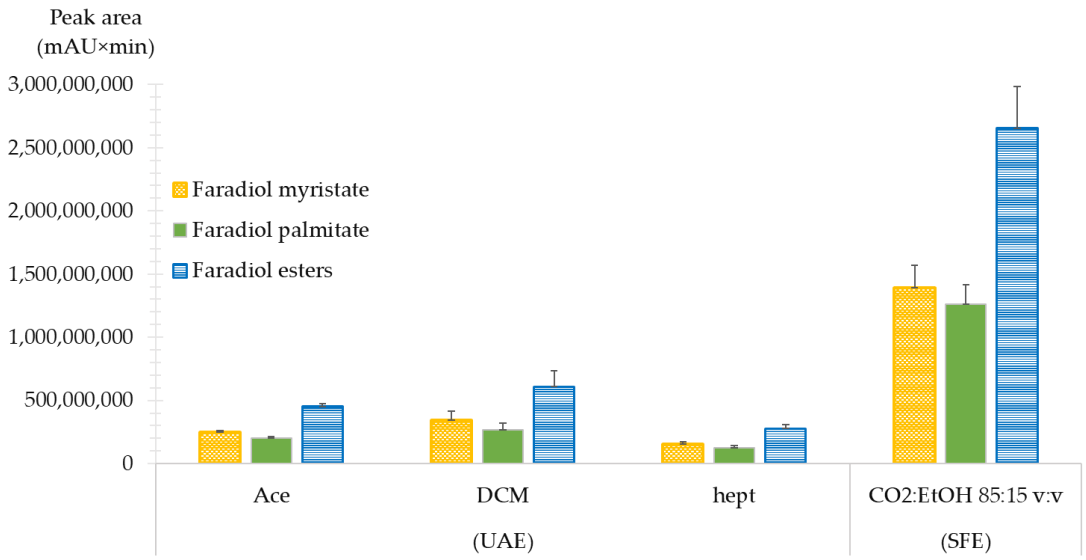


Figure 5: Comparison between supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) for CNPE faradiol esters (myristate and palmitate).

For all extracts, faradiol myristate was the major faradiol ester. However, significantly different yields were obtained from each technique.

The comparison between SFE and UAE demonstrated that SFE had a significantly higher extraction yield compared to UAE regardless of the solvent. The extraction recovery of faradiol esters with UAE using Ace, DCM and hept resulted in a relative yield percentage of 17, 23 and 10.4% respectively, compared to the SFE yield considered as 100%.

This indicated that SFE is highly advantageous for the recovery of high yield of non-polar compounds from *Calendula officinalis* L. and additionally it required the use of non-toxic solvents.

2.2.Optimization of marigold polar compounds extraction (CPE)

2.2.1. Statistical analysis, model, and factor significance

Based on the second BBD, SFE applied to marigold flowers polar compounds was optimized. The temperature was selected because its ability to impact the solubility of compounds in the SC-CO_2, and the modifier and water percentages were chosen because of their capacity to increase the polarity of the extraction phase (**Table 3**).

Table 3: Design matrix for the selective SFE design of experiment (BBD) and responses for polar compounds of interest from marigold flowers. (total polar molecules represent all polar compounds quantified at 354 nm including narcissin).

Experiment N°	Coded levels			Results (concentration mg/g of biomass after 15 min of extraction at 354nm)	
	X'_1	X'_2	X'_3	Narcissin (Y') (mg/g of biomass)	Total polar compounds (mg/g of biomass)
	Temperature (°C)	Modifier (%)	H_2O in modifier (%)		
1	40 (-1)	10 (-1)	10 (0)	0.00	0.01
2	80 (+1)	10 (-1)	10 (0)	0.00	0.00
3	40 (-1)	30 (+1)	10 (0)	1.10	2.52
4	80 (+1)	30 (+1)	10 (0)	1.71	3.85
5	40 (-1)	20 (0)	0 (-1)	0.01	0.03
6	80 (+1)	20 (0)	0 (-1)	0.04	0.12
7	**40 (-1)**	**20 (0)**	**20 (+1)**	**3.31**	**9.18**
8	**80 (+1)**	**20 (0)**	**20 (+1)**	**2.82**	**8.14**
9	60 (0)	10 (-1)	0 (-1)	0.00	0.00
10	60 (0)	30 (+1)	0 (-1)	0.05	0.12
11	60 (0)	10 (-1)	20 (+1)	0.00	0.00
12	**60 (0)**	**30 (+1)**	**20 (+1)**	**5.30**	**14.64**
13	60 (0)	20 (0)	10 (0)	0.11	0.33
14	60 (0)	20 (0)	10 (0)	0.13	0.37
15	60 (0)	20 (0)	10 (0)	0.14	0.42
16	60 (0)	20 (0)	10 (0)	0.21	0.57

After the pilot-scale extraction of non-polar compounds (see part 2.15), the plant mass was collected and used as treated biomass for the optimization of polar compounds extraction. The model's determination coefficient (R^2) was equal to 0.97, while the value of adjusted determination coefficient (adj R^2) was 0.94. This signifies that the model was unable to account for almost 3% of the total variance. The R^2 value demonstrates a highly strong correlation between the model's expected and experimental response values and indicated that the empirical model shows a good fit with experimental data. The Dixon statistical test was applied to the data set and displayed the presence of one experiment as an outlier, this was validated by the analysis of the residuals between predicted and experimental values, this point represented a repetition of the central point and was removed from the model. The relative standard deviation (RSD%) of the central point replicates (4 repetitions), for the concentration of narcissin (mg/g of plant), and after the elimination of one outlier was equal to 29.19%.

The results are shown in **Table 3** with the 16 experimental points left to create the model. The correlation between the three variables (X'_1, X'_2, and X'_3) and the response Y' (mg/g) is presented in **Equation 3**. The terms presented in this equation were chosen to obtain the highest possible predicted R^2, therefore increasing the predictive ability of the model for the response. The quadratic terms and the interaction between the

modifier percentage and water percentage in modifier were included, and the predicted R^2 was equal to 0.72.

$$Y' (mg/g) = 4.402 - 0.1134X'_1 - 0.09875X'_2 - 0.3241X'_3 + 0.0009531X'^2_1 + 0.001737\ X'^2_2 + 0.01016\ X'^2_3 + 0.01313X'_2\ X'_3 \quad (3)$$

The results of the polynomial model's analysis of variance (ANOVA) are summarized in **Table 4**, and the regression results indicated that the model is significant. Each factor's significance was assessed using the probability associated with its F-value and p-value, and the percentage of each factor's contribution to the model's response was examined. The marigold regression model's narcissin yield (mg/g) had an F-value of 34.3206 and a corresponding p-value of 0.0000. A highly significant model is presented by both statistical terms.

Table 4: ANOVA for response surface regression model for narcissin yield from selective CPE.

Source	DF	SS	MS	F value	p-value	Contribution (%)	Conclusion
Regression model	7	36.096	5.1566	34.3206	0.0000		Very significant
X'_1- Temperature (°C)	1	0.0028125	0.56408	3.7544	0.0887	3.02	Limit
X'_2- Modifier (%)	1	8.3232	0.22289	1.4835	0.2579	1.19	Not significant
X'_3- H2O in modifier (%)	1	16.046	4.9439	32.9049	0.0004	26.5	Very significant
$X'_1{}^2$	1	0.58141	0.58141	3.8697	0.0847	3.12	Limit
$X'_2{}^2$	1	0.12076	0.12076	0.8037	0.3962	0.647	Not significant
$X'_3{}^2$	1	4.1311	4.1311	27.4951	0.0008	22.1	Very significant
$X'_2 \times X'_3$	**1**	**6.8906**	**6.8906**	**45.8620**	**0.0001**	**36.9**	**Very significant**
Residuals	8	1.202	0.15025			6.44	
Total	15	37.298					
	Degree of freedom	Sum of squares	Mean square				

The factor X'_3 (H_2O percentage in modifier) and its quadratic term $X'_3{}^2$ ($H_2O\% \times H_2O\%$) were significant with 26.5% and 22.1% of contribution respectively. Their respective p-values of 0.0004 and 0.0008 validated this as well. However, the interaction term between the modifier's percentage and water's percentage ($X'_2 \times X'_3$) displayed the highest significance, contributed 36.9% to the model's response and presented a 0.0001 p-value. The temperature (X'_1) had a limited significance to the model's response with and 3.02% of contribution and p-values of 0.0887, as for the separate term of the modifier's percentage (X'_2), it was not significant with a contribution of only 1.19% and p-value of 0.2579.

2.2.2. Extraction parameters influence on CPE assessed with Box-Behnken design

Heat-maps were used as a graphical representation of the independent factors and dependent response interactions, they were correlated to the response of marigold flowers polar compounds narcissin (mg/g of plant), for 15 min of extraction time.

Since the temperature presented minimal influence on the response, **Figure 6** presented the influence of the total modifier percentage and water percentage included as an additive in the modifier on the narcissin yield. This demonstrated that both the water content and the modifier's percentage had a favorable effect on the extraction yield of narcissin. When the temperature was set at 60°C and no water was added to the modifier, narcissin yield is very low, 0.0 mg/g and 0.05 mg/g for the modifier percentage equal to 10% (exp n° 9) and 30% (exp n° 10).

However, the increase of modifier percentage at 20% H_2O in the modifier, increased

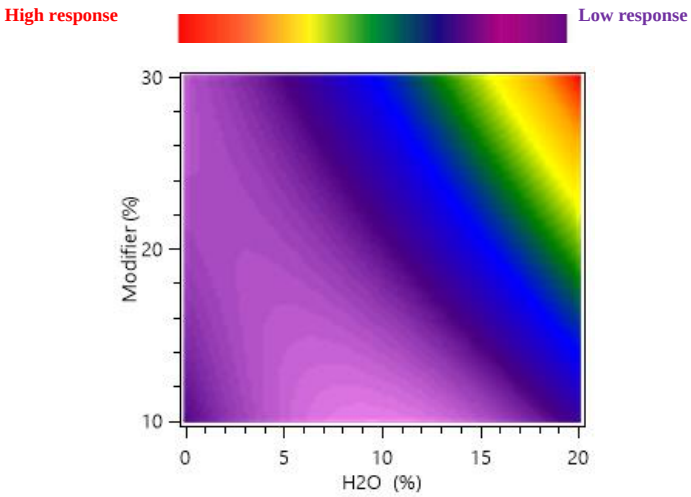


Figure 6: Response surface heat-maps showing the effect of the factor's variation on the narcissin yield (mg/g). Variation of total modifier percentage (*X*'2) and water percentage (*X*'3).

the predicted yield from 0.42 mg/g to 5.07 mg/g (1107.14% increase). These results confirmed the significant effect of water as an additive to extract polar compounds from marigold flowers, the same conclusion was made for the total polar compounds. The high polarity of narcissin is most likely responsible for this behavior. Indeed, SC-CO_2's low polarity limits its ability to extract non-polar or moderately polar compounds. However, the extraction phase's polarity was boosted by the addition of ethanol and water as modifiers, which increased the affinity of polar molecules like the target polyphenols and raised the recovery yield.

2.2.3. *Optimal selective extraction of narcissin*

Based on **Equation 3**, the computed optimal extraction conditions to recover the maximum amount yield are the flowing: temperature of 80°C, 30% EtOH:H_2O 80:20 *v:v*, this allowed for the recovery of 5.5 mg/g of narcissin and 14.9 mg/g of total polar compounds. These values are higher than the ones obtained by using ethanol/water/propane diol mixtures [63]. However, since the temperature had a low significance to the model's response, the yield for 40°C and the same amount and composition of modifier, allowed for the recovery of 5.45 mg/g of narcissin and 14.8 mg/g of total polar compounds. This showed a minimal decrease in the yield, but it allowed a significate reduction in energy consumption for the SFE of polar *Calendula officinalis* L. compounds.

To validate the experimental design results and determine the optimal extraction time, one hour extraction kinetics was done at 40,60 and 80°C with the use of 30% EtOH:H_2O 80:20 *v:v* as a modifier (**Figure 7**) .

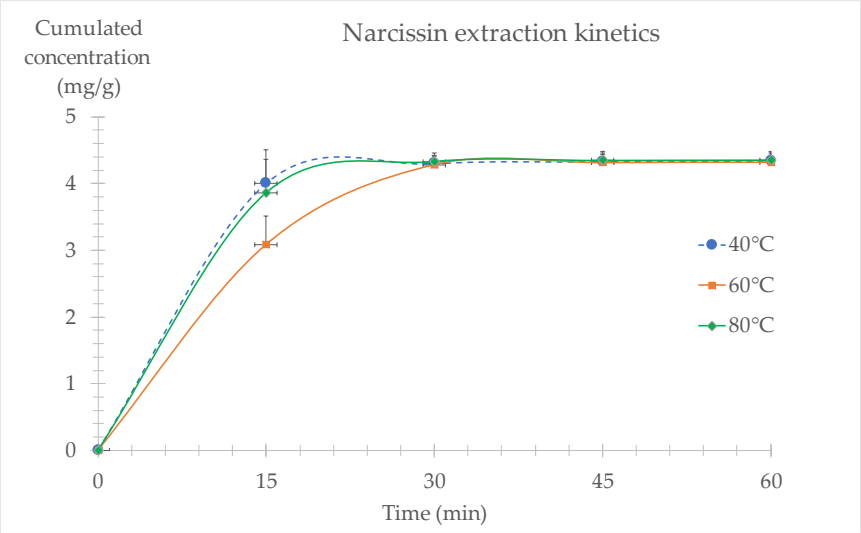


Figure 7: SFE kinetics of narcissin (mg/g) from CPE, variation of temperature 40,60 and 80°C constant conditions: 30% EtOH:H2O 80:20 v:v as a modifier, 15 MPa, 3 mL/min

It showed that after 15 min of extraction time for a set temperature of 40,60 and 80°C the narcissin yield was of 4.00, 3.08 and 3.80 respectively.

In addition, the cumulated yield at 30 min was 4.30, 4.28 and 4.33 respectively. The yield did not increase after this point, demonstrating that 30 min of extraction is sufficient for the extraction of polar compounds from *Calendula officinalis* L. and validating that the use of 40°C can recover similar amounts of narcissin with a lower consumption of energy.

2.2.4. *Comparison between SFE and UAE for CPE*

The UAE extraction of treated and non-treated biomass (the same batch of plant without a prior SFE of non-polar compounds) was conducted in equivalent condition to the optimized SFE extraction of polar compounds from marigold, 27 mL of EtOH:H_2O solvents with 50:50 and 80:20 *v:v* were used, which is equivalent to a 30 min extraction with 30% modifier at 3 mL/min flow rate. The results were compared with optimized SFE extraction conditions at 40°C and 30% EtOH:H_2O 80:20 *v:v* (**Figure 8**).

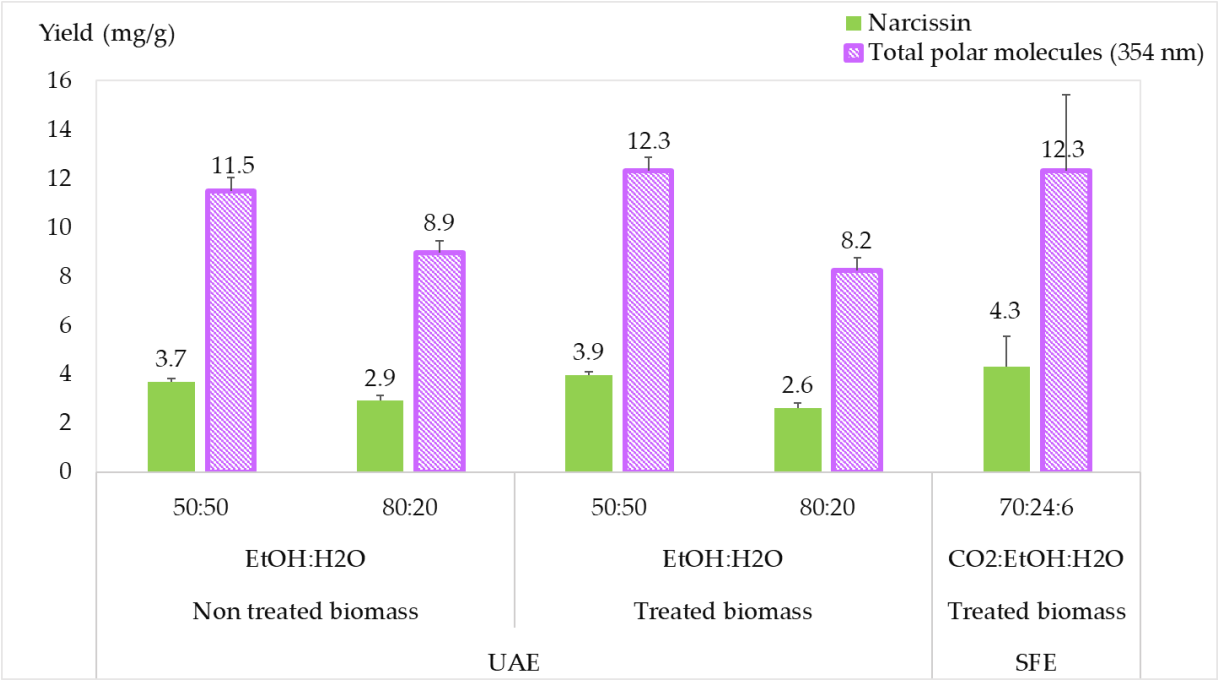


Figure 8: Comparison between supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) for polar compounds of CPE (total polar molecules represent all polar compounds quantified at 354 nm including narcissin).

The comparison between the treated and non-treated biomass for UAE extraction, demonstrated that the prior treatment with the pilot-scale extraction of faradiol esters did not influence the extraction of narcissin or total polar compounds from *Calendula officinalis* L. flowers.

Besides, for the UAE of treated biomass the increase of water percentage in the extraction solvent from 20 to 50% resulted in the increase of the extraction yield of total polar compounds and specifically narcissin by 50%, producing a yield that is comparable to that of the SFE extraction. This increase is due to the polarity of the compounds and mainly narcissin which is a glycosylated polyphenol with a log P of -1, making it a highly polar compound with a considerable affinity for extraction solvents with higher percentages of water.

However, extracts with high water content (50%) are generally less stable microbiologically and require evaporation of the extraction solvent and consumption of energy. Consequently, SFE, which only uses 6% of water in the extraction fluid can offer extract with higher concentration of bioactive compounds while reducing the final water% in the extracts, that subsequently favors the stability of the extract and decreases the need for further evaporation.

Validation of S³FE

The selective extraction of both non-polar compounds and polar compounds in *Calendula officinalis* L. have been optimized using two separate BBD. The results showed that a high temperature of 80°C combined with 15% EtOH allowed for the recovery of a high yield of faradiol esters (myristate and palmitate), and the residue could be extracted with a using 30% EtOH:H_2O 80:20 *v:v* at 40°C to recover the polar compounds.

To validate the sequential extraction of both steps, a sequential selective supercritical extraction (S³FE) was applied to marigold flowers non-treated powder. The first step used 15% EtOH at 80°C and the second step 30% EtOH:H_2O 80:20 *v:v* at 40°C, both steps were of 30 min making a total extraction time of one hour, fractions were collected every 15 minutes and a 5 min cool time was required between the two steps. All fractions were analyzed using SFC-ELSD, UC-DAD and UHPLC-DAD, to determine their content of molecules of interest. The results were illustrated by the means of percentage of yield for the total one hour of extraction (**Figure 9**), the results showed that a full selective and sequential extraction was achieved with both steps.

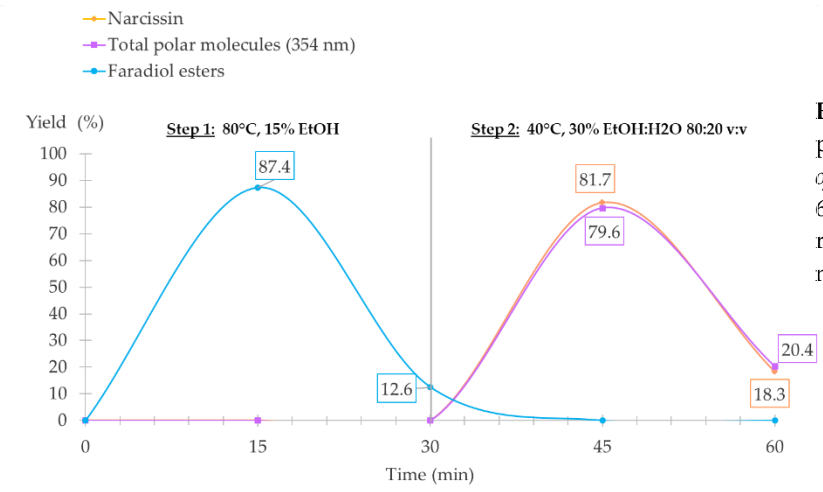


Figure 9: Extraction kinetics of the S3FE of non-polar and polar compounds from *Calendula officinalis* L. represented in yield percentage for 60 min of extraction (total polar molecules represent all polar compounds quantified at 354 nm including narcissin).

The first step of 30 min assured a faradiol esters rich extract with the first 15 min, 87.4% of faradiol esters were extracted, the remaining amount was collected in the second 15

min fraction with 12.6%. The UC-DAD analysis showed no co-extraction of polar compounds, which validates the results of the experimental design. In the second step, the following 30 min allowed for the extraction of polar compounds including narcissin. Similar extraction kinetics were noticed for the polar compounds. The first 15 min of the second step allowed for the extraction of 79.6% of polar compounds and 81.7% of the narcissin yield both compared to the total yield recovered at the end of the extraction. The final 15 min of the second step allowed for the recovery of the remaining molecules of interest with a yield 20.4% of polar compounds and 18.3% for narcissin, the yield of the polar compounds was equivalent to the amount of optimized determined conditions of the BBD with 4.63 mg/g and 13.85 mg/g of narcissin and total polar compounds respectively.

Consequently, the first step of CNPE did not significantly influence the recovery of polar compounds, regarding the extraction kinetic in **Figure 7**, this validates that the S^3FE approach is applicable for fast and sequential recovery of both non-polar and polar compounds from *Calendula officinalis* L. flowers.

3. Methodology

3.1.Plant material

The dried flower of the marigold (*Calendula officinalis* L.) plant consisted of a light brown, yellow powder, which was provided by PMA 28 (Varize, France), it was kept at room temperature in an airtight container.

3.2.Chemical and reagents

Air Liquide (Fleury-les-Aubrais, France) provided the CO_2 gas. Acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM), acetone (Ace), heptane (hept), dimethylsulfoxide (DMSO) and ethanol (EtOH) were provided by VWR (Fontenay-sous-Bois, France). Formic acid (FA) and methanesulfonic acid (MSA) were purchased from Sigma-Aldrich (Merck, Semoy, France). Ultra-pure water (H_2O) was purified using a Milli-Q system from Sigma-Aldrich with a resistance >18 MΩ.cm. Narcissin (Isorhamnetin-3-*O*-rutinoside) standard was used to identify and quantify the compound (Purity (HPLC) ≥99%), it was supplied by Extrasynthese (Genay, France).

3.3.SC-CO_2 extraction

For the analytical scale SFE, a Waters MV-10® ASFE (Milford, Massachusetts, United States) was used for all extractions. 1 g of plant powder was combined with 1 g of diatomaceous earth powder from Sigma-Aldrich (Merck, Semoy, France) in a stainless-steel extraction vessel (5 mL). To filter the extract and completely fill the extraction cell, cotton was positioned at the top and bottom part. A continuous dynamic extraction was used, with a 3 mL/min flow rate. The amount of modifier added was calculated based on this overall extraction flow.

For the pilot-scale extraction of non-polar compounds, an SFE process (Tomblaine, France) extractor was used. The extractor is equipped with a CO_2 and co-solvent pump. A gravity separator (S_1) and a cyclone separator (S_2) were used to collect the extracts. The extraction conditions were set to 60°C temperature, 15 MPa pressure, total flow of 60 mL/min and 15% of EtOH (96% purity) added as a modifier to the SC-CO_2. One litre stainless-steel extraction vessel was used, it was filled with 100 g of plant material combined with 100 g of diatomaceous earth. The total duration of the EtOH modified extraction was 57 min, 6 fractions were weighed and collected from S_1. Approximately 50 g of extract were collected for each fraction (around 9 min for each fraction). A final step (fraction 7) of 90 min with 100% SC-CO_2 was applied to dry the plant mass, removing the residual EtOH and collect extract residues. At the end of the extraction the content of the S_2 of the total extraction duration was collected for analysis.

3.4.Ultra-sound assisted extraction (UAE)

The UAE was performed in a Branson 3510 (Bransonic® ultrasonic) bath from Sigma-Aldrich (Merck, Semoy, France), the power of the extractor was equal to 130 W. Using 1 g of C*alendula offininalis* L. flower powder mixed with solvents in equivalent volume consumption to the determined SFE optimized conditions, an extraction duration of 30 min was applied for all extractions.

To examine the extraction yields of calendula polar extracts (CPE), EtOH:H_2O *v:v* in 80:20 and 50:50 composition were utilized as extraction solvents, with a volume of 27 mL. As for calendula non-polar extracts (CNPE), three non-polar solvents were used: heptane (hept), acetone (Ace) and dichloromethane (DCM) with a volume of 13.5 mL. All extractions were done in duplicate to evaluate the repeatability.

After sonication, the extracts were centrifuged at 10000 rpm for 10 min at 25°C and filtered using a 0.45 μm polyvinylidene difluoride (PVDF) syringe filter from Agilent Technologies (Les Ulis, France).

3.5.SC-CO_2 extracts sample treatment

To compare and determine the analytical-scale extract yields for the target compounds, all extracts were evaporated using a nitrogen flow for a maximum of 24 hours. The non-polar extracts (CNPE) were diluted with 1 mL of a mixture of DCM:MeOH 1:1 *v:v* for SFC analysis. The polar extracts (CPE) were diluted in 12 mL of MeOH:DMSO:H_2O 9:2:1 *v:v:v*, then they were sonicated for 40 minutes for total solubilization, they were filtered using 0.45 μm PVDF and later diluted 4 times using H_2O for UHPLC analysis.

For the pilot-scale extracts of non-polar compounds, all fractions volume was adjusted to 80 mL of EtOH to compare and determine the extraction yield. 1 mL of each fraction was collected and evaporated using a nitrogen stream. The extracts were analyzed using the CNPE chromatographic method, and the results were normalized to 1 g of extract to compare with the analytical-scale extractor.

3.6.Calendula non-polar extracts (CNPE) chromatographic analysis

3.6.1. Ultra-high efficiency low-pressure / supercritical fluid chromatography (UHLP/SFC-ELSD): terpenoid esters analysis in CNPE

Shimadzu Corporation's (Kyoto, Japan) Nexera UC system was used for all analysis of terpenoid esters. This system included an autosampler (SIL-30AC), column ovens (CT0-20AC), a photodiode array (PDA) detector (SPD-M20A), a back-pressure regulator (BPR) (SFC-30A), a carbon dioxide pump (LC-30ADsf), a modifier pump (LC-30AD), and an Evaporative light scattering detector (ELSD-LT-III). Shimadzu Corporation, LabSolutions LCMS version 5.93 was used to record all chromatograms. The CNPE triterpenoid esters were separated using UHLP/SFC and detected using an ELSD, due to the absence of chromophore groups.

Five octadecyl-bonded silica columns (75 cm of total length) used for the separation, including four Kinetex C18 (150×4.6 mm), 2.6 μm superficially porous particles, from Phenomenex (Le Pecq, France) and one Accucore C18 (150×4.6 mm) 2.6 μm

superficially porous particles, from Thermo-electron (les Ulis, France) were used in tandem based on previously published work [64–66]. Some of the conditions were modified from the above-mentioned method, the column oven temperature set at 15°C. Isocratic analyses were performed during 55 min with 80% of SC-CO_2 and 20% of co-solvent composed of MeOH:ACN 75:25 (*v:v*). The total flow rate was 1.6 mL/min. Injection volume was set at 5 µL for all samples. The back-pressure regulator was set at 100 bar and heated at 60°C to limit the effect of CO_2 cold depressurization. The automated sampler was kept at 25°C to avoid the precipitation of the extracts. The compounds were detected using an ELSD with a temperature of 40°C, filter of 4 seconds, a nitrogen pressure of 3 bars and a gain set on wide.
To compare the yields of the CNPE, the peak areas of the two major terpenoid esters (faradiol-3-O-palmitate, faradiol-3-O-myristate) were evaluated (**Figure 10**).

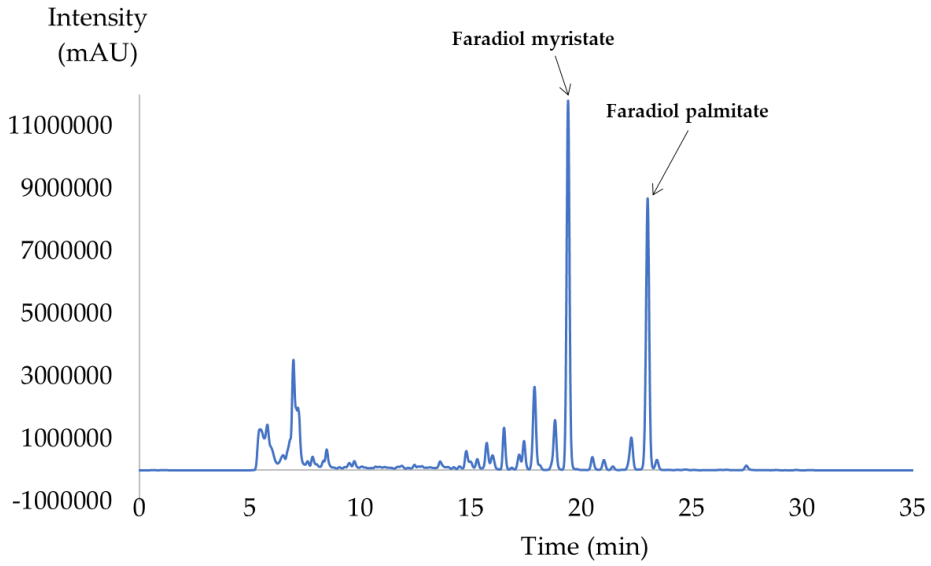


Figure 10: UHLP/SFC-ELSD analysis of calendula non-polar extract (CNPE). Peak identification (1) faradiol-3-O-myristate (tr =19.78 min), (2) fardiol-3-O-palmitate (tr=23.46 min).

3.6.2. Unified chromatography UC-DAD: analysis of polar compounds in CNPE

To achieve a selective sequential SFE of non-polar compounds without the co-extraction of polar molecules, the CNPE needed to be evaluated for their content in targeted polar compounds. The analysis of polar compounds (narcissin) solubilized in pure organic solvent (MeOH:DCM 1:1 *v:v*) in reversed phase UHPLC may lead to the precipitation of the non-polar compounds in the column, due to the presence of water in the mobile phase.

Therefore, to prevent this matter all non-polar extract were additionally analyzed using UC-DAD with an adapted gradient based on previously published work [67]. This was done using a Waters Corporation (Milford, Massachusetts, United States) ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) system equipped with a diode-array (ACQUITY PDA®). The analysis was carried out using a Torus DEA (100 × 2.1 mm; 1.7 µm) commercialized by Waters. The mobile phase was composed of CO_2 and methanol acidified with 0.1% MSA, the column oven was heated at 25°C. Similarly, the automated sampler was also kept at 25°C to avoid the precipitation of the extracts during the analysis sequence. The gradient is represented in **Figure 11a**.

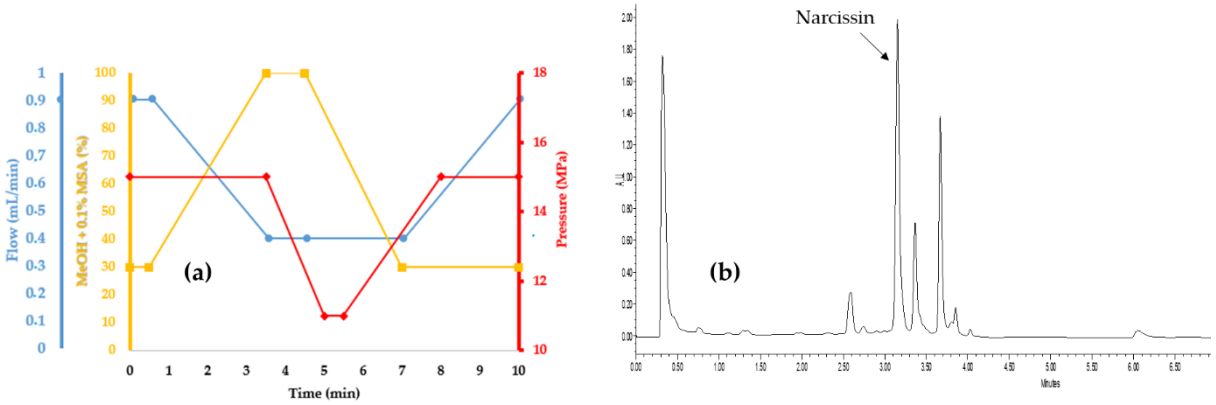


Figure 11: (a) UC-DAD gradient of polar compounds in CPE. Bleu: flow (mL/min), yellow: MeOH+0.1% MSA (%) and red: pressure (MPa). (b) UC-DAD analysis of polar compounds in CNPE (354 nm)/ SFE extract solubilized in MeOH:DCM 1:1 v:v. narcissin (tr=3.15 min).

Due to the high inlet pressure generated at the end of the gradient by the high viscosity of the mobile phase that reached 100% of organic solvent, a reversed pressure and flow rate gradients were applied. The chromatograms were recorded with DAD in the 190–800 nm range. Visualization and peak integration were done at 354 nm **Figure 11b**. Since the column is of polar nature (Diethylamine), only the polar compounds (narcissin and other flavonoids) interacted with the stationary phase and therefore eluted later in the gradient with higher percentages of the modifier, while the non-polar compounds were not retained and eluted in the dead time.

3.7.Calendula polar extracts (CPE): chromatographic analysis of polyphenols

All polar extracts were analyzed with a Nexera-LC40 system of Shimadzu Corporation (Kyoto, Japan). This system was equipped with a photodiode array (PDA) detector (SPD-40), a solvent delivery unit (LC-40), an auto-sampler (SIL-40), a column oven (CTO-40) and a system controller SCL-40. All chromatograms were recorded on Lab-Solutions LC-UV 5.97 SP1 version (Shimadzu Corporation, Kyoto, Japan).
A Cortecs C18 (100 ×3.0 mm) column coupled to a Cortecs C18 VanGuard Cartridge (5 ×2.1 mm) both packed with 2.7 μm superficially porous particles, from Waters Corporation (Milford, Massachusetts, United States) were used for the analysis of all extracts. The column temperature was maintained at 30°C. The injection volume was 5 μL and the automated sampler was kept at 10°C. The flow rate was maintained at 1 mL/min. Equilibration time between two injections was 5 min.
The total time of each analysis was 11 min, the mobile phase consisted of a combination of H_2O acidified with 0.1% of FA (solvent A) and ACN (solvent B). The percentage of solvent B varied as follows: 0-1 min: 10-25% B, 1-3 min: 25% B, 3-6 min: 90% B, 6.1-11 min: 10% B. The separation of CPE is presented in **Figure 12**.

Quantitative analysis of narcissin was performed by injecting a standard at 15 different concentrations from 0.001 mg/mL to 0.8 mg/mL. The calibration curve was obtained at 354 nm ($y=10^7x+48684$, $R^2 = 0.9937$), the equation was used to estimate the concentrations from peak area for narcissin (y = concentration; x = peak area). The total polar compounds yield was estimated using the same equation as an equivalent to narcissin, this approximation was possible due to their similar molar absorption coefficient (ε) at 354 nm.

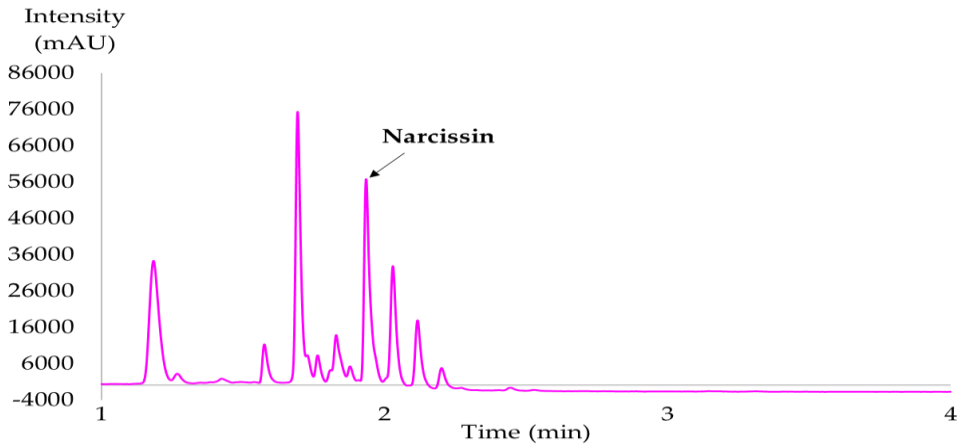


Figure 12: UHPLC-DAD analysis of polar extracts (354 nm); narcissin (tr=1,93 min).

3.8.Experimental design and statistical analysis

Ellistat software 6.4 2020/11 version (Poisy, France) was used for the experimental design and data analysis. A Box–Behnken design (BBD) with response surface methodology (RSM) was chosen to establish the model and to determine the response pattern. It was used to optimize the supercritical fluid extraction of both polar and non-polar targeted compounds from *Calendula officinalis* L. flowers. For the optimization of CNPE extraction three independent factors used in this study were temperature (X_1), pressure (X_2) and EtOH percentage as a polarity modifier (X_3). As for CPE extraction the three independent factors used were temperature (X'_1), total modifier percentage that consisted of EtOH or EtOH:H_2O *v:v* mixture (X'_2) and water percentage added as an additive to the modifier (X'_3), while the pressure was kept constant at 15 MPa. The three levels that were used for both experimental designs were coded (+1) for the highest, (0) for the middle and (-1) for the lowest level (**Table 1 & 3**). Both the regression model and the graphical analysis of the data were performed using the same software. The significance of the independent factors on the response was evaluated using an ANOVA analysis of variance. Significant factors were identified by a p-value of 0.05 or lower. This was done using statistical tests such as Fisher's test (F-value), the classification of the significance of the model was done following the contribution percentage of the terms to the model. The fitness of the design model was evaluated using the correlation coefficient (R^2), the adjusted correlation coefficient (adj R^2) and predicted R^2 (prd R^2). Two-dimensional heat-map plots were used to illustrate the interaction between the factors. The regression equation that represented the predictive model was solved to obtain the optimal extraction conditions.

Flow rate and plant mass were kept constant in all SFE experiments. Extraction durations were determined after a kinetics of extraction at the central level

experimental conditions (X_1, X_2, X_3, X'_1, X'_2 and X'_3 equal to 0). 30 minutes were used for the CNPE design and 15 min for the CPE design.

For CNPE the dependent response or output (Y) was the sum of peak area of both major terpenoid esters (faradiol-3-*O*-palmitate, faradiol-3-*O*-myristate) found in marigold (mAU×min) for 30 minutes of extraction duration. As for CPE the dependent response (Y') was the yield of the targeted compound narcissin (mg/g) for 15 minutes of extraction duration. The experiments were randomized to maximize the effect of the variability in the response. Five replicates at the central level experimental conditions (X_1 *and* $X'_1=0$, X_2 *and* $X'_2=0$, *and* $X'_3=0$) of the design were conducted to evaluate the experimental repeatability. The relative standard deviation (RSD) was estimated to confirm the reproducibility of the extraction model. The Dixon statistical test was applied to determine the presence of an outlier in the results with a 95% range. The polynomial equations 3 and 4 for CNPE and CPE respectively, represented the relationship between the responses (Y *and* Y') and the corresponding three independent variables (X_1, X_2, X_3, X'_1, X'_2, *and* X'_3). The equation terms were selected to represent the regression of the model while avoiding the overfitting of the data. Consequently, for equations 4 and 5 the identified terms were chosen to optimize the prediction of the model and therefore increase the predicted R^2.

$$Y\,(mAU\times min) = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_2+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{13}X_1X_3 \quad \textbf{(4)}$$

$$Y'\,(mg/g) = \delta_0+\delta_1X'_1+\delta_2X'_2+\delta_3X'_2+\delta_{11}X'^2_1+\delta_{22}X'^2_2+\delta_{33}X'^2_3+\delta_{23}X'_2X'_3 \quad \textbf{(5)}$$

With β_{11}, β_{22}, β_{33}, δ_{11}, δ_{22}, and δ_{33} representing the quadratic coefficients, and β_{13} and δ_{23} representing the interaction coefficients between the factors. The terms X_1, X_2 *and* X_3 represented the variables: temperature, pressure and EtOH% used as a modifier respectively. As for X'_1, X'_2 *and* X'_3 represented the factors: temperature, modifier percentage and water percentage in modifier respectively.

4. Conclusion

This study demonstrated the feasibility of a sequential selective extraction of triterpendiol esters and polyphenols from *Calendula officinalis* L. flowers with supercritical fluids. Two experimental designs adapted to the polarity of the targeted molecules, were used for a simple optimization of their extraction. First, the optimization non-polar molecules allowed to recover high yield extracts (around 90 mg/g), rich in major anti-edematous compounds (faradiol myristate and faradiol palmitate). This was achieved with the use of a high temperature of 80°C combined with a pressure of 15 MPa and 15% EtOH in carbon dioxide reaching a dried extract mass of 100.3 mg/g. The extraction of these non-polar compounds was scaled-up to a pilot-scale extractor using 100 g of plant mass, the results of both analytical and pilot scales were similar when comparing solvent-to-feed ratios. Third, the optimization of polar compounds was conducted using a BBD and high concentration of polyphenols was reached in SFE at 40°C, with 30% EtOH:H_2O 80:20 *v:v*, for which 12.3 mg/g of polar compounds were recovered. This allowed for the sequential selective extraction of both non-polar and polar compounds of marigold within a 60 min extraction with only two steps on-line. Finally, the SFE results were compared to UAE using comparable solvents and time conditions, it showed that for non-polar extraction SFE was highly advantageous compared to UAE both in terms of extractions yields and the use of green solvents. For the polar UAE extraction, SFE had comparable results to UAE showing the interest of using SFE for the extraction of polar molecules, in addition to reducing water percentage in final extract, favoring the stability of the extracts, and reducing the energy cost of water evaporation.

5. References

[1] W. Xiao, L. Han, B. Shi, *Separation and Purification Technology*. **2008**, *62* (3), 614–618. DOI: 10.1016/j.seppur.2008.03.025.

[2] 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, *Journal of Food Engineering*. **2013**, *117* (4), 426–436. DOI: 10.1016/j.jfoodeng.2013.01.014.

[3] N. Mezzomo, J. Martínez, S. R. S. Ferreira, *The Journal of Supercritical Fluids*. **2009**, *51* (1), 10–16. DOI: 10.1016/j.supflu.2009.07.008.

[4] J. M. Prado, I. Dalmolin, N. D. D. Carareto, R. C. Basso, A. J. A. Meirelles, J. Vladimir Oliveira, E. A. C. Batista, M. A. A. Meireles, *Journal of Food Engineering*. **2012**, *109* (2), 249–257. DOI: 10.1016/j.jfoodeng.2011.10.007.

[5] J. M. Prado, G. H. C. Prado, M. A. A. Meireles, *The Journal of Supercritical Fluids*. **2011**, *56* (3), 231–237. DOI: 10.1016/j.supflu.2010.10.036.

[6] M. T. Fernández-Ponce, B. R. Parjikolaei, H. N. Lari, L. Casas, C. Mantell, E. J. Martínez de la Ossa, *Chemical Engineering Journal*. **2016**, *299*, 420–430. DOI: 10.1016/j.cej.2016.04.046.

[7] N. Vafaei, C. B. Rempel, M. G. Scanlon, P. J. H. Jones, M. N. A. Eskin, *AppliedChem*. **2022**, *2* (2), 68–92. DOI: 10.3390/appliedchem2020005.

[8] V. Larocca, M. Martino, M. Trupo, R. A. Magarelli, A. Spagnoletta, A. Ambrico, *Biomass Conv. Bioref.* **2023**. DOI: 10.1007/s13399-023-04434-z.

[9] I. Solaberrieta, A. C. Mellinas, J. Espagnol, M. Hamzaoui, A. Jiménez, M. C. Garrigós, *Foods*. **2022**, *11* (16), 2408. DOI: 10.3390/foods11162408.

[10] S. Rajput, S. Kaur, P. S. Panesar, A. Thakur, *Biomass Conv. Bioref.* **2022**. DOI: 10.1007/s13399-022-02807-4.

[11] S. Atwi-Ghaddar, L. Zerwette, E. Destandau, E. Lesellier, *Molecules*. **2023**, *28* (14), 5485. DOI: 10.3390/molecules28145485.

[12] K. A. Avilés-Betanzos, M. Scampicchio, G. Ferrentino, M. O. Ramírez-Sucre, I. M. Rodríguez-Buenfil, *Processes*. **2023**, *11* (7), 2055. DOI: 10.3390/pr11072055.

[13] C. Dauber, T. Carreras, A. Fernández Fernández, B. Irigaray, S. Albores, A. Gámbaro, E. Ibáñez, I. Vieitez, *The Journal of Supercritical Fluids*. **2022**, *180*, 105460. DOI: 10.1016/j.supflu.2021.105460.

[14] S. Atwi-Ghaddar, E. Destandau, E. Lesellier, *Journal of CO2 Utilization*. **2023**, *70*, 102440. DOI: 10.1016/j.jcou.2023.102440.

[15] R. John, D. Jan, *Proceedings of the Indian National Science Academy*. **2017**, *93*. DOI: 10.16943/ptinsa/2017/49126.

[16] K. Shahane, M. Kshirsagar, S. Tambe, D. Jain, S. Rout, M. K. M. Ferreira, S. Mali, P. Amin, P. P. Srivastav, J. Cruz, et *al.*, *Pharmaceuticals*. **2023**, *16* (4), 611. DOI: 10.3390/ph16040611.

[17] E. Saffari, S. Mohammad-Alizadeh-Charandabi, M. Adibpour, M. Mirghafourvand, Y. Javadzadeh, *Women & Health*. **2017**, *57* (10), 1145–1160. DOI: 10.1080/03630242.2016.1263272.

[18] C. De Angelis, A. Di Stadio, S. Vitale, G. Saccone, M. C. D. Angelis, B. Zizolfi, A. Di Spiezio Sardo, *The Journal of Maternal-Fetal & Neonatal Medicine*. **2022**, *35* (10), 1860–1864. DOI: 10.1080/14767058.2020.1770219.

[19] M. Buzzi, F. de Freitas, M. de Barros Winter, *J Wound Care*. **2016**, *25* (12), 732–739. DOI: 10.12968/jowc.2016.25.12.732.

[20] G. S. Giostri, E. M. Novak, M. Buzzi, L. C. Guarita-Souza, *Tissue Barriers*. **2022**, *10* (3), 1994822. DOI: 10.1080/21688370.2021.1994822.

[21] N. Babaee, D. Moslemi, M. Khalilpour, F. Vejdani, Y. Moghadamnia, A. Bijani, M. Baradaran, M. T. Kazemi, A. Khalilpour, M. Pouramir, et *al.*, *DARU J Pharm Sci*. **2013**, *21* (1), 18. DOI: 10.1186/2008-2231-21-18.

[22] P. Pommier, F. Gomez, M. p. Sunyach, A. D'Hombres, C. Carrie, X. Montbarbon, *JCO*. **2004**, *22* (8), 1447–1453. DOI: 10.1200/JCO.2004.07.063.

[23] Y. Panahi, M. R. Sharif, A. Sharif, F. Beiraghdar, Z. Zahiri, G. Amirchoopani, E. T. Marzony, A. Sahebkar, *The Scientific World Journal*. **2012**, *2012*, e810234. DOI: 10.1100/2012/810234.

[24] Z. Kasprzyk, J. Pyrek, *Phytochemistry*. **1968**, *7* (9), 1631–1639. DOI: 10.1016/S0031-9422(00)88616-1.

[25] H. Neukirch, M. D'Ambrosio, J. D. Via, A. Guerriero, *Phytochemical Analysis*. **2004**, *15* (1), 30–35. DOI: 10.1002/pca.739.

[26] G. Reznicek, K. Zitterl-Eglseer, *Scientia Pharmaceutica*. **2003**, *71* (2), 121–128. DOI: 10.3797/scipharm.aut-03-13.

[27] M. Ukiya, T. Akihisa, K. Yasukawa, H. Tokuda, T. Suzuki, Y. Kimura, *J. Nat. Prod.* **2006**, *69* (12), 1692–1696. DOI: 10.1021/np068016b.

[28] T. Naved, S. H. Ansari, H. M. Mukhtar, M. Ali, *ChemInform*. **2005**, *36* (39). DOI: 10.1002/chin.200539165.

[29] B. Wiłkomirski, *Phytochemistry*. **1985**, *24* (12), 3066–3067. DOI: 10.1016/0031-9422(85)80062-5.

[30] S. KISHIMOTO, T. MAOKA, K. SUMITOMO, A. OHMIYA, *Bioscience, Biotechnology, and Biochemistry*. **2005**, *69* (11), 2122–2128. DOI: 10.1271/bbb.69.2122.

[31] A. Pintea, *Acta Biologica Szegediensis*. **2003**, *47* (1–4), 37–40.

[32] N. T. Ul'chenko, A. I. Glushenkova, Kh. S. Mukhamedova, *Chem Nat Compd*. **1998**, *34* (3), 272–274. DOI: 10.1007/BF02282400.

[33] B. Król, T. Paszko, *Industrial Crops and Products*. **2017**, *97*, 242–251. DOI: 10.1016/j.indcrop.2016.12.029.

[34] R. J. Martin, R. J. Martin, N. G. Porter, N. G. Porter, B. Deo, B. Deo, **2005**, *35*, 129–137.

[35] M. Garaiová, Q. Hua, R. Holič, *Journal of Agricultural and Food Chemistry*. **2023**. DOI: 10.1021/acs.jafc.2c08967.

[36] A. K. Shakib, A. R. Nejad, A. Khalighi, *Research on Crops*. **2010**, *11* (3), 728–732.

[37] B. Wilkomirski, Z. Kasprzyk, *Phytochemistry*. **1979**, *18* (2), 253–255. DOI: 10.1016/0031-9422(79)80064-3.

[38] T. C. S. P. Pires, M. I. Dias, L. Barros, I. C. F. R. Ferreira, *Food Chemistry*. **2017**, *220*, 337–343. DOI: 10.1016/j.foodchem.2016.10.026.

[39] M. Miguel, L. Barros, C. Pereira, R. C. Calhelha, P. A. Garcia, M. Á. Castro, C. Santos-Buelga, I. C. F. R. Ferreira, *Food Funct.* **2016**, *7* (5), 2223–2232. DOI: 10.1039/C6FO00398B.

[40] B. Niżyński, A. S. M. Alsoufi, C. Pączkowski, M. Długosz, A. Szakiel, *Phytochemistry Letters*. **2015**, *11*, 410–417. DOI: 10.1016/j.phytol.2014.12.017.

[41] K. Zitterl-Eglseer, S. Sosa, J. Jurenitsch, M. Schubert-Zsilavecz, R. Della Loggia, A. Tubaro, M. Bertoldi, C. Franz, *Journal of Ethnopharmacology*. **1997**, *57* (2), 139–144. DOI: 10.1016/S0378-8741(97)00061-5.

[42] M. R. García-Risco, L. Mouhid, L. Salas-Pérez, A. López-Padilla, S. Santoyo, L. Jaime, A. Ramírez de Molina, G. Reglero, T. Fornari, *Plant Foods Hum Nutr*. **2017**, *72* (1), 96–102. DOI: 10.1007/s11130-016-0596-8.

[43] L. M. A. S. Campos, E. M. Z. Michielin, L. Danielski, S. R. S. Ferreira, *The Journal of Supercritical Fluids*. **2005**, *34* (2), 163–170. DOI: 10.1016/j.supflu.2004.11.010.

[44] M. Hamburger, S. Adler, D. Baumann, A. Förg, B. Weinreich, *Fitoterapia*. **2003**, *74* (4), 328–338. DOI: 10.1016/S0367-326X(03)00051-0.

[45] L. Danielski, L. M. A. S. Campos, L. F. V. Bresciani, H. Hense, R. A. Yunes, S. R. S. Ferreira, *Chemical Engineering and Processing: Process Intensification*. **2007**, *46* (2), 99–106. DOI: 10.1016/j.cep.2006.05.004.

[46] A. López-Padilla, A. Ruiz-Rodriguez, G. Reglero, T. Fornari, *The Journal of Supercritical Fluids*. **2017**, *130*, 292–300. DOI: 10.1016/j.supflu.2017.03.033.

[47] D. Villanueva-Bermejo, E. Vázquez, M. Villalva, S. Santoyo, T. Fornari, G. Reglero, M. Rodriguez García-Risco, *Applied Sciences*. **2019**, *9* (11), 2245. DOI: 10.3390/app9112245.

[48] V. Kaškonienė, P. Kaškonas, M. Jalinskaitė, A. Maruška, *Chromatographia*. **2011**, *73* (1), 163–169. DOI: 10.1007/s10337-011-1910-0.

[49] N. Crabps, B. Marongiu, A. Piras, T. Pivetta, S. Porcedda, *Journal of Essential Oil Research*. **2003**, *15* (5), 350–355. DOI: 10.1080/10412905.2003.9698610.

[50] V. A. Kurkin, O. V. Sharova, *Chem Nat Compd*. **2007**, *43* (2), 216–217. DOI: 10.1007/s10600-007-0084-3.

[51] D. Silva, M. S. Ferreira, J. M. Sousa-Lobo, M. T. Cruz, I. F. Almeida, *Cosmetics*. **2021**, *8* (2), 31. DOI: 10.3390/cosmetics8020031.

[52] G. Ak, G. Zengin, K. I. Sinan, M. F. Mahomoodally, M. C. N. Picot-Allain, O. Cakır, S. Bensari, M. A. Yılmaz, M. Gallo, D. Montesano, *Applied Sciences*. **2020**, *10* (17), 5920. DOI: 10.3390/app10175920.

[53] H. Kırmızıbekmez, C. Bassarello, S. Piacente, C. Pizza, İ. Çalış, *Zeitschrift für Naturforschung B*. **2006**, *61* (9), 1170–1173. DOI: 10.1515/znb-2006-0920.

[54] A. I. Derkach, N. F. Komissarenko, V. T. Chernobai, *Chem Nat Compd*. **1986**, *22* (6), 722–723. DOI: 10.1007/BF00598365.

[55] B. BEKDEŞER, *Turkish Journal of Chemistry*. **2019**, *43* (5), 1457–1471. DOI: 10.3906/kim-1905-48.

[56] G. S. Ćetković, S. M. Djilas, J. M. Čanadanović-Brunet, V. T. Tumbas, *Food Research International*. **2004**, *37* (7), 643–650. DOI: 10.1016/j.foodres.2004.01.010.

[57] T. Ercetin, F. S. Senol, I. Erdogan Orhan, G. Toker, *Industrial Crops and Products*. **2012**, *36* (1), 203–208. DOI: 10.1016/j.indcrop.2011.09.007.

[58] T. Lefebvre, E. Destandau, E. Lesellier, *Journal of Chromatography A*. **2021**, *1639*, 461709. DOI: 10.1016/j.chroma.2020.461709.

[59] T. Lefebvre, E. Destandau, E. Lesellier, *Journal of Chromatography A*. **2021**, *1635*, 461770. DOI: 10.1016/j.chroma.2020.461770.

[60] S. Atwi-Ghaddar, E. Destandau, E. Lesellier, *Journal of CO2 Utilization*. **2023**, *70*, 102440. DOI: 10.1016/j.jcou.2023.102440.

[61] D. Baumann, S. Adler, S. Grüner, F. Otto, B. Weinreich, M. Hamburger, *Phytochemical Analysis*. **2004**, *15* (4), 226–230. DOI: 10.1002/pca.772.

[62] H. Sovová, *The Journal of Supercritical Fluids*. **2012**, *66*, 73–79. DOI: 10.1016/j.supflu.2011.11.004.

[63] M. L. L. Moraes, H. D. T. da Silva, L. Blanes, P. Doble, M. F. M. Tavares, *Journal of Pharmaceutical and Biomedical Analysis*. **2016**, *125*, 408–414. DOI: 10.1016/j.jpba.2016.04.017.

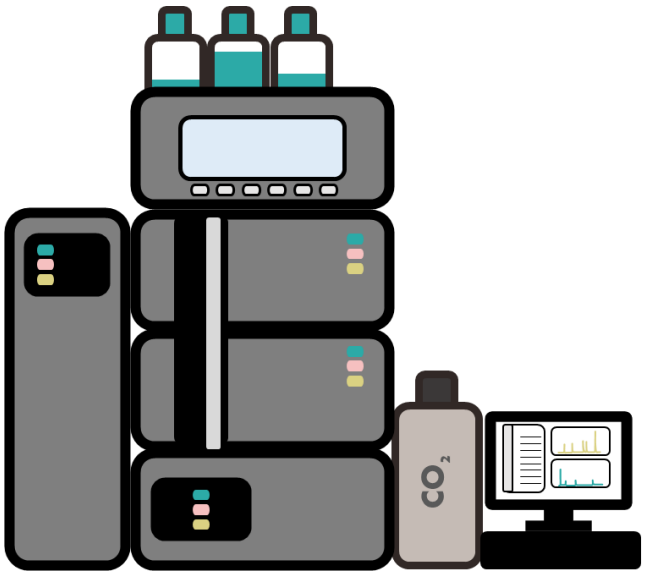
[64] Q. Gros, M. Wolniaczyk, J. Duval, C. West, S. Horie, Y. Toyota, Y. Funada, E. Lesellier, *Journal of Food Composition and Analysis*. **2023**, *115*, 104960. DOI: 10.1016/j.jfca.2022.104960.

[65] E. Lesellier, A. Latos, C. West, *Analytical Science Advances*. **2021**, *2* (1–2), 33–42. DOI: 10.1002/ansa.202000156.

[66] E. Lesellier, A. Latos, A. L. de Oliveira, *Journal of Chromatography A*. **2014**, *1327*, 141–148. DOI: 10.1016/j.chroma.2013.12.046.

[67] J. Molineau, M. Meunier, A. Noireau, L. Fougère, A.-M. Petit, C. West, *Anal Bioanal Chem*. **2020**, *412* (24), 6595–6609. DOI: 10.1007/s00216-020-02798-z.

Chapter IV: Separation of *Calendula officinalis* L esterified triterpenoids with ultra-high efficiency / low-pressure supercritical fluid chromatography (UHE-LP/SFC)



The quality control of the extracts is an essential step that follows the extraction process. The total quantity of secondary metabolites recovered during extraction is generally correlated with its overall activity. Thus, the quantification of these metabolites is of considerable interest. Considering the intricate nature of metabolites' structure and abundance, this process is not always simple or ecological, and it may be challenging. To get results with a high degree of resolution and accuracy, high performance analytic techniques are needed.

In chapter III, the lipophilic extracts of *Calendula officinalis* L. are rich in esterified triterpenoids. These compounds present numerous isomers and require the use of ultra-high efficiency/ low pressure SFC technique to be separated. Therefore, the separation of these compounds and the method optimization will be described, based on the use of various statistical tools.

1. *Introduction*

Supercritical fluid chromatography (SFC) can be defined as a separation technique employing the use of SC-CO_2 mixed with an organic solvent as a mobile phase. The instrumentation is relatively identical to a common HPLC system, with the addition of CO_2 tank to be used as a mobile phase and a back pressure regulator to pressurize the system, keeping the mobile phase at supercritical or subcritical state (related to the analytical temperature). The use of SFC offers many advantages for the analysis of non-polar compounds. Other chromatographic techniques are available for the analysis of highly lipophilic compounds, like Gas chromatography (GC), non-aqueous reversed phase liquid chromatography (NARP-LC) or simply reversed phase liquid chromatography (RPLC). However, the use of these techniques requires either the derivatization of such compounds (GC) or the use of high volumes of organic solvents (NARP-LC or RPLC). Therefore, the use of SFC can be beneficial for the analysis of lipophilic compounds, since they are soluble in SC-CO_2 due to its low polarity. Its elution strength can be tuned with the use of organic solvents (MeOH, EtOH or ACN…), allowing a high separation versatility of moderately polar and non-polar compounds.

As mentioned in chapter 3, *Calendula officinalis* L. is used in traditional medicine for its healing and soothing properties. Phytochemical studies have highlighted its richness in triterpenoids, especially faradiol, maniladiol, arnidiol and calenduladiol. These isomers can be esterified with saturated fatty acids: lauric (C12), myristic (C14), and

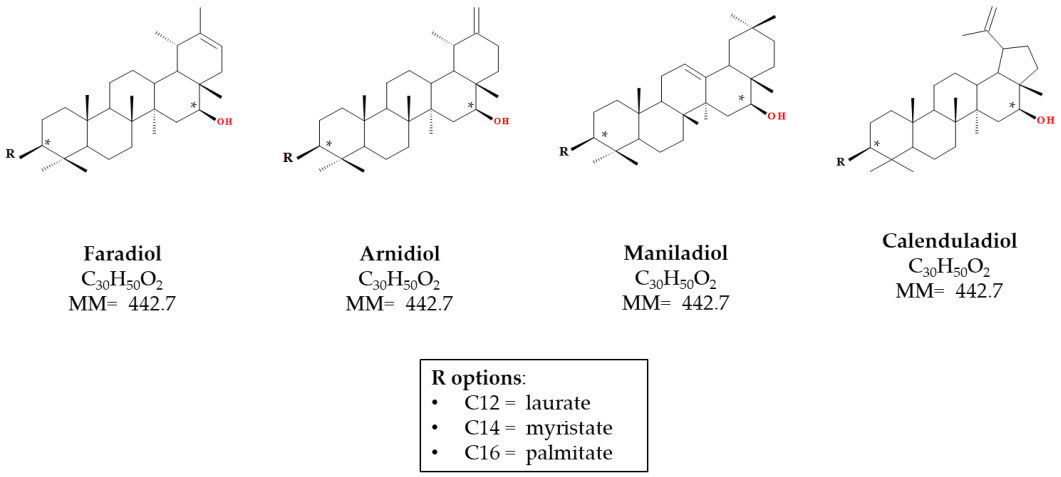


Figure 1: List of commonly found triterpenoids in *Calendula officinalis* L. non-polar compounds.

palmitic (C16) acids with log P values ranging from 7.5 (non-esterified) to 15.4 (esterified) (**Figure 1**).

These triterpenoids esterified or not have the same chemical formula and many possibilities of esterification are possible mainly on the C*3 with C12, C14 and C16 chains. It was mentioned in the literature the possibility of double esterification on C*16 with saturated fatty acids in addition to C*3, however these compounds are very minor compared to one esterification on C*3 [1] . Their similar structure makes their separation and characterization complex. Currently, they are analyzed by NARP-LC or with high percentages of organic solvents [2–4], which is suitable for separating non-polar molecules. However, this technique does not always offer efficient separation, and requires the consumption of large quantities of organic solvent.

This study involved the separation of these compounds using UHE/LP-SFC. In the objective of reducing organic solvents consumption and increasing separation performance. The use of tandem columns and C18-bonded stationary phases based on core-shell particles can improve efficiency to achieve ultra-high resolution of complex mixtures separation without the need for high pressures, this is thanks to the low viscosity of the mobile phase compared ultra-high performance LC systems. A Box-Behnken (BBD) experimental design with three variables: temperature, percentage of modifier , and relative percentage of ACN mixed with MeOH (used as modifiers). Three levels were applied (-1, 0 and +1) to optimize the choice of separation conditions with 5 coupled C18 columns (total length 75 cm). Indeed, the UHE/LP-SFC has already demonstrated its performance for the analysis of natural non-polar compounds like triglycerides in vegetable oils [5–7].

In addition, Derringer desirability functions were used to select optimal analysis conditions based on 5 criteria: retention time of last peak, number of peaks (with peak area greater than 1% of total integrated peaks), number of resolved peaks and discrimination factors (d_0) [8] of the two major compounds (Faradiol myristate and Faradiol palmitate.

2. *Methodology*

2.1.Chemical and reagents

The CO_2 gas was provided by Air Liquide (Fleury-les-Aubrais, France). HPLC grade Acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM), ethanol (EtOH) were provided by VWR (Fontenay-sous-Bois, France).

2.2.Plant material and extract

The dried flower of the marigold (*Calendula officinalis* L.) plant consisted of a light brown/ dark yellow powder, provided by PMA 28 (Varize, France), it was kept at room temperature in an airtight container. The extract used for chromatographic separation was obtained using SFE (Waters MV-10® ASFE) of 5 g of plant material mixed with 5 g of diatomaceous earth in the following conditions: pressure (15 MPa), temperature (60°C), flow rate (3 mL/min), extraction solvent (CO_2:EtOH 90:10 *v:v*) and time (60 min) The extract was later evaporated under nitrogen stream and solubilized in 5 mL of DCM:MeOH 1:1 *v:v* mixture.

2.3.Instrumentation

Shimadzu Corporation's (Kyoto, Japan) Nexera UC system was used for all analysis of terpenoid esters. This system included an autosampler (SIL-30AC), column ovens (CT0-20AC), a photodiode array (PDA) detector (SPD-M20A), a back-pressure regulator (BPR) (SFC-30A), a carbon dioxide pump (LC-30ADsf), a modifier pump (LC-30AD), and an Evaporative light scattering detector (ELSD-LT-III). Shimadzu Corporation, LabSolutions LCMS version 5.93 was used to record all chromatograms. The triterpenoid esters were detected using an ELSD, due to the absence of chromophore groups.

Five octadecyl-bonded silica columns (75 cm of total length) used for the separation, including four Kinetex C18 (150×4.6 mm), 2.6 μm superficially porous particles, from Phenomenex (Le Pecq, France) and one Accucore C18 (150 × 4.6 mm) 2.6 μm superficially porous particles, from Thermo-electron (les Ulis, France) were used in tandem based on previously published work [6,7]. The total flow rate was 1.6 mL/min. Injection volume was set at 5 μL for all samples. The back-pressure regulator was set at 10 MPa and heated at 60°C to limit the effect of CO_2 cold depressurization. The

automated sampler was kept at 25°C to avoid the precipitation of the extract. The compounds were detected using an ELSD with a temperature of 40°C, filter of 4 seconds, a nitrogen pressure of 3 bars and a gain set on wide.

2.4.Experimental design and statistical analysis

Ellistat software 6.4 2020/11 version (Poisy, France) was used for the experimental design and data analysis. A Box–Behnken design (BBD) with response surface methodology (RSM) was chosen to establish the model and to determine the response pattern. The three independent factors used in this study were temperature (X_1), total modifier percentage that consisted of MeOH:ACN mixture (X_2) and percentage of ACN (X_3). The three levels that were used for both experimental designs were coded (+1) for the highest, (0) for the middle and (-1) for the lowest level (**Table 1**).

Table *1*: Coded levels and independent variables (factors) for the experimental model to optimize the SFC separation of *Calendula officinalis* L non-polar compounds.

	Factors		
Coded Levels	**Temperature (°C) X_1**	**Modifier (%) X_2**	**ACN in Modifier (%) X_3**
-1	15	10	25
0	20	20	50
1	25	30	75

A total of 17 randomized experiments were conducted with 5 repetitions of the central level conditions, allowing an evaluation of the repeatability of the model. Derringer functions data analysis was performed using Excel 2021 (Microsoft Corporation, Redmond, Washington, USA).

3. Results

3.1.Criteria for separation optimization

One of the main objectives of this optimization was to obtain an efficient, relatively fast (for five coupled columns), and high-resolution separation of calendula's triterpenoids (esterified or not). The use of SFC for the analysis of these compounds was due to

several reasons: the low viscosity of the mobile phase and the excellent solubility of non-polar compounds in CO_2 provided high separation performances. Additionally, the use of an isocratic elution mode can be beneficial for quantification using ELSD detector sensitive to mobile phase composition. Indeed, due to the lack of UV absorbance of the studied esterified triterpenoids, ELSD detection was selected for this study. The temperature and modifier percentage (MeOH:ACN mixture) were chosen as variable for optimization due to their influence on retention behavior and selectivity of the triterpenoid aglycon, and on the chain length as observed in the case of triglycerides separation [7]. In theory, this can be applied to the different esters (C12, C14 of C16) in the case of esterified triterpenoids, separating the compounds in relation to the fatty acid chain. To evaluate the quality of the separation five different responses were considered (**Table 2**).

Table 2: BBD experimental design list with coded level, values, factors. The results or dependant responses. With Y_1 -LPRT (last peak retention time), Y_2 - d_0P_1 (discrimination factor of faradiol myristate), Y_3 - d_0P_2 (discrimination factor of faradiol palmitate), Y_4 - TPN (total peak number) and Y_5 - (total resolved peak number). Maximal and minimal responses are in bold.

Exp N°	Coded Levels and factors			Results (Response Y)				
	X_1	X_2	X_3					
	Temperature (°C)	Modifier (%)	ACN in Modifier (%)	Y_1- LPRT (min)	Y_2-d_0P_1	Y_3-d_0P_2	Y_4-TPN	Y_5-TRPN
1	15 (-1)	10 (-1)	50 (0)	**36.186**	0.9218	0.8228	18	8
2	25 (+1)	10 (-1)	50 (0)	28.962	**0.5532**	0.8346	18	5
3	15 (-1)	30 (+1)	50 (0)	28.485	**0.9758**	0.2367	15	**13**
4	25 (+1)	30 (+1)	50 (0)	20.559	0.9404	0.6239	16	7
5	15 (-1)	20 (0)	25 (-1)	25.555	0.9586	0.7855	**14**	12
6	25 (+1)	20 (0)	25 (-1)	**19.525**	0.8824	0.7414	**14**	**5**
7	15 (-1)	20 (0)	75 (+1)	27.014	0.9639	0.6886	**14**	10
8	25 (+1)	20 (0)	75 (+1)	20.276	0.9125	0.7094	16	8
9	20 (0)	10 (-1)	25 (-1)	32.248	0.7721	**0.9451**	**20**	7
10	20 (0)	30 (+1)	25 (-1)	22.644	0.9523	0.6980	**14**	**13**
11	20 (0)	10 (-1)	75 (+1)	31.746	0.7115	0.8866	16	5
12	20 (0)	30 (+1)	75 (+1)	25.613	0.9692	**0.2262**	15	7
13	20 (0)	20 (0)	50 (0)	22.458	0.9420	0.7037	**14**	8
14	20 (0)	20 (0)	50 (0)	22.486	0.9443	0.7552	**14**	8
15	20 (0)	20 (0)	50 (0)	22.54	0.9417	0.7599	**14**	8
16	20 (0)	20 (0)	50 (0)	22.085	0.9458	0.7222	**14**	8
17	20 (0)	20 (0)	50 (0)	22.534	0.9423	0.7490	**14**	8

3.1.1. Last peak retention time (LPRT)

The first chosen response was last peak retention time (detected in ELSD), equivalent for a shorter analysis time to reduce energy and solvent consumption (at a constant flow rate). Therefore, the objective was to minimize this response. The same extract was injected for all experimental conditions. As expected, the LPRT varied according to each experiment. With the exp n°1 (15°C, 10% MeOH:ACN 50:50) having the

maximum response with 36.186 min, and exp n°6 (25°C, 20% MeOH:ACN 75:25) the lowest with 19.525 min.

3.1.2. Discrimination factor - resolution (d_0)

Discrimination factor indicating the resolution of separation was evaluated for the two major peaks found in *Calendula officinalis* L. lipophilic fraction: faradiol-3-*O*-myristate (d_0 P_1) and faradiol-3-*O*-palmitate (d_0 P_2), these two compounds have been reported to be the major esterified triterpenoids found in non-polar extracts of calendula flowers (chapter III, section 1). Therefore, the resolution of their separation is highly important for quantification and extraction monitoring.

The discrimination factor is related to the separation power originally described by Kaiser [8,9]. It's based on the relative heights of the smaller peak and the valley above the baseline, and is graphically determined as shown in **Figure 2**, following **Equation 1** : $do = \frac{h\,v}{hp}$

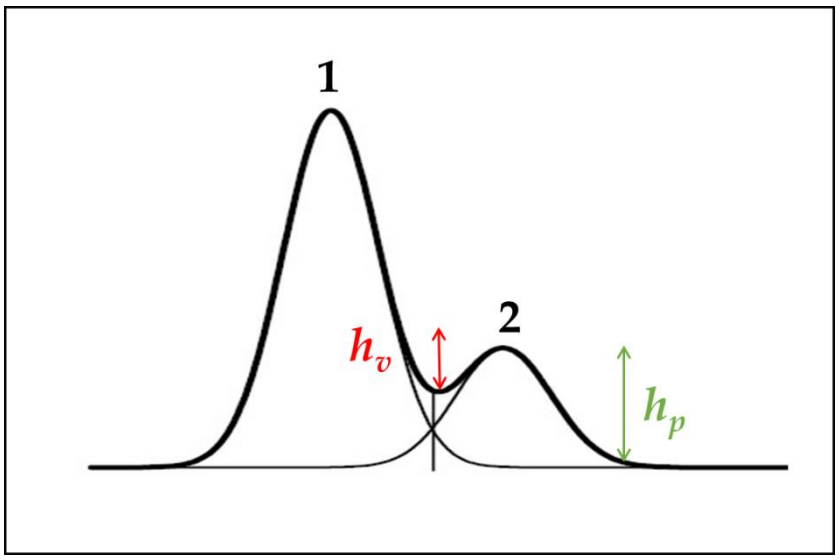


Figure 2: representation of co-eluted peaks with the hv (valley above the baseline) and hp (hight of small peak).

The value of d_0 varies between 0 and 1, with 1 indicating a full separation between two peaks and 0 a full coelution. The maximization of d_0 indicates a high resolution of separation. The exp n°2 and 12 had the lowest value for peak 1 and 2 with 0.553 and 0.226 respectively, and exp n°3 and 9 had the highest do value for peak 1 and 2 with 0.975 and 0.945 respectively. The calculation of d_0 P_1 (faradiol myristate) and d_0 P_2 (faradiol palmitate) is presented in the supplementary data of chapter IV.

3.1.3. Total peak number (TPN)

To achieve a high-resolution separation, the maximum number of compounds possible must be separated according to the experiment's condition range. Therefore, the objective was to maximize this response. The non retained peaks at the dead time were not considered for this result and only retained compounds were considered. In addition, after the full integration of all peaks, only the ones with a percentage of peak area higher than 1% compared to the total integrated peaks areas were considered. Therefore, excluding minor peaks. The TPN for exp n° 5, 6, 7, 10, 13, 14, 15, 16 and 17 had the lowest score with 14 peaks, as for exp n° 9 had the highest score with 20 peaks.

3.1.4. Total resolved peak number (TRPN)

Peak separation is an important factor when optimizing chromatographic analysis, however other factors should be considered, like peak return to the base line (Rs =1.5). Indeed, just counting peak sums will not ensure that these peaks are not co-eluted with other compounds. Therefore, TRPN was evaluated with exp n° 6 and 11 scoring the lowest value with only 5 peaks, and exp n° 3 and 10 the highest value with 13 peaks.

3.2.Statistical analysis of the experimental model

Opposed to SFE optimization using experimental design with only one response and one objective: the maximization of the yield, separation optimization is more delicate. Five responses needed to be considered to achieve an efficient and high-resolution separation of *Calendula officinalis* L. esterified triterpenoids and non-polar compounds.

Therefore, to analyze the experimental model all 5 responses were considered, with the aim of minimization of LPRT and the maximization of d_0 P_1, d_0 P_2, TPN and TRPN. The determination coefficient (R^2), adjusted determination coefficient (R^2_{adj}), predictive determination coefficient (R^2_{pred}) and the model p-values were tested to establish the significance of the regression. The significative factors were determined according to the polynomial model's analysis of variance or ANOVA (supplementary data chapter IV).

The polynomial **Equation 2** represents the relationship between the response and the three independent variables. Due to the reduced experiment number, the equation did not include the interactions terms between the three factors, to avoid the overfitting the data.

$$Y_{(1,2,3,4\ or\ 5)} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad \textbf{(2)}$$

In this equation, Y = the dependent response. X_1, X_2 and X_3 = the coded values of the independent variables : temperature, modifier (%) and ACN (%) respectively.

β_0 = constant coefficient; β_1, β_2 and β_3 = the coefficient of the linear terms and β_{11}, β_{22} and β_{33} = coefficients of the quadratic terms.

The RSD values were calculated according to the 5 repetitions of the central point, the calculated optimized conditions and model's equations were computed by the software. These values (**Table 3**) indicated a high repeatability of the system, where Y_1, Y_2, Y_4 and Y_5 had an RSD lower than 1%, only Y_3 had the highest variation of 3.26%, an acceptable value lower than 5%.

A summary of the results presented in **Table 3** demonstrated that all responses (from Y_1 to Y_5) had $R^2 > 0.7$ and a p-values < 0.05 indicating significant models.

Table 3: summary of the statistical analysis of BBD experimental design for each response (Y).

Response	R^2	R^2 adj	R^2 pred	P-value model	significative factors	RSD (%)	objective	Calculated optimized conditions	model's equation
Y_1- LPRT (min)	0.989	0.982	0.955	0	X_1 X_2 X_2^2	0.85	MIN	25°C 24.48% Modifier 25% ACN	**LPRT**= 75.02 - 1.626X_1 - 2.617X_2 + 0.00851X_3 + 0.02321X_1^2 + 0.05548X_2^2 + 0.0001484X_3^2
Y_2-d_0 P_1	0.809	0.695	0.059	0.0038	X_2 X_2^2	0.19	MAX	25°C 10% Modifier 25%ACN	**d_0 P_1** = 0.512 - 0.0001X_1 - 0.04552X_2 - 0.00091X_3 + 0.00034X_1^2 + 0.00086X_2^2 + 9.6×10^{-06}X_3^2
Y_3-d_0 P_2	0.789	0.662	0	0.006	no significative factors	3.26	MAX	15°C 30% Modifier 75% ACN	**d_0 P_2** = 0.598 - 0.0654X_1 - 0.00885X_2 + 0.00806X_3 + 0.00141X_1^2 + 0.0007525X_2^2 - 4.76×10^{-05}X_3^2
Y_4-TPN	0.79	0.664	0	0.0059	X_2 X_2^2	0	MAX	25°C 10% Modifier 25% ACN	**TPN** = 32.75 - 0.725X_1 - 1.05X_2 - 0.005X_3 + 0.02X_1^2 + 0.0225×X_2^2 + 1.197×10^{-17}×X_3^2
Y_5-TRPN	0.77	0.633	0	0.0088	no significative factors	0	MAX	15°C 30% Modifier 25% ACN	**TRPN** = 23 - 1.25×X_1 + 0.2875×X_2 - 0.075×X_3 + 0.02×X_1^2 - 0.0025×X_2^2 + 0.0004×X_3^2

However, only Y_1 demonstrated a high R^2_{pred} with 0.955 value indicating a high predictivity of the model, where the other responses Y_2, Y_3, Y_4 and Y_5 had very low R^2_{pred} indicating a significantly low capacity of prediction.

The calculated optimized conditions varied for each response. For Y_1, Y_2 and Y_4 displaying significative factors, the optimized temperature was 25°C. The percentage

of modifier varied between 10% (Y_2 and Y_4), 24.48% (Y_1). As for the composition of modifier was more homogeneous, where MeOH:ACN 75:25 *v:v* was considered as an optimal composition for Y_1,Y_2, Y_4. Additionally, Y_3 and Y_5 demonstrated no significant factors.

Therefore, the results summary concluded that statistical analysis of the design of experiments alone was not sufficient to find the optimal separation conditions and additional statistical evaluation was needed using different tools and approaches.

3.3.Derringer desirability functions and application to experimental design

Since the statistical analysis alone was not enough to deduct one experimental condition that optimized separation resolution and analysis time. Derringer desirability functions were applied to the data set. These functions offer a flexible, convenient approach to rank unrelated responses while comparing them. They were first described by Derringer and Markham [10] to select polymeric materials based on varied properties [11]. Functions were defined following the minimal and maximal value for each response in the data set (**Table 2**). According to the objective of each response a score of 1 was attributed to the desired value in the response list, and the lowest score of 0 to the undesired value.

The Derringer desirability functions indeed allow to compare the chromatographic conditions based on different criteria and without preconception [12,13]. First, it was necessary to define the comparison criteria and to translate them into desirability functions (d_1, d_2, d_3, d_4 and d_5).

3.3.1. Definition of desirability functions (d_i)

The first function **d_1** represented the LPRT. The use of 5 coupled columns favors the chromatographic efficiency of the esterified triterpenoids isomers by increasing the number of theoretical plates up to 100.000. However, it comes with the simultaneous increase of the analysis time. Therefore, to decrease organic solvents and energy consumption, the analysis time needed to be reduced and the final detected peak retention time was evaluated. Consequently, a score of 0 was given for the longest analysis time corresponding to exp n°1 (15°C, 10% MeOH:ACN 50:50 *v:v*) with 36.19 min, and a score of 1 was given for the shortest time corresponding to exp n°6 (25°C, 20% MeOH:ACN 75:25 *v:v*) with 19.52 min. This function was later applied to the total data set of experiment for response Y_1, allowing us to obtain a score for each experimental point.

The second and third functions $\mathbf{d_2}$ and $\mathbf{d_3}$ represented the discrimination factors of the two major esterified triterpenoids faradiol myristate (d_0 P_1) and faradiol palmitate (d_0 P_2) respectively. To ensure the highest resolution of separation of these compounds, the discrimination factor d_0 was to be maximized. Therefore, a derringer function for both responses Y_2 and Y_3 was applied for highest (for Y_2 exp n°3, and Y_3 exp n°9) and lowest score (for Y_2 exp n°2, and Y_3 exp n°12), giving a score of 1 and 0 respectively.

The fourth function $\mathbf{d_4}$ presented TPN. Since this value is closely related to the efficiency of the separation where a lower TPN indicates that the separation was not efficient, and more compounds were co-eluted therefore a score was attributed to Y_4. A value of 0 was attributed to separation where only 14 peaks were observed (exp n°5, 6, 7, 10, 13, 14, 15, 16 and 17). As for the maximum value of 20 peaks, indicated the highest separation efficiency and was attributed the score of 1 (exp n°9).

The fifth and final function $\mathbf{d_5}$ was attributed to the TRPN, representing the quality of separation. Since the increase of peak number without considering the resolution of separation is not convenient for quantifications. A score of 0 was given to the lowest value of the data set of Y5 with only 5 resolved peaks (exp n°6) and 1 for the highest value with 13 peaks (exp n°3).

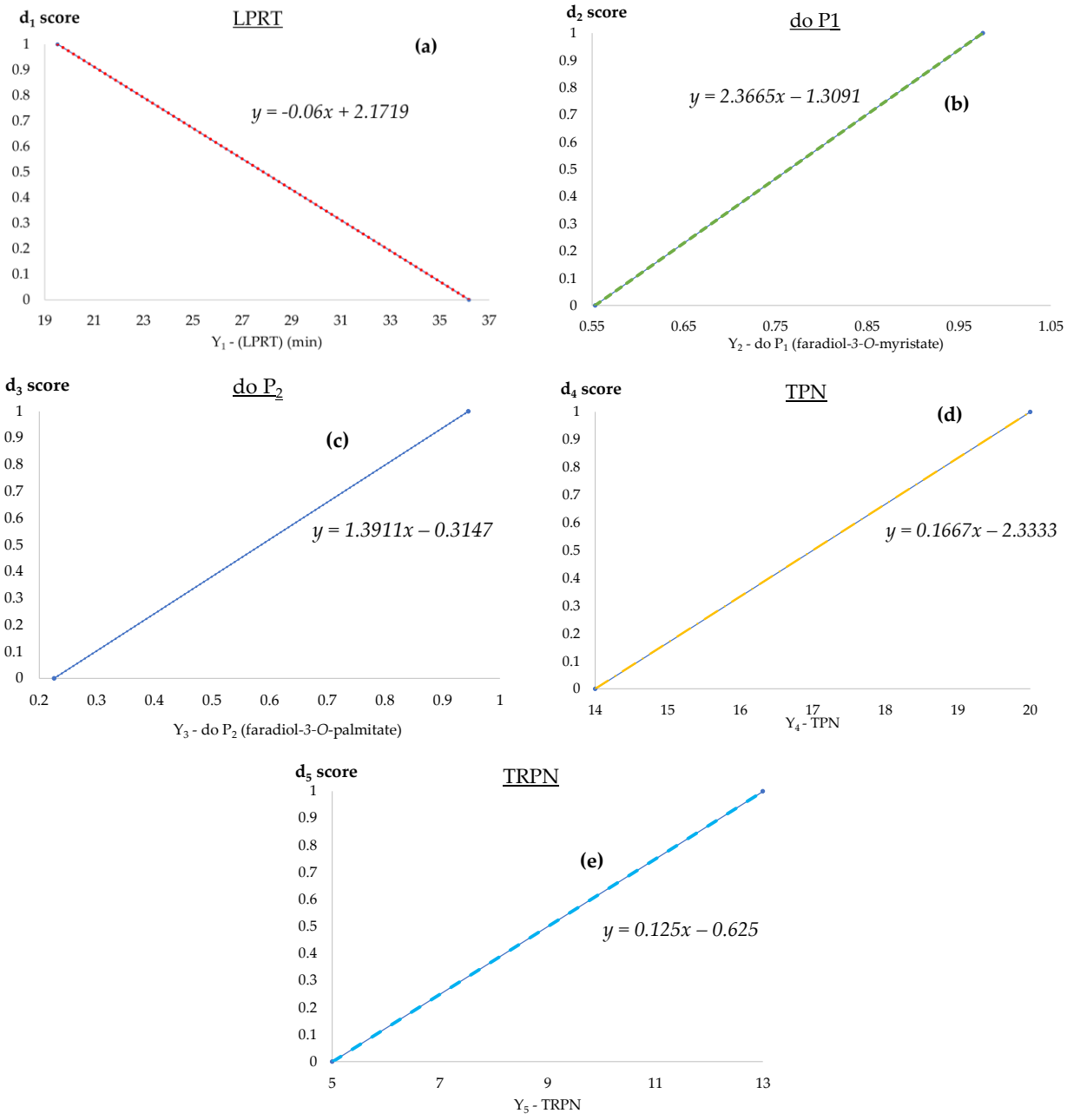


Figure 3: Derringer functions representation. (a): LPRT, (b) d_0P_1, (c) d_0P_2, (d): TPN and (e): TRPN.

3.3.2. *Global desirability (D)*

After applying the desirability functions to the experimental data, the equations were correlated to each response (Y). It is useful to verify that all functions are not correlated to each other, and therefore no redundancies in results are found.

The total desirability value (D) was then calculated as the arithmetic mean of the five functions, where all the functions were attributed, the same weight following **Equation 3**:

$$D = \frac{d_1 + d_2 + d_3 + d_4 + d_5}{5}$$

The desirability calculation to the DoE data set was presented in **Table 4.** After the analysis of the global desirability score (D mean), it was clear that exp n°10 (20°C, 30% MeOH: ACN 75:25 *v:v*) provided the highest score with 0.683, as for exp n°2 (25°C, 10% MeOH: ACN 50:50 *v:v*) it had the lowest D mean with a value of 0.390.

The comparison of two experiments chromatograms with the highest (exp n°10), lowest (exp n°2) D mean was presented in **Figure 4**, all other chromatograms are presented in the supplementary data chapter IV.

Table 4: BBD experimental design list with coded level and Derringer values for each experiment. Experiment de highest (exp n° 10) and lowest (exp n°2) global Derringer score are in bold.

Exp N°	Coded Levels			Desirability values					
	X_1	X_2	X_3						
	Temperature (°C)	Modifier (%)	ACN in Modifier (%)	LPRT (d_1)	$d_0 P_1$ (d_2)	$d_0 P_2$ (d_3)	TPN (d_4)	TRPN (d_5)	D mean
1	15 (-1)	10 (-1)	50 (0)	0.001	0.667	0.872	0.830	0.375	0.549
2	**25 (+1)**	**10 (-1)**	**50 (0)**	**0.434**	**0.667**	**0.000**	**0.846**	**0.000**	**0.390**
3	15 (-1)	30 (+1)	50 (0)	0.463	0.167	1.000	0.015	1.000	0.529
4	25 (+1)	30 (+1)	50 (0)	0.938	0.334	0.916	0.553	0.250	0.598
5	15 (-1)	20 (0)	25 (-1)	0.639	0.000	0.959	0.778	0.875	0.650
6	25 (+1)	20 (0)	25 (-1)	1.000	0.000	0.779	0.717	0.000	0.499
7	15 (-1)	20 (0)	75 (+1)	0.551	0.000	0.972	0.643	0.625	0.558
8	25 (+1)	20 (0)	75 (+1)	0.955	0.334	0.850	0.672	0.375	0.637
9	20 (0)	10 (-1)	25 (-1)	0.237	1.001	0.518	1.000	0.250	0.601
10	**20 (0)**	**30 (+1)**	**25 (-1)**	**0.813**	**0.000**	**0.945**	**0.656**	**1.000**	**0.683**
11	20 (0)	10 (-1)	75 (+1)	0.267	0.334	0.375	0.919	0.000	0.379
12	20 (0)	30 (+1)	75 (+1)	0.635	0.167	0.985	0.000	0.250	0.407
13	20 (0)	20 (0)	50 (0)	0.824	0.000	0.920	0.664	0.375	0.557
14	20 (0)	20 (0)	50 (0)	0.823	0.000	0.926	0.736	0.375	0.572
15	20 (0)	20 (0)	50 (0)	0.820	0.000	0.919	0.742	0.375	0.571
16	20 (0)	20 (0)	50 (0)	0.847	0.000	0.929	0.690	0.375	0.568
17	20 (0)	20 (0)	50 (0)	0.820	0.000	0.921	0.727	0.375	0.569

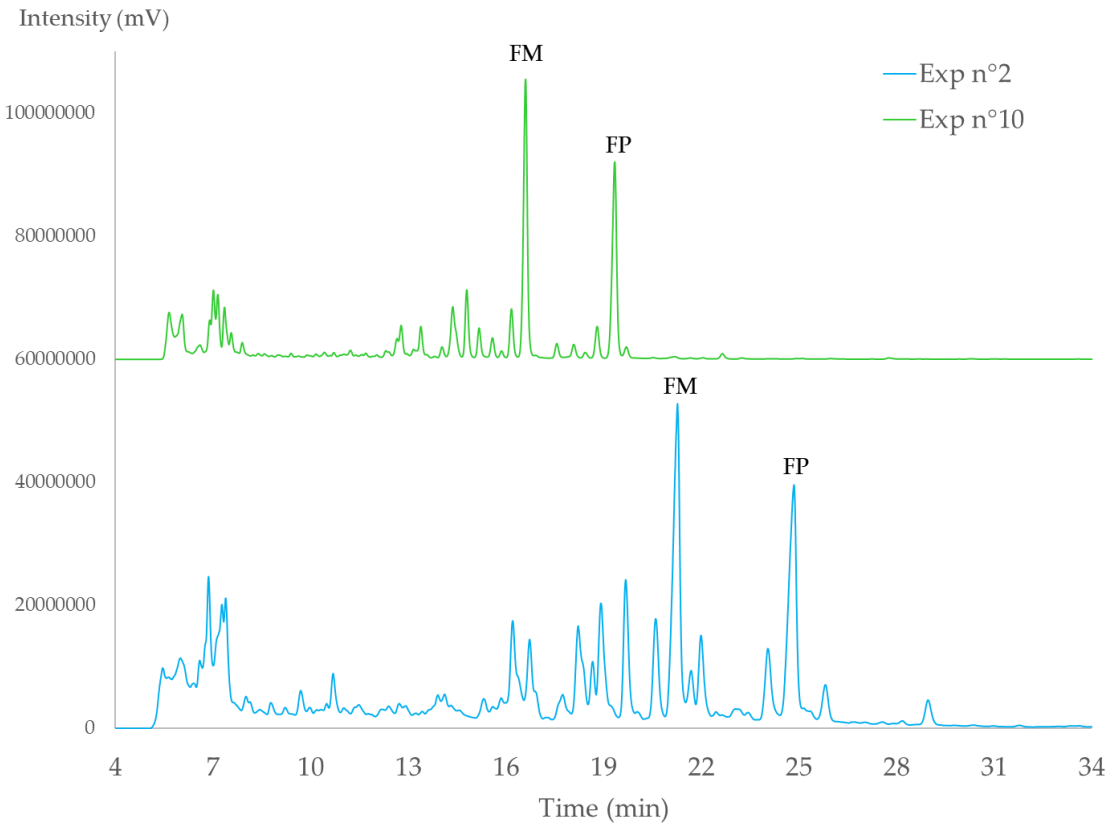


Figure 4: UHE-LP/SFC-ELSD chromatograms for exp n°10 (green) representing highest D mean and exp n°2 (bleu) with the lowest D mean. FM (faradiol myristate) and FP (faradiol palmitate)

The differences in the two experiments are noteworthy, exp n°10 had the most resolved peaks and a shorter analysis time and less coelutions compared to exp n°2. In addition, two separate groups of 4 peaks eluted before the major peaks faradiol myristate and faradiol palmitate can be identified in exp n°10, probably related to other minor triterpenoids esterified with a C14 chain before faradiol myristate and a C16 chain for faradiol palmitate. Representing a high resolution of separation of isobars found in lipophilic fractions. This demonstrated that the use of desirability functions allowed us to reach a rationalized conclusion to optimize the separation of complex extracts like *Calendula officinalis* L lipophilic one.

4. *Conclusion*

The use of statistical tools was applied to optimize the separation of esterified triterpenoids using UHE-LP/SDC-ELSD. First the experimental design allowed us to produce an experiment list following a BBD. The statistical analysis of the 5 responses

(LPRT, d_0P_1, d_0P_2, TPN and TRPN) was insufficient to produce one optimized condition following the results. Therefore, a rationalized approach using Derringer function allowed to calculate one condition that offered the highest score. Experiment n°10 with 20°C, 30% MeOH:ACN 75:25 *v:v* modifier composition allowed to reach high separation resolution, while reducing the consumption of organic solvent.

5. References

[1] C. Nicolaus, A. Sievers-Engler, R. Murillo, M. D'Ambrosio, M. Lämmerhofer, I. Merfort, *J. Pharm. Biomed. Anal.* **2016**, *118*, 195–205. DOI: 10.1016/j.jpba.2015.10.025.

[2] C. Nicolaus, A. Sievers-Engler, R. Murillo, M. Lämmerhofer, I. Merfort, *Planta Med.* **2015**, *81* (16), PW_177. DOI: 10.1055/s-0035-1565801.

[3] M. Hamburger, S. Adler, D. Baumann, A. Förg, B. Weinreich, *Fitoterapia*. **2003**, *74* (4), 328–338. DOI: 10.1016/S0367-326X(03)00051-0.

[4] K. Zitterl-Eglseer, G. Reznicek, J. Jurenitsch, J. Novak, W. Zitterl, C. Franz, *Phytochem. Anal.* **2001**, *12* (3), 199–201. DOI: 10.1002/pca.582.

[5] E. Lesellier, A. Latos, C. West, *Anal. Sci. Adv.* **2021**, *2* (1–2), 33–42. DOI: 10.1002/ansa.202000156.

[6] E. Lesellier, A. Latos, A. L. de Oliveira, *J. Chromatogr. A*. **2014**, *1327*, 141–148. DOI: 10.1016/j.chroma.2013.12.046.

[7] Q. Gros, M. Wolniaczyk, J. Duval, C. West, S. Horie, Y. Toyota, Y. Funada, E. Lesellier, *J. Food Compos. Anal.* **2023**, *115*, 104960. DOI: 10.1016/j.jfca.2022.104960.

[8] M. Z. El Fallah, M. Martin, *Chromatographia*. **1987**, *24* (1), 115–122. DOI: 10.1007/BF02688473.

[9] J. V. Hinshaw, *LCGC N. Am.* **2010**, *28* (11), 952–958.

[10] G. C. Derringer, R. L. Markham, *J. Appl. Polym. Sci.* **1985**, *30* (12), 4609–4617. DOI: 10.1002/app.1985.070301208.

[11] C. West, *LC-GC N. Am.* **2016**, *34* (6), 420–422.

[12] Q. Gros, L. Réset, J. Duval, S. Horie, Y. Toyota, Y. Funada, Y. Hayakawa, E. Lesellier, C. West, *J. Chromatogr. A*. **2023**, *1687*, 463667. DOI: 10.1016/j.chroma.2022.463667.

[13] J. Molineau, Y. Hamel, M. Hideux, P. Hennig, S. Bertin, F. Mauge, E. Lesellier, C. West, *J. Chromatogr. A*. **2021**, *1658*, 462631. DOI: 10.1016/j.chroma.2021.462631.

Conclusion

The aim of this thesis project was to develop the extraction of bioactive compounds from plants using green methods specifically CO_2 based supercritical fluid extraction, to produce natural cosmetic ingredients. In the objective of replacing maceration, a traditional extraction technique currently employed by our industrial partner. Even though this last technique is relatively simple, it requires the use of great amounts of solvents, filtration, and finally solvent evaporation to reach the final extract ready to be formulated. This created the need to replace it with other extraction methods. The ecological impact of the process was considered in all the steps while seeking to reduce unit operations, organic solvent, and energy consumption.

In the opening chapter, the key ideas we intended to emphasize, whether on a theoretical or experimental basis, were presented. Starting with a brief classification of secondary metabolites, the six green extraction principles, followed by an explanation of the concepts, advantages, and disadvantages of modern methods of extraction (UAE, MAE, PLE, and NaDESs). SFE was thoroughly discussed both theoretically and experimentally. Finally, modern extraction techniques for extracting plants employing integrated methodologies were described.

Among the various methods available for eco-extraction, SFE answers our needs. However, the approach required the examination of a wide range of plants. Furthermore, a variety of factors affect the results of SFE, as described in the first chapter. Each parameter would need to be optimized independently, which would involve numerous trials over extended periods of time. So, to speed up the optimization, a quick and straightforward process was needed. Therefore, the use of chemometric and statistical methods, and especially the design of experiment (DoE) was essential.

Many variations and types of experimental design exist. One type of DoE that allows a high reduction of the experimental points number is response surface methodology (RSM), and this was a great candidate for this work.

RSM is used to obtain a fine predictive model, presenting interactions between variables (when applicable), non-linear response, and proposes a digital filling of the space when needed. The Box-Behnken design was chosen mainly for its particularity of ensuring that all the factors are not set at their highest levels at the same time,

allowing their gradual screening. In addition, it offered a low number of experiences as 13 points were chosen for screening of a model with three variables/levels and additional 4 repetitions of the central points allowed to determine the repeatability of the system and the plant matrix itself. Where a full factorial design with the same variables/levels number would require 27 experiments.

Before diving into the extraction results, we would like to highpoint that numerous UHPLC methods were developed in the goal to guarantee a rapid and efficient separation and quantification. All these methods were achieved by using the same stationary phase, a C18 bonded silica with partially porous particles of 2.5 μm, and the mobile phase composition varied according to each study. A gradient elution mode was frequently employed, and quantification of the targeted compounds was achieved thanks to the use of calibration curves. These developed methods served in at least three other studies related to the main PIERIC project, including a PhD student and a postdoctoral contract, showing the cross-functionality of the studies in this project, and the central place of this thesis.

One of the challenges of this project was the extraction of polar molecules. Given that CO_2 is non-polar, it's broadly used for non-polar compounds extraction (e.g., lipids, carotenoids, etc.). The dynamic mode used in SFE offered a crucial advantage for fractionated extraction with the help of modifier, make-up, and the tuning of other parameters. Finally, the inherent properties of CO_2 allowed to recover concentrated extracts, in solvents adapted for formulation.

Accordingly, the second chapter is constructed to present all the applications of BBD for the extraction of polar compounds from plants. In the aim of standardizing the methodology and facilitating the use of SFE.

The first plant was the **black locust**, the tree's heartwood is rich in many secondary metabolites but in this study's framework two compounds were targeted, dihydrorobinetin (DHR) and robinetin (Rob), two major flavonoids with high potential for cosmetic applications. Employing a Box-Behnken design (BBD) with three variables/levels, their extraction was optimized. Parameters like flow rate and plant mass were kept constant corresponding to the extraction system, and extraction time employed for the experiences was determined following extraction kinetics with the conditions of the model's central level. This permitted the reduction of the variables to only three (temperature, pressure, and modifier percentage that consisted of EtOH:H_2O 80:20 *v:v* mixture) and with it the number of experimental points. As a result, we established that the pressure had minimal effect on the yield within the

applicable range, this was due to the high density of the extraction phase due to the high modifier percentage that ranged from 10 to 30%, and therefore was kept at a constant value of 10 MPa. The extraction yield for both flavonoids was higher using SFE. When comparing the optimized SFE conditions (20% of modifier EtOH:H_2O 80:20 *v:v*, 10 MPa, 80°C, for 30 minutes) with the classical ultrasonic extraction with a EtOH:H_2O 80:20 *v:v* with an identical extraction duration and organic solvent volume, the capacity of SFE to achieve high polar compounds recovery was demonstrated. Since, DHR is more soluble in water while Rob is more soluble in ethanol, additional experiments were conducted, by studying the water content mixed to ethanol from 5 to 30% at two different temperature 40 and 80°C, to investigate a selective extraction between both flavonoids. Nonetheless, the two compounds exhibited no tendencies of selective extraction between each other. It is important to mention that the selective extraction of these compounds was also not possible with solid-liquid maceration with EtOH:H_2O *v:v* mixtures, even with a H_2O percentage that ranged from 0 to 100%. This demonstrated that SFE did not enhance the selective extraction of these compounds with such similar structures, the difference between these two compounds is only one double bound (Rob) and polarities.

In the second part, the extraction of catechins and caffeine (Log P= -0.1) from **green tea leaves** was optimized also using a BBD, these compounds exhibit a lower Log P when compared to DHR (Log P= 0.9) and Rob (Log P= 1.6). On the other hand, this time based on the previous study, the pressure was replaced as variable with the H_2O percentage added in the modifier mixed with EtOH and its value was kept constant at 15 MPa. This was chosen in the aims of achieving both selectivity and yield optimization directly in the same experimental design, in addition to the determination if a high percentage of water is needed as additive for all polar compounds' extraction. According to the results, a temperature of 60°C combined with a high modifier and water content (20% in ethanol) favored the extraction of the polar green tea caffeine and catechins. After the analysis of the experimental results, it was clear that using 10% EtOH as modifier induced a selective recovery of pure caffeine compared to EtOH:H_2O 80:20 *v:v* that allowed a more exhaustive extraction of caffeine and catechins. Thus, a sequential, partially selective extraction of caffeine was also realized.

In the third part, punicalagins and ellagic acid represented the compounds of interest in the pomegranate pericarp. The same experimental design employed for green tea leaves was utilized. This offered fast optimization like the previous DoE and validated

its use on other plant matrixes. A lower temperature, higher percentages of modifier and water were needed to maximize the yield of the polar studied compounds. Moreover, the solubilization of the dried extracts prior to chromatographic analysis presented a significant challenge when preparing the samples because no solvent other than DMSO (50%) could provide a homogeneous mixture (*i.e.*, to solubilize the bioactive compounds). However, if this was easily resolved for chromatographic analysis, real issues exist regarding the use of DMSO to solubilize extracts in cosmetic ingredients.

Consequently, an integrated pre-formulation of the SFE extracts was carried out using cosmetic-approved solvents, maintaining the bioactive compounds in a liquid useable form without the addition of DMSO. This resulted in the addition of glycerol after the extraction step, to store the extracts in a liquid form rather than completely evaporating them. In that case, at the end of the SFE process the extract is composed by EtOH:H_2O 80:20 *v:v*, and glycerol was added to the extract after the depressurization of the CO_2 in the same amount of EtOH. After the evaporation of EtOH, the bioactive compounds are stabilized in a mixture glycerol:H_2O 80:20 *v:v*, a composition that is commonly used as ingredient in cosmetic pre-formulation. In addition, glycerol offers many advantages for cosmetic use since it reduces the risk of microorganisms' development and offers humectant and hydrating properties to the skin. This approach reduced energy consumption related to evaporation because water is maintained in a liquid extract. Furthermore, we demonstrated that these extracts were stable and showed little degradation when kept at 5°C without the use of preservatives.

The question in the case of pomegranate extracts was what is the reason for this issue of solubilization? This problem was not observed with all the experimental points, revealing that it is possibly due to the composition, or the structure of the compounds extracted. This behavior was prominent in the extracts with higher yields, especially PNGs. Indicating the possibility of a correlation between the glycosylation of polyphenols and the size of the compounds with this difficulty. Another issue we faced was the high variation value of this model compared to green tea and the black locust, this required further examination of the extraction time and flow rate. The use of a lower flow rate increased the repeatability and thus required a longer extraction time. The need for a low flow rate for pomegranate and not for other plants can be related to various elements in pomegranate plant matrix or the targeted compounds (*i.e.*, the position of the metabolites in the cellular space, the particle size of the biomass, the structure of the compounds, or their stability). Consequently, demonstrating that the

standardized model is applicable to another matrix but requires nonetheless further investigation of other elements.

In the third chapter, marigold flowers served as a case study of selective sequential SFE based on the polarity of the targeted compounds. It was shown that a high temperature combined with 15% EtOH as a modifier offered the best yield of lipophilic esterified triterpenoids using a BBD constructed for non-polar molecules. These conditions were scaled up on a pilot-scale extractor utilizing 100 g of plants after being optimized on an analytical scale extractor (1 g). The results showed that both extractors had comparable performance when evaluating the solvent-to-feed ratio. The previous standardized extraction design for polar compounds was employed to maximize the extraction of narcissin present in marigold utilizing the treated biomass (free of esterified triterpenoids). Later, a non-treated biomass was used to validate the results with a full sequential selective extraction, and the outcomes were comparable proving the effectiveness of this method.

The objective of a fully selective extraction required the development of several analysis techniques. Since the lipophilic compounds precipitated in solvents compatible with reverse phase liquid chromatography (*i.e.*, MeOH) and the injection of highly non-polar compounds resulted in the precipitation of the extract at the column and false negatives. Hence, the use of unified chromatography was very beneficial in this case, showing the usefulness of supercritical fluids for the analysis of polar compounds in lipophilic extracts.

The chromatographic separation of the non-polar chemicals found in marigolds was investigated using a design of experiments in the fourth and final chapter. However, the statistical analysis of the model was insufficient to identify the optimal conditions. This indicated that the use of RSM in the case of separation optimization is maybe not the best approach. Still, the BBD allowed the production of large amounts of data that needed interpretation. This is where the Derringer functions were very beneficial, and their use was helpful to rationalize the generated data and determine the ideal analysis conditions of ultra-high efficiency/low pressure SFC using 5 coupled core-shell columns.

Perspectives

This thesis was part of the PIERIC project from Cosmétosciences and funded by the Centre-Val-de-Loire region with one industrial partner. Several thesis and post-doctoral contracts were working on the same set of plants, application using UAE, continuous flow extraction (CFE) and polar and non-polar NaDESs. The aim was to compare modern sustainable extraction techniques using the same plants mass while extracting the same compounds of interest. Therefore, during this PhD we focused only on extracting the compounds using SFE and compared with UAE to determine the interest of applying SFE for polar and non-polar compounds.

When comparing UAE and SFE the sole focus was on retaining the same liquid consumption, composition, and extraction time. A fully optimized UAE could indicate altering the mass to solvent ratio, solvent volume, temperature, and extraction duration. This would allow for a thorough comparison of two optimized techniques.

As for the general applications of SFE, in this thesis we only focused on natural plant compounds for cosmetic use, however the use of other matrixes like cell cultures for metabolomic approaches, the food industry or pharmaceutical applications is of great interest. In addition, the application of SFE on an industrial level allows for a great advantage on the ecological level, reducing global carbon print with the recycling of CO_2 and sequential selective extraction that allows the reduction of unit operation and to valorize a large scale of molecules found in the biomass with generally a high yield.

Concerning additives in the modifier for polar extraction, only water has been added to improve the yield of polar chemicals during extraction. The use other environmentally friendly solvents mixed SC-CO_2 such as NaDESs may offer an increase of the yield and is interesting to test. However, this use of NaDESs can induce some difficulties when mixed with CO_2, like the precipitation of the NaDESs due to the loss the eutectic mixtures after its dilution with other solvents or the high viscosity of the solvent that may reduce the miscibility with the supercritical fluid and influence the pumping system.

The use of integrated pre-formulation procedures for SFE was employed for polar compounds using cosmetic solvents (*i.e.*, glycerol, PG, DPG, etc.). The use of polar and non-polar NaDESs can be of interest as a pre-formulation agent for both hydrophilic and lipophilic extracts.

An efficient and straightforward method for extraction optimization was provided using standardized SFE optimization models. But extract sample preparation for chromatographic analysis required a lot of time and significantly extended the optimization period. Therefore, an online-SFE-SFC coupling can be valuable since it can reduce the overall optimization time significantly while also lowering the possibility of any sample deterioration or contamination caused by evaporation or sample preparation.

The use of SFE is not limited to extraction, other application can be explored for example the use of trapping system can allow a selective purification of certain impurities within a dynamic extraction mode. Furthermore, SC-CO_2 can be used in many ways to produce microencapsulated products depending on the properties of the active ingredient, coating material, and appropriate solvent to be used. An integrated approach of extraction and microencapsulation can allow to achieve high quality ingredients and, in some cases, high bioavailability (depending on the encapsulation type). In addition, the dynamic mode of SFE can offer a high advantage for an online functionalization of extracted natural compounds like a continuous flow extraction, adapting certain chemical functions for natural compounds to increase their bioavailability or bioactivity for human health, or formability in final products.

Finally, the methodology used in chapter 4 permitted the separation of the lipophilic compounds found in *Calendula officinalis* L, but further work is required to identify each isomer using high-resolution mass spectroscopy. Another option is purifying each compound using centrifugal partition chromatography and characterizing the compounds with NMR. The purification can be conducted with the use of the five columns in SFC connected to fraction collector, allowing to recover the pure compounds without the use of hazardous solvents.

Finally, this thesis project has developed a novel strategy for the optimization process of selective extraction of natural polar and non-polar compounds from plants. Opening many perspectives to expand the application range of SC-CO_2 for natural bioactive molecules.

Conclusion et perspectives (FR)

L'objectif de ce projet de thèse était de développer l'extraction de composés bioactifs de plantes en utilisant des méthodes respectueuses de l'environnement, notamment l'extraction par fluides supercritique à base de CO_2. Nous souhaitions produire des ingrédients cosmétiques naturels en remplaçant la macération, une technique d'extraction traditionnelle, actuellement utilisée par notre partenaire industriel. Bien que cette dernière technique soit relativement simple, elle nécessite l'utilisation de grandes quantités de solvants, une étape de filtration et d'évaporation du solvant pour obtenir l'extrait final prêt à être formulé. Cela a créé la nécessité de remplacer la macération par d'autres méthodes d'extraction. L'impact écologique du processus a été pris en compte à toutes les étapes tout en cherchant à réduire les opérations unitaires, les solvants organiques et la consommation d'énergie.

Dans le premier chapitre, les idées clés ont été présentées d'un point de vue théorique ou expérimental en commençant par une brève classification des métabolites spécialisés, les six principes de l'extraction verte, suivis d'une description des concepts, avantages et inconvénients des méthodes modernes d'extraction (UAE, MAE, PLE et NaDESs). L'extraction par fluide supercritique (SFE) a été discutée en détail (historique, propriétés physico-chimiques, instrumentation, paramètres…). Enfin, des techniques modernes d'extraction utilisant des méthodologies intégrées ont été décrites.

Parmi les diverses méthodes disponibles pour l'éco-extraction, la SFE répondait à nos critères mais l'approche nécessitait l'examen d'une large gamme de plantes. De plus, une variété de facteurs affecte les résultats de la SFE, comme décrit dans le premier chapitre. Chaque paramètre devrait être optimisé indépendamment, ce qui impliquerait de nombreux essais expérimentaux sur des périodes de travail prolongées. Ainsi, pour accélérer l'optimisation, l'utilisation de méthodes chimiométriques et statistiques, et en particulier le plan d'expériences (design of experiments ou DoE) a été un grand atout car il permet une réduction significative du nombre de points expérimentaux et la méthodologie des surfaces de réponse (RSM).

La RSM est utilisée pour obtenir un modèle prédictif, présentant des interactions entre les variables (quand celles-ci sont applicables), une réponse non linéaire et proposant un remplissage numérique de l'espace, si nécessaire. Le modèle Box-Behnken a été choisi principalement pour sa particularité de ne pas fixer tous les facteurs

expérimentaux à leurs niveaux les plus élevés en même temps, ce qui permet leur investigation progressive. De plus, il propose un faible nombre d'expériences, 13 points étaient suffisants pour l'analyse d'un modèle avec trois variables à trois niveaux et quatre répétitions des points centraux, pour déterminer la reproductibilité du système et de la matrice végétale elle-même. Un plan factoriel complet avec le même nombre de variables et de niveaux aurait nécessité 27 expériences.

Avant de discuter des résultats liés à l'extraction, nous tenons à souligner que de nombreuses méthodes UHPLC ont été développées, dans le but de garantir une séparation et une quantification rapides et efficaces des composés ciblés. Toutes ces méthodes ont été réalisées en utilisant la même phase stationnaire, une silice greffée C18 avec des particules partiellement poreuses de 2,5 μm. L'utilisation de la même colonne a facilité l'analyse des extraits des différents plantes sur une même séquence d'analyse simplifiant ainsi le suivi des composés en diminuant la contrainte de temps. La composition de la phase mobile variait selon chaque étude et un temps de stabilisation nécessaire a été mis en œuvre à chaque étape. Un mode d'élution en gradient était fréquemment utilisé et la quantification des composés ciblés était réalisée grâce à l'utilisation de courbes d'étalonnage établies à partir de molécules standard. La détection et l'identification des composés ont été assurées par UV et DEDL et comparaison des temps de rétention à ceux des composés standards et dans certains cas par l'utilisation des techniques d'identification comme la spectrométrie de masse ou la spectroscopie par résonance magnétique nucléaire.

Ces méthodes développées ont été utilisées dans au moins trois autres études liées au projet PIERIC et ont permis la quantification des composés d'intérêt dans les extraits réalisés en flux continu et avec différents types de NaDEs, ce qui démontre la transversalité des études de ce projet et la place centrale de cette thèse.

Un des défis de ce projet était l'extraction de molécules polaires. Étant donné que le CO_2 est non polaire, il est largement utilisé pour l'extraction de composés non polaires (par exemple, les lipides, les caroténoïdes, etc.). Le mode dynamique utilisé en SFE offrait un avantage crucial pour l'extraction fractionnée et sélective avec l'aide de modificateurs ou de co-solvants et le réglage d'autres paramètres. Enfin, les propriétés inhérentes du CO_2 ont permis de récupérer des extraits concentrés, dans des solvants adaptés à la formulation.

C'est dans cet objectif que, le deuxième chapitre est construit sur toutes les applications du BBD (Box-Behnken Design) pour l'extraction de composés polaires à partir de

plantes, dans le but de standardiser la méthodologie et de faciliter l'utilisation de la SFE.

La première plante examinée était le robinier faux-acacia dont le bois de cœur est riche de nombreux composés phénoliques. Dans le cadre de cette étude, deux composés ont été ciblés, la dihydrorobinétine (DHR) et la robinétine (Rob), deux flavonoïdes majeurs avec un fort potentiel pour des applications cosmétiques. Leur extraction a été optimisée par un design de Box-Behnken (BBD) avec trois variables à trois niveaux. Les paramètres tels que le débit et la masse végétale ont été maintenus constants (optimisé pour l'instrument), correspondant au système d'extraction, et le temps d'extraction employé pour les expériences a été déterminé en suivant la cinétique d'extraction avec les conditions du point central du modèle. Cela a permis de réduire à trois variables (la température, la pression et le pourcentage de modificateur composé d'un mélange EtOH:H_2O 80:20 *v:v*) et de fait, le nombre de points expérimentaux.

Nous avons établi que la pression avait un effet minimal sur le rendement (jusqu'à 20 MPa) en raison de la densité élevée de la phase d'extraction due au pourcentage élevé de modificateur qui varie de 10 à 30%. Elle a été maintenue à une valeur constante de 10 MPa. Le rendement d'extraction pour les deux flavonoïdes était supérieur avec la SFE comparer à l'extraction assistée par ultrasons. Les conditions optimisées de la SFE (20% de modificateur EtOH:H_2O 80:20 *v:v*, 10 MPa, 80 °C, pendant 30 minutes) comparées à l'extraction ultrasonore classique avec un mélange EtOH:H_2O 80:20 v:v avec une durée d'extraction et un volume de solvant organique identiques, ont démontré la capacité de la SFE à atteindre une récupération élevée de composés polaires. Des expériences supplémentaires ont été menées en étudiant la teneur en eau mélangée à l'éthanol (de 5 à 30%) à deux températures différentes (40 et 80 °C) pour une extraction sélective des deux flavonoïdes. La conclusion est qu'aucune condition n'a montré une tendance à une extraction sélective entre ces deux composés.

Notre choix d'utiliser ce co-solvant (EtOH: H_2O 80:20 *v:v)* en présence des fluides supercritiques pendant cette étude est lié à une recherche effectuée précédemment au sein de l'équipe. Cette composition de co-solvant était la plus intéressante pour extraire les deux composés avec des rendement élevés. Nous nous sommes limités à 20% d'eau ajouté dans le co-solvant afin d'éviter des problèmes de précipitation ou de bouchage dans le système d'extraction mais aussi à l'oxydation ou au changement de couleur des extraits. Ces variations organoleptiques peuvent être un désavantage esthétique pour l'industriel cosmétique ou provoquer une dégradation des composés d'intérêt en diminuant le rendement ou l'activité biologique de l'extrait final. De plus,

une précipitation nécessite une étape en plus de filtration ou de centrifugation ce qui a un coût à grande échelle.

La pression appliquée pendant cette étude est limitée à une plage de 10 à 20 MPa maximum. Ce choix est lié à plusieurs facteurs que sont le cout énergétique et économique excessif de l'augmentation de pression notamment dans un cadre industriel. Dans la littérature et le premier chapitre de ce manuscrit, de nombreuses publications ont démontré l'influence des pressions élevées, autour de 50 MPa et au-dessus, sur le rendement d'extraction. Cela pourrait être très intéressant à l'échelle analytique mais tout ceci devient couteux et dans certains cas dangereux dans le cadre réglementaire de l'usine à plus grande échelle. Dans ce contexte, une pression de 15 MPa permet d'atteindre des densités suffisantes de fluides en évitant ces désavantages.

Dans la deuxième partie, l'extraction de catéchines et de caféine (LogP = -0,1) à partir des feuilles de thé vert a également été optimisée à l'aide d'un BBD. Ces composés ont un LogP plus faible que la DHR (LogP = 0,9) et la Rob (LogP = 1,6). Cette fois, sur la base de l'étude précédente, la pression a été remplacée en tant que variable par le pourcentage d'eau ajoutée dans le modificateur associé à l'éthanol et la valeur de la pression a été maintenue constante à 15 MPa. Le but est d'optimiser le rendement d'extraction des composés d'intérêt et de voir l'influence de la variation de la composition du co-solvant sur le rendement et la sélectivité en plus de déterminer si un pourcentage élevé d'eau (20%) est nécessaire comme additif pour l'extraction de tous les composés polaires.

L'eau est nécessaire pour extraire ces composés très polaires mais les désavantages cités auparavant lors de l'augmentation du pourcentage d'eau en présence des fluides supercritiques, tels que son évaporation plus difficile, plus couteuse et énergivore comparer à l'éthanol, rend son utilisation limitée. A partir de ce constat, une diminution du pourcentage d'eau pourrait être intéressant à étudier à condition que le rendement ne soit pas affecté.

A partir des résultats, une température de 60 °C combinée à un pourcentage de modificateur élevé et à une teneur en eau élevée (20% dans l'éthanol) favoriserait l'extraction de la caféine et des catéchines polaires du thé vert. Après l'analyse des résultats, il apparait que l'utilisation de 10% d'éthanol comme co-solvant induit une récupération sélective de la caféine pure par rapport à un mélange EtOH:H_2O 80:20 *v:v* qui permettait une extraction plus exhaustive de la caféine et des catéchines. Ainsi, une extraction séquentielle partiellement sélective de la caféine a également été réalisée.

Dans la troisième partie, les punicalagines et l'acide ellagique représentaient les composés d'intérêt dans le péricarpe de grenade. Le même plan d'expérience utilisé pour les feuilles de thé vert a été utilisé. Cela a permis une optimisation rapide et a validé l'utilisation de ce plan sur d'autres matrices végétales. Une température plus basse, des pourcentages plus élevés de modificateur et d'eau étaient nécessaires pour maximiser le rendement des composés polaires étudiés. De plus, la solubilisation des extraits séchés avant l'analyse chromatographique présentait un défi important lors de la préparation des échantillons car aucun solvant autre que le DMSO (50 %) ne pouvait fournir un mélange homogène (c'est-à-dire solubiliser l'ensemble des composés bioactifs). Cependant, même si cela a été résolu pour l'analyse chromatographique, des problèmes persistent concernant l'utilisation du DMSO pour solubiliser des extraits dans les ingrédients cosmétiques.

Par conséquent, une pré-formulation intégrée des extraits de SFE a été réalisée en utilisant des solvants approuvés pour les produits cosmétiques, maintenant les composés bioactifs sous une forme liquide utilisable sans l'ajout de DMSO. Cela a abouti à l'ajout de glycérol après l'étape d'extraction pour stocker les extraits sous forme liquide plutôt que de les évaporer complètement. Dans ce cas, à la fin du processus de SFE, l'extrait est solubilisé dans un mélange EtOH:H_2O 80:20 *v:v* et le glycérol a été ajouté à l'extrait après la dépressurisation du CO_2 dans la même quantité d'éthanol. Après évaporation de l'éthanol, les composés bioactifs sont stabilisés dans un mélange de glycérol et d'eau 80:20 *v:v*, une composition couramment utilisée comme ingrédient dans la pré-formulation cosmétique.

Le glycérol offre de nombreux avantages pour une utilisation cosmétique car il réduit le risque de développement de micro-organismes et offre des propriétés hygroscopiques et hydratantes à la peau. Cette approche a réduit la consommation d'énergie liée à l'évaporation car l'eau est maintenue dans un extrait liquide. De plus, nous avons démontré que ces extraits étaient stables lorsqu'ils sont conservés à 5 °C pendant trois mois sans utiliser de conservateurs.

La question liée au cas des extraits de grenade était la suivante : Quelle est la raison de ce problème de solubilisation ? Ce problème n'a pas été observé pour tous les points expérimentaux probablement lié à la composition ou à la structure des composés extraits. Ce comportement était particulièrement prononcé dans les extraits avec des rendements plus élevés, en particulier les punicalagines. Cela indique la possibilité d'une corrélation entre la glycosylation des polyphénols et la taille des composés avec cette difficulté.

Dans le troisième chapitre, les fleurs de souci ont servi d'étude de cas pour une SFE séquentielle sélective basée sur la différence de polarité des composés ciblés. Il a été démontré qu'une température élevée combinée à 15 % d'éthanol en tant que co-solvant offrait le meilleur rendement des triterpénoïdes estérifiés lipophiles par l'utilisation d'un BBD construit pour les molécules non polaires. Ces conditions ont été transférées à l'échelle pilote sur un extracteur utilisant 100 g de plantes après l'optimisation sur un extracteur à l'échelle analytique avec 1 g. Les résultats ont montré que les deux extracteurs avaient des performances comparables lors de l'évaluation du rapport solvant/substrat. La conception d'une extraction standardisée pour les composés polaires a été utilisée pour maximiser l'extraction de la narcissine présente dans le souci en utilisant la biomasse traitée (libre de triterpénoïdes estérifiés). Ultérieurement, une biomasse non traitée a été utilisée pour valider les résultats par une extraction séquentielle sélective complète et les résultats ont prouvé l'efficacité de cette méthode.

L'objectif d'une extraction entièrement sélective nécessite le développement de plusieurs techniques d'analyse. Comme les composés lipophiles précipitaient dans des solvants compatibles avec la chromatographie liquide en phase inverse (ex : MeOH) et que l'injection de composés très apolaires entraînait la précipitation de l'extrait dans la colonne et des faux négatifs, l'utilisation de la chromatographie unifiée a été très bénéfique dans ce cas, démontrant l'utilité des fluides supercritiques pour l'analyse de composés polaires dans des extraits lipophiles.

La séparation chromatographique des composés non polaires trouvés dans les fleurs de soucis a été étudiée en utilisant un plan d'expériences dans le quatrième et dernier chapitre. Cependant, l'analyse statistique du modèle était insuffisante pour identifier les conditions optimales, puisque dans le cas de l'optimisation de séparation plein de critères sont pris en compte afin d'identifier une séparation optimale, dans notre cas c'était : nombre de pics résolus, nombre total de pics, facteur de discrimination des deux pics majoritaires des esters de faradiol (d_0) et temps de rétention du dernier pic. Chacun de ces critères était considéré comme une réponse et analysé statistiquement afin de trouver les conditions optimales de séparation. Pourtant, cette stratégie à générer des optimums différents pour chaque réponse.

Cela indique que l'utilisation de la RSM pour l'optimisation de la séparation n'est peut-être pas la meilleure approche, dans ce cas. Néanmoins, le BBD a permis la production de grandes quantités de données sur les critères de séparation. C'est là que les fonctions de Derringer ont été très bénéfiques, et leur utilisation a permis de rationaliser les données générées et de déterminer les conditions optimales d'analyse

de la SFC ultra haute efficacité à basse pression à l'aide de 5 colonnes couplées core-shell.

Finalement, cette thèse a apporté une contribution significative au domaine du développement de méthodes d'extraction de composés bioactifs utilisant du dioxyde de carbone supercritique. Plus précisément, elle a étendu l'utilisation de la SFE pour l'extraction de composés polaires, tout en développant des méthodes séquentielles permettant l'extraction sélective et fractionnée de composés non polaires, suivie de composés polaires, à partir d'une même source de biomasse végétale. Ces méthodes, qui se basent exclusivement sur l'utilisation de solvants verts, ont démontré des performances comparables, voire supérieures, à celles des méthodes traditionnelles d'extraction, et sont susceptibles d'être adaptées à une mise en œuvre industrielle.

Supplementary data

Chapter II: Part I (Black locust)

Flavonoids yield (mg/g)

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	2153.5	2153.5	2153.5	358.91	18.7600	0.0003		Very significant
P	1	65.345	12.623	12.623	12.623	0.6598	0.4401	0.827%	Not significant
T°	1	287.82	87.082	87.082	87.082	4.5517	0.0654	5.71%	Limit
%M	1	1301.1	737.9	737.9	737.9	38.5694	0.0003	48.4%	Very significant
P*P	1	5.955	20.169	20.169	20.169	1.0542	0.3346	1.32%	Not significant
T°*T°	1	34.1	55.807	55.807	55.807	2.9170	0.1260	3.66%	Not significant
%M*%M	1	459.12	459.12	459.12	459.12	23.9974	0.0012	30.1%	Very significant
Residuals	8	153.05	153.05	153.05	19.132			10%	
Total	14	2306.5							

Figure 1: ANOVA for response surface regression model for total flavonoids yield (DHR and Rob) mg/g of biomass.

Parameters	nb df	S	R^2	R^2 Adjusted	R^2 Predicted
Values	8	4.3740	93.4%	88.4%	73.9%

Flavonoids yield (mg/g) = -96.66 + 0.2233*P + 1.466*T° + 5.736*%M - 0.0009349*P*P - 0.009719*T°*T° - 0.1115*%M*%M

Figure 2: model's R^2. adjusted R^2 and predicted R^2 and equation.

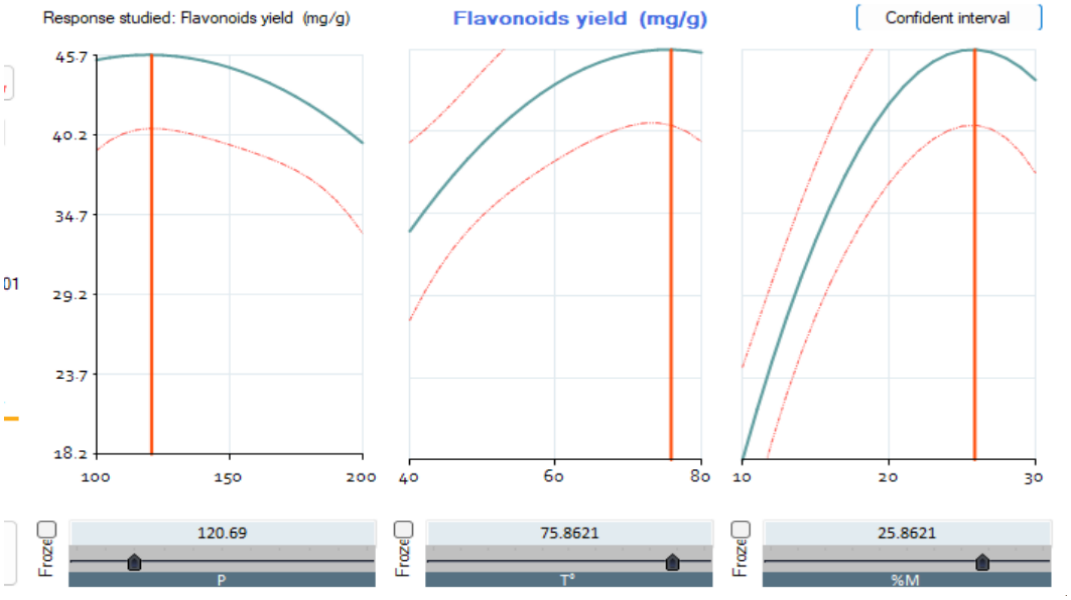


Figure 3: Predicted values for the maximization of the response .

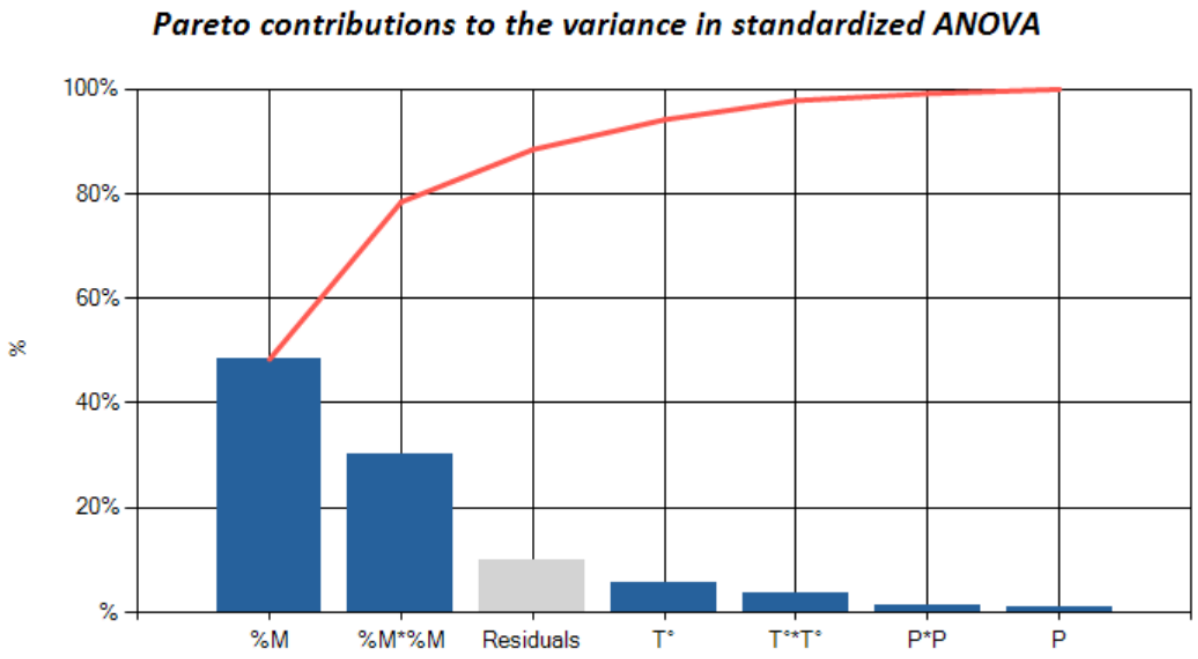


Figure 4: Pareto contributions to variance in standardized ANOVA.

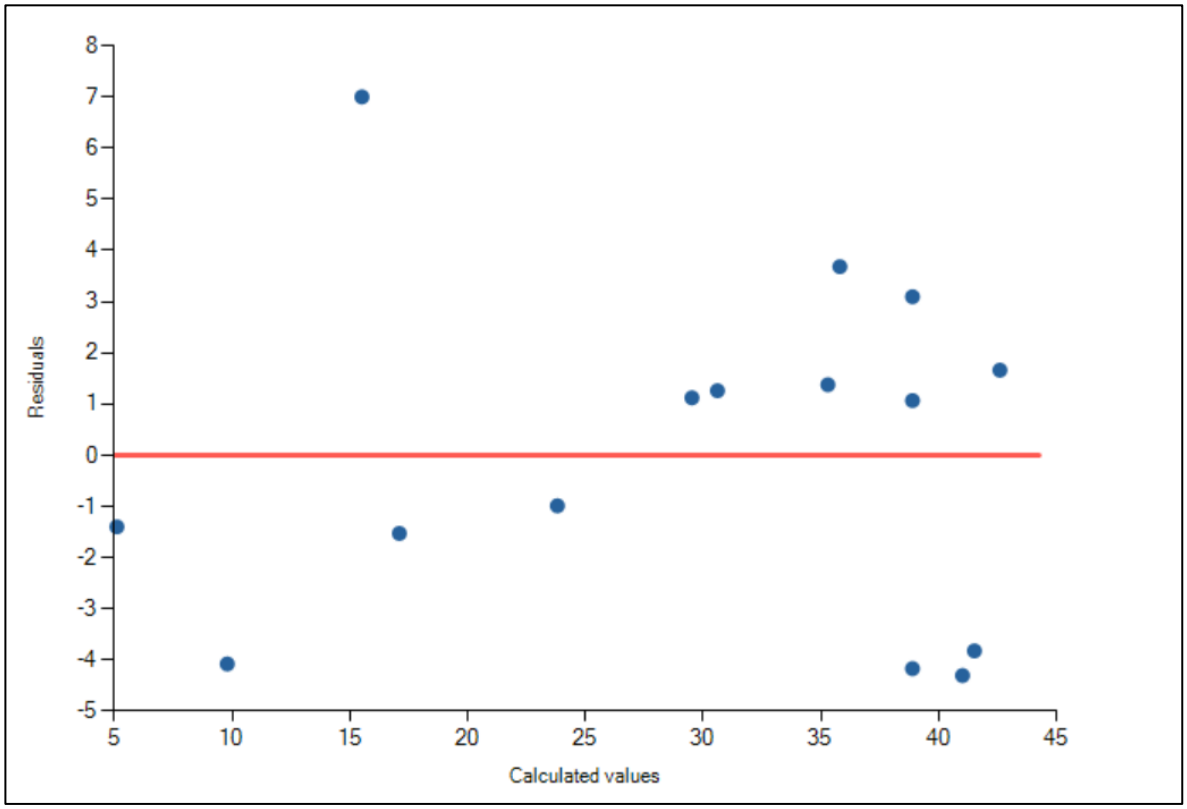


Figure 5: Residuals vs fitted values.

Residuals | Cartography

	P	T°	%M	Flavonoids yield (mg/g)	Flavonoids yield (mg/g)~	Error	Res std
1	150	60	20	39.961	38.892	1.0685	0.24
2	100	40	20	30.65	29.527	1.1225	0.26
3	200	40	20	22.825	23.811	-0.98651	-0.23
4	100	80	20	37.704	41.523	-3.8196	-0.87
5	150	60	20	34.727	38.892	-4.1655	-0.95
6	200	80	20	39.491	35.807	3.6836	0.84
7	100	60	10	22.506	15.509	6.9972	1.60
8	200	60	10	5.7189	9.7929	-4.074	-0.93
9	150	60	20	41.989	38.892	3.0969	0.71
10	100	60	30	36.715	41.015	-4.3001	-0.98
11	200	60	30	36.676	35.299	1.3769	0.31
12	150	40	10	3.7054	5.1022	-1.3968	-0.32
13	150	80	10	15.572	17.099	-1.5264	-0.35
14	150	40	30	31.869	30.608	1.2608	0.29
15	150	80	30	44.267	42.605	1.6624	0.38

Colour Key

Very high residual ==> This is probably an outlier

high residual (greater than 2σ) ==> check this run

Low residual (less than 2σ) ==> normal situation

Figure 6: : experimental data (Y=grey column) vs predicted values (Y~). deviation and residuals.

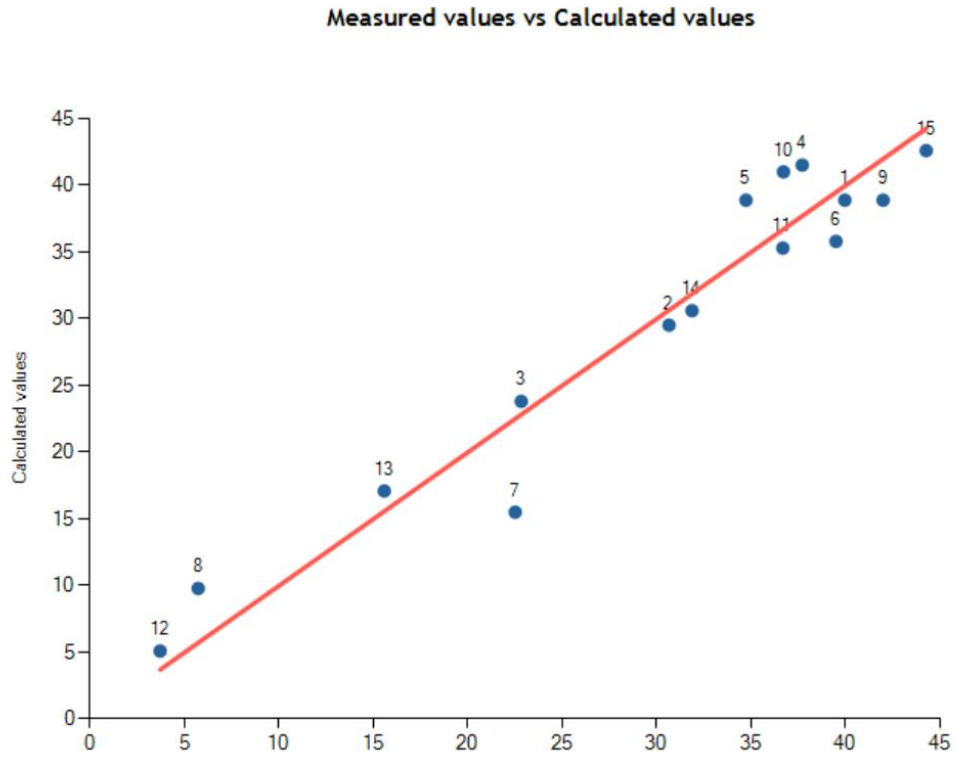


Figure 7: Distribution of measured vs calculated values.

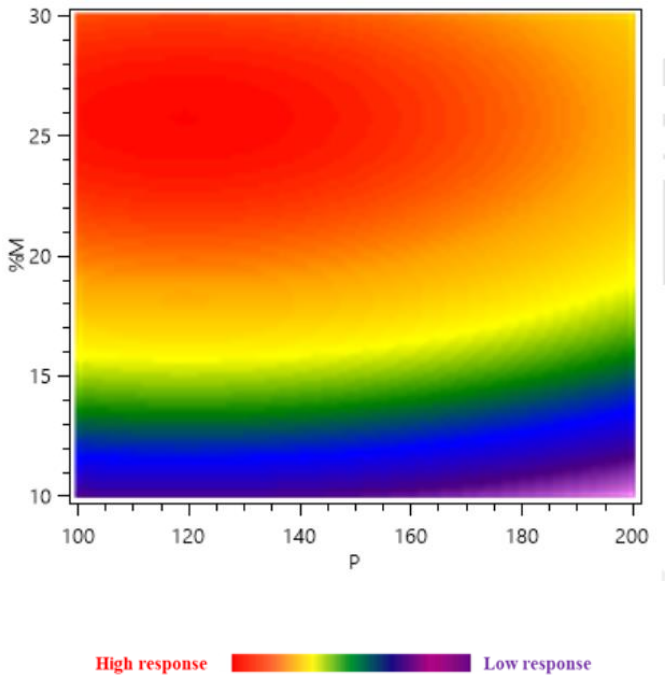


Figure 8: : Response surface heat-maps showing the effect of pressure (MPa) and modifier (%) variation on the total flavonoids yield (mg/g).

Chapter II: Part II (green tea)

Total molecules (mg/g of biomass)

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	16426	16426	16426	2737.6	12.5291	0.0004		Very significant
Temperature (°C)	1	54.601	475.15	475.15	475.15	2.1746	0.1711	4.54%	Not significant
Modifier percentage (%)	1	4446.2	1127.9	1127.9	1127.9	5.1618	0.0464	10.8%	Significant
Water percentage (%)	1	9473.8	4472.9	4472.9	4472.9	20.4708	0.0011	42.7%	Very significant
Temperature (°C)*Temperature (°C)	1	592.92	443.66	443.66	443.66	2.0305	0.1846	4.24%	Not significant
Modifier percentage (%) *Modifier percentage (%)	1	573.96	485.67	485.67	485.67	2.2227	0.1668	4.64%	Not significant
Water percentage (%)*Water percentage (%)	1	1284.3	1284.3	1284.3	1284.3	5.8778	0.0358	12.3%	Significant
Residuals	10	2185	2185	2185	218.5			20.9%	
Total	16	18611							

Figure 9: ANOVA for response surface regression model for total compounds (mg/g of biomass).

Parameters	nb df	S	R^2	R^2 Adjusted	R^2 Predicted
Values	10	14.7818	88.3%	81.2%	46.9%

Total molecules (mg/g of biomass) = -166.5 + 3.21*Temperature (°C) + 6.653*Modifier percentage (%) + 6.934*Water percentage (%) - 0.02566*Temperature (°C)*Temperature (°C) - 0.1074*Modifier percentage (%) *Modifier percentage (%) - 0.1747*Water percentage (%) *Water percentage (%)

Figure 10: : model's R^2. adjusted R^2 and predicted R^2 and equation.

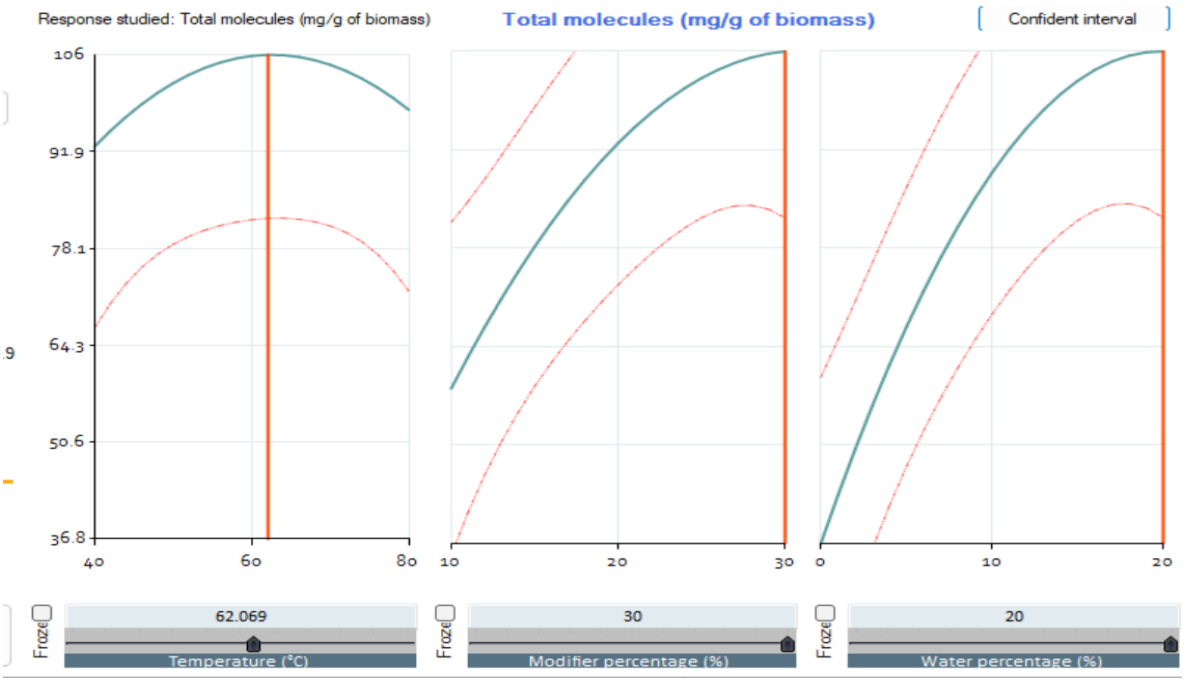


Figure 11: Predicted values for the maximization of the response.

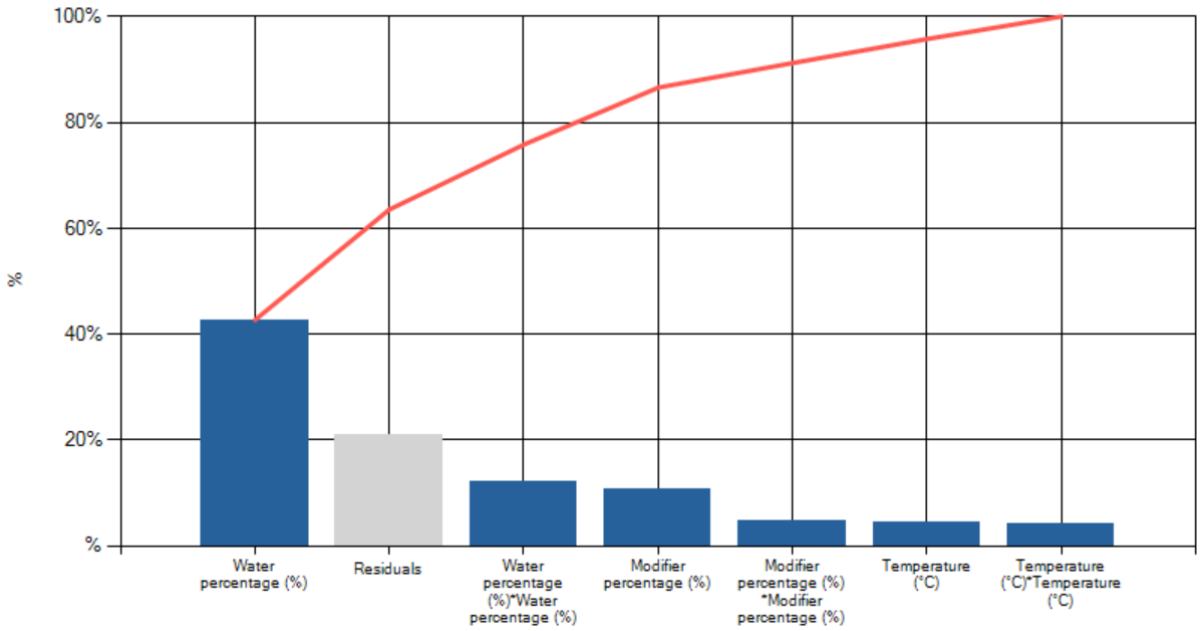


Figure 12: Pareto contributions to variance in standardized ANOVA.

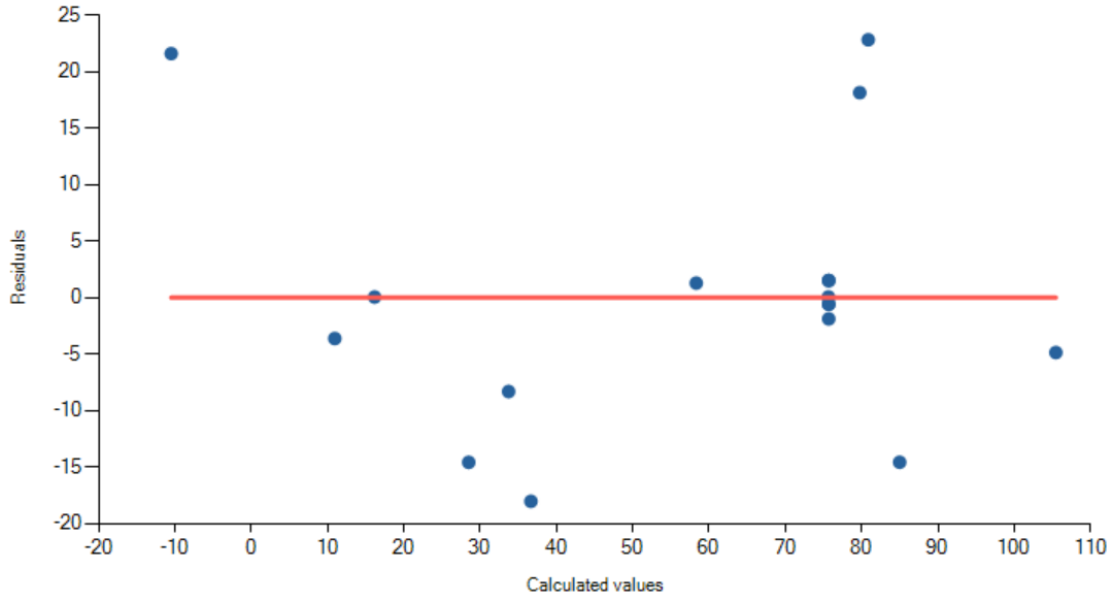


Figure 13: Residuals vs fitted values.

	Temperature (°C)	Modifier percentage (%)	Water percentage (%)	Total molecules (mg/g of biomass)	Total molecules (mg/g of biomass) ~	Error	Res std
1	40	10	10	13.9	28.488	-14.588	-0.99
2	80	10	10	25.4	33.713	-8.3125	-0.56
3	40	30	10	75.7	75.637	0.0625	0.00
4	80	30	10	103.7	80.863	22.838	1.54
5	40	20	0	7.3	10.925	-3.625	-0.25
6	80	20	0	16.2	16.15	0.05	0.00
7	40	20	20	97.9	79.75	18.15	1.23
8	80	20	20	70.4	84.975	-14.575	-0.99
9	60	10	0	11.1	-10.512	21.612	1.46
10	60	30	0	18.6	36.638	-18.038	-1.22
11	60	10	20	59.6	58.313	1.2875	0.09
12	60	30	20	100.6	105.46	-4.8625	-0.33
13	60	20	10	73.8	75.68	-1.88	-0.13
14	60	20	10	77.2	75.68	1.52	0.10
15	60	20	10	77.2	75.68	1.52	0.10
16	60	20	10	75.1	75.68	-0.58	-0.04
17	60	20	10	75.1	75.68	-0.58	-0.04

Colour Key

Very high residual ==> This is probably an outlier

high residual (greater than 2σ) ==> check this run

Low residual (less than 2σ) ==> normal situation

Figure 14: experimental data (Y=grey column) vs predicted values (Y~). deviation and residuals.

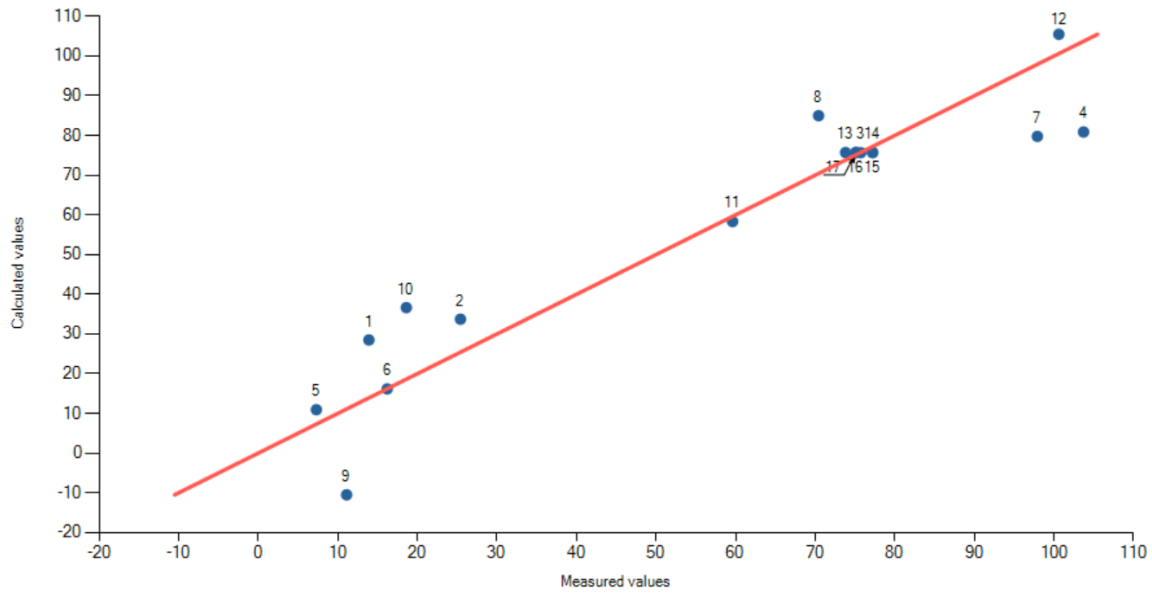


Figure 15: Distribution of measured vs calculated values.

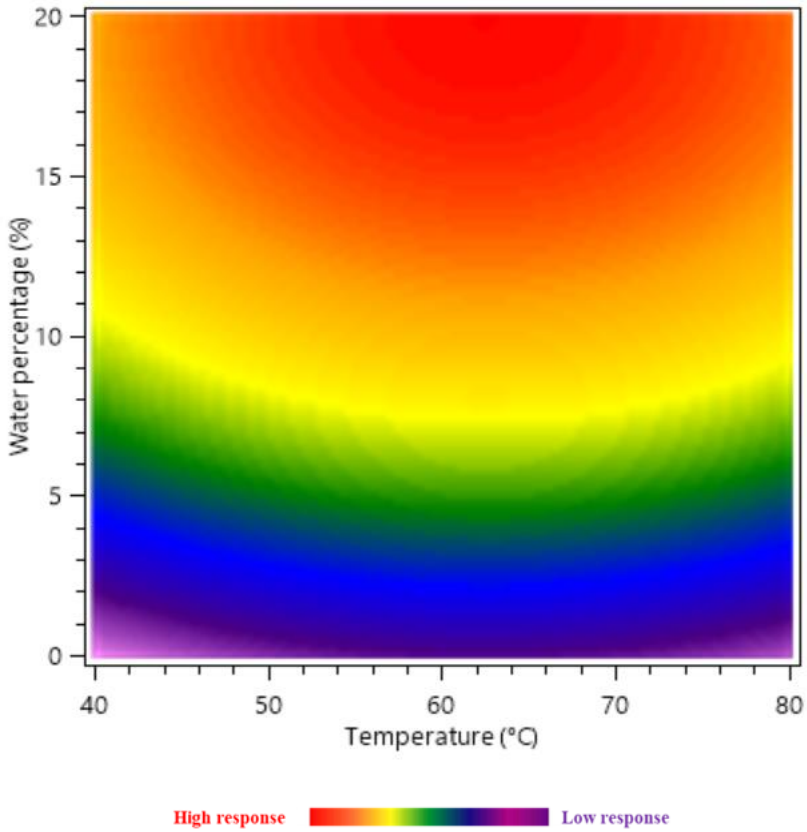


Figure 16: Response surface heat-maps showing the effect of temperature and water percentage variation on the total yield.

Chapter II: Part III (pomegranate)

Total molecules (mg/g)

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	236.34	236.34	236.34	39.39	6.4223	0.0054		Very significant
Temperature (°C)	1	3.4716	26.716	26.716	26.716	4.3560	0.0634	11.9%	Limit
% Modifier	1	64.128	45.62	45.62	45.62	7.4381	0.0213	20.4%	Significant
% H2O	1	85.805	4.5228	4.5228	4.5228	0.7374	0.4106	2.02%	Not significant
Temperature (°C)*Temperature (°C)	1	25.075	24.812	24.812	24.812	4.0454	0.0720	11.1%	Limit
% Modifier *% Modifier	1	26.247	29.29	29.29	29.29	4.7756	0.0538	13.1%	Limit
% H2O*% H2O	1	31.611	31.611	31.611	31.611	5.1540	0.0466	14.1%	Significant
Residuals	10	61.333	61.333	61.333	6.1333			27.4%	
Total	16	297.67							

Figure 17: ANOVA for response surface regression model for total compounds (mg/g of biomass).

Parameters	nb df	S	R^2	R^2 Adjusted	R^2 Predicted
Values	10	2.4765	79.4%	67.0%	0.7%

Total molecules (mg/g) = 10.22 - 0.7612*Temperature (°C) + 1.338*% Modifier - 0.2205*% H2O + 0.006069*Temperature (°C)*Temperature (°C) - 0.02638*% Modifier *% Modifier + 0.0274*% H2O*% H2O

Figure 18: : model's R^2. adjusted R^2 and predicted R^2 and equation.

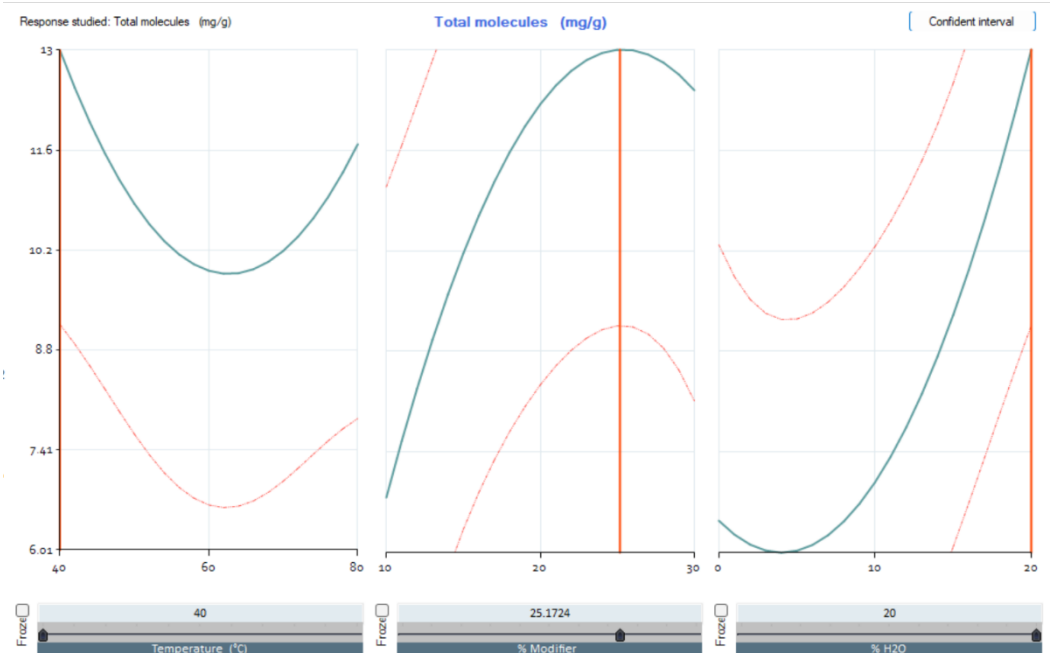


Figure 19: Predicted values for the maximization of the response.

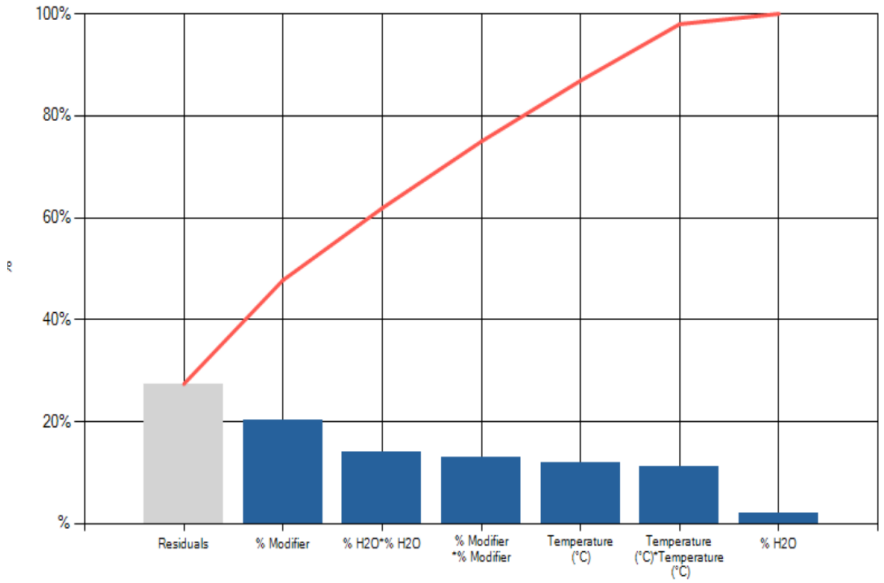


Figure 20: Pareto contributions to variance in standardized ANOVA.

	Temperature (°C)	% Modifier	% H2O	Total molecules (mg/g)	Total molecules (mg/g) ~	Error	Res std
1	40	10	10	0.02	0.7575	-0.7375	-0.30
2	80	10	10	0.57	-0.56	1.13	0.46
3	60	20	10	2.85	3.14	-0.29	-0.12
4	40	30	10	7.76	6.42	1.34	0.54
5	80	30	10	3.37	5.1025	-1.7325	-0.70
6	60	20	10	3	3.14	-0.14	-0.06
7	40	20	0	2.72	5.6913	-2.9713	-1.20
8	80	20	0	2.57	4.3738	-1.8038	-0.73
9	60	20	10	4.13	3.14	0.99	0.40
10	40	20	20	14.61	12.241	2.3687	0.96
11	80	20	20	13.33	10.924	2.4062	0.97
12	60	20	10	4.12	3.14	0.98	0.40
13	60	10	0	0.31	-2.8637	3.1737	1.28
14	60	30	0	4.4	2.7988	1.6012	0.65
15	60	20	10	1.6	3.14	-1.54	-0.62
16	60	10	20	0.12	3.6863	-3.5663	-1.44
17	60	30	20	8.14	9.3488	-1.2088	-0.49

Colour Key
Very high residual ==> This is probably an outlier
high residual (greater than 2σ) ==> check this run
Low residual (less than 2σ) ==> normal situation

Figure 21: experimental data (Y=grey column) vs predicted values (Y~). deviation and residuals.

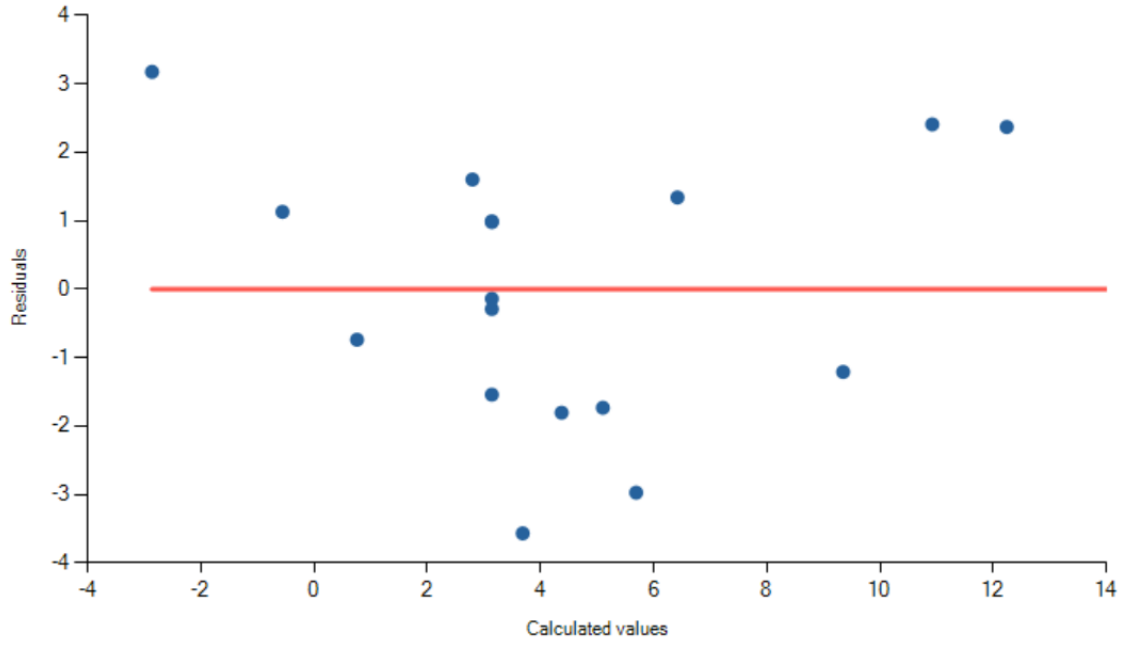


Figure 22: Residuals vs fitted values.

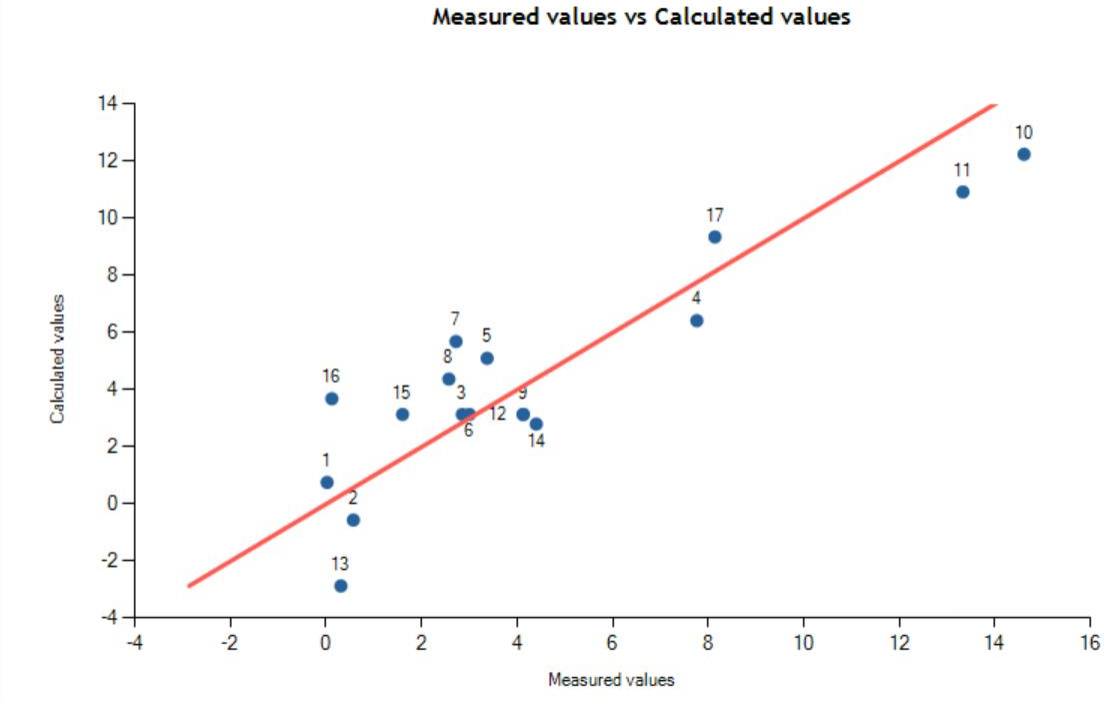


Figure 23: Distribution of measured vs calculated values.

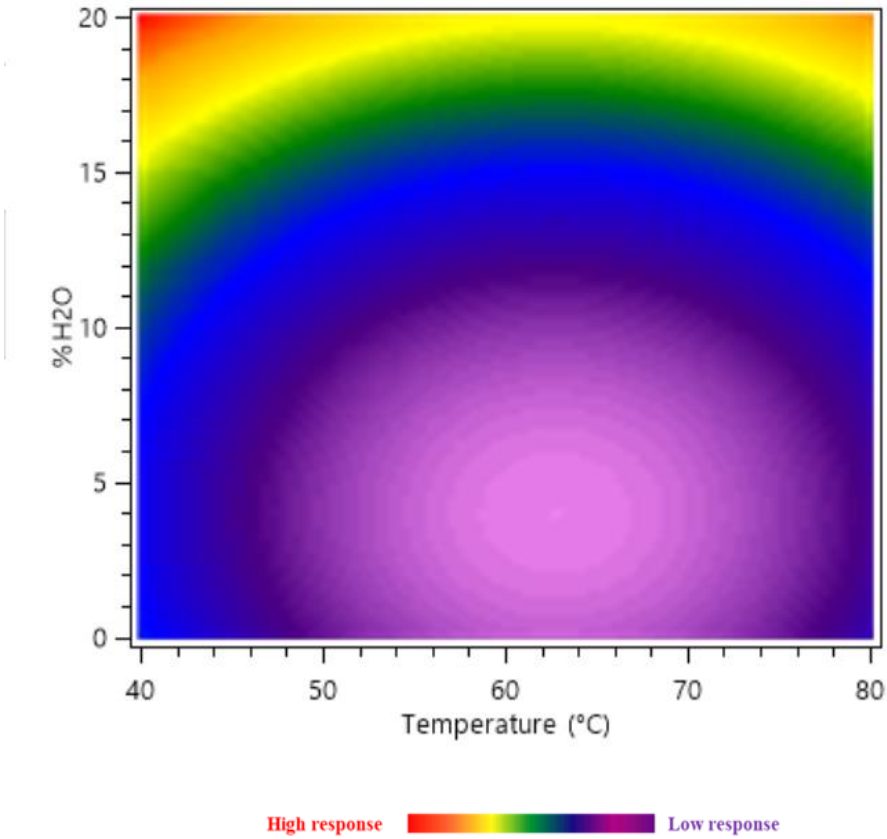


Figure 24: influence of water percentage and temperature on the total yield.

Chapter III: Calendula non-polar extracts (CNPE)

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	7	4.3485E+18	4.3485E+18	4.3485E+18	6.2121E+17	17.5737	0.0001		Very significant
X1	1	5.9963E+17	9.8515E+16	9.8515E+16	9.8515E+16	2.7869	0.1294	3.75%	Not significant
X2	1	1.9942E+16	4.0094E+17	4.0094E+17	4.0094E+17	11.3423	0.0083	15.3%	Very significant
X3	1	2.2417E+18	3.0974E+17	3.0974E+17	3.0974E+17	8.7624	0.0160	11.8%	Significant
X1*X1	1	2.3401E+15	5.2683E+15	5.2683E+15	5.2683E+15	0.1490	0.7084	0.2%	Not significant
X2*X2	1	3.7452E+17	3.8531E+17	3.8531E+17	3.8531E+17	10.9002	0.0092	14.7%	Very significant
X3*X3	1	3.3276E+16	3.3276E+16	3.3276E+16	3.3276E+16	0.9414	0.3573	1.27%	Not significant
X1*X3	1	1.0771E+18	1.0771E+18	1.0771E+18	1.0771E+18	30.4700	0.0004	41%	Very significant
Residuals	9	3.1814E+17	3.1814E+17	3.1814E+17	3.5349E+16			12.1%	
Total	16	4.6666E+18							

Figure 25: ANOVA for response surface regression model for total peak area of faradiol esters (faradiol myristate and faradiol palmitate) in selective CNPE.

Y Y^0.2777 Y^0.5 Y^2 Ln(Y) Response studied: Y (mAUxmin)

Parameters	nb df	S	R^2	R^2 Adjusted	R^2 Predicted
Values	9	188012861.3631	93.2%	87.9%	54.2%

Y (mAUxmin) = 7.976E+07 - 4.881E+07*X1 + 3.73E+08*X2 - 2.766E+08*X3 + 8.843E+04*X1*X1 - 1.21E+07*X2*X2 + 3.556E+06*X3*X3 + 5.189E+06*X1*X3

Figure 26: model's R^2. adjusted R^2 and predicted R^2 and equation.

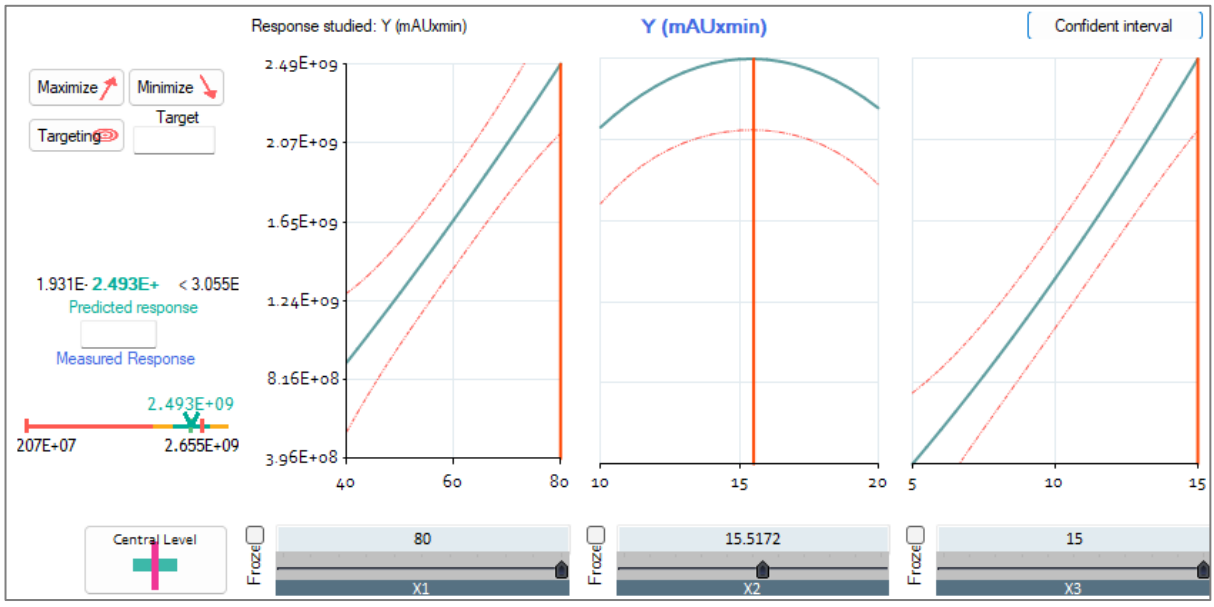


Figure 27: Predicted values for the maximization of the response (Y = faradiol esters peak area mAU×min)

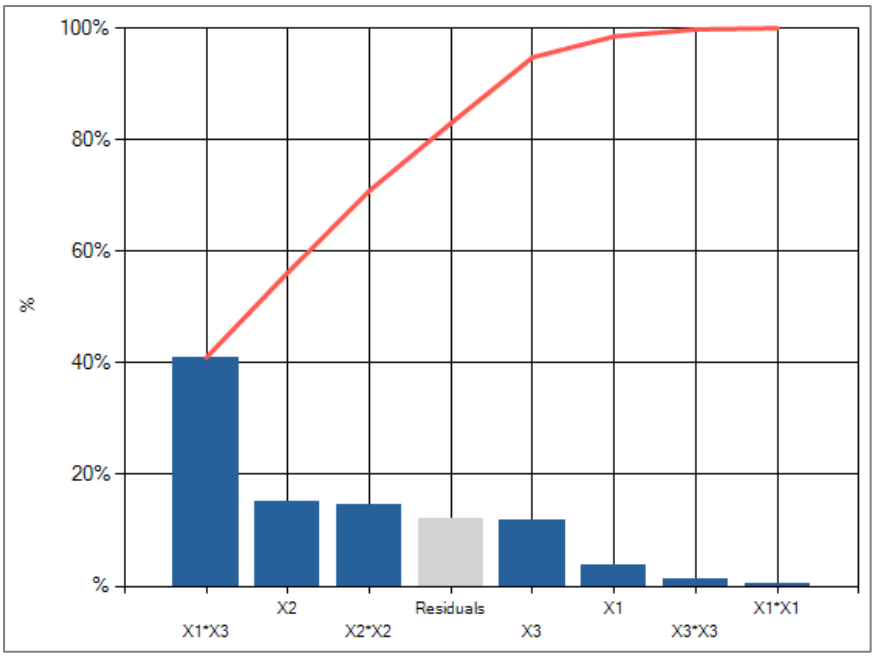


Figure 28: Pareto contributions to variance in standardized ANOVA.

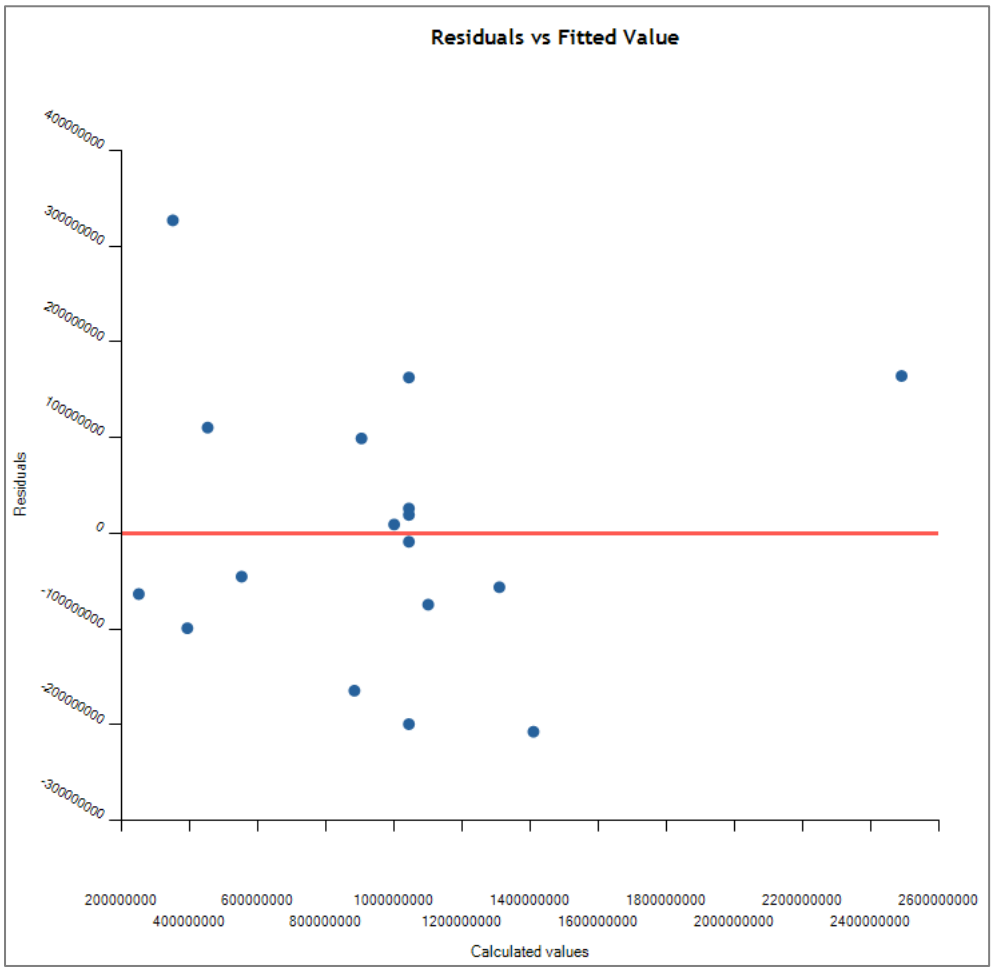


Figure 29: Residuals vs fitted values.

Residuals | Cartography

	X1	X2	X3	Y (mAUxmin)	Y (mAUxmin) ~	Error	Res std
1	40	10	10	5.6438E+08	4.5388E+08	1.1051E+08	0.59
2	80	10	10	1.0106E+09	1.0014E+09	9.2182E+06	0.05
3	40	20	10	5.0847E+08	5.5373E+08	-4.5259E+07	-0.24
4	80	20	10	1.0268E+09	1.1013E+09	-7.4465E+07	-0.40
5	40	15	5	7.2036E+08	8.8477E+08	-1.6442E+08	-0.87
6	80	15	5	2.9533E+08	3.945E+08	-9.9173E+07	-0.53
7	40	15	15	1.0048E+09	9.0565E+08	9.9173E+07	0.53
8	80	15	15	2.6554E+09	2.491E+09	1.6442E+08	0.87
9	60	10	5	1.8839E+08	2.5183E+08	-6.3441E+07	-0.34
10	60	20	5	6.7872E+08	3.5169E+08	3.2703E+08	1.74
11	60	10	15	1.2542E+09	1.3105E+09	-5.6283E+07	-0.30
12	60	20	15	1.2031E+09	1.4104E+09	-2.0731E+08	-1.10
13	60	15	10	1.0707E+09	1.0447E+09	2.6002E+07	0.14
14	60	15	10	1.0359E+09	1.0447E+09	-8.8063E+06	-0.05
15	60	15	10	1.064E+09	1.0447E+09	1.9292E+07	0.10
16	60	15	10	1.2076E+09	1.0447E+09	1.6286E+08	0.87
17	60	15	10	8.4537E+08	1.0447E+09	-1.9935E+08	-1.06

Very high residual ==> This is probably an outlier
high residual (greater than 2σ) ==> check this run
Low residual (less than 2σ) ==> normal situation

Figure 30: experimental data (Y=grey column) vs predicted values (Y~). deviation and residuals.

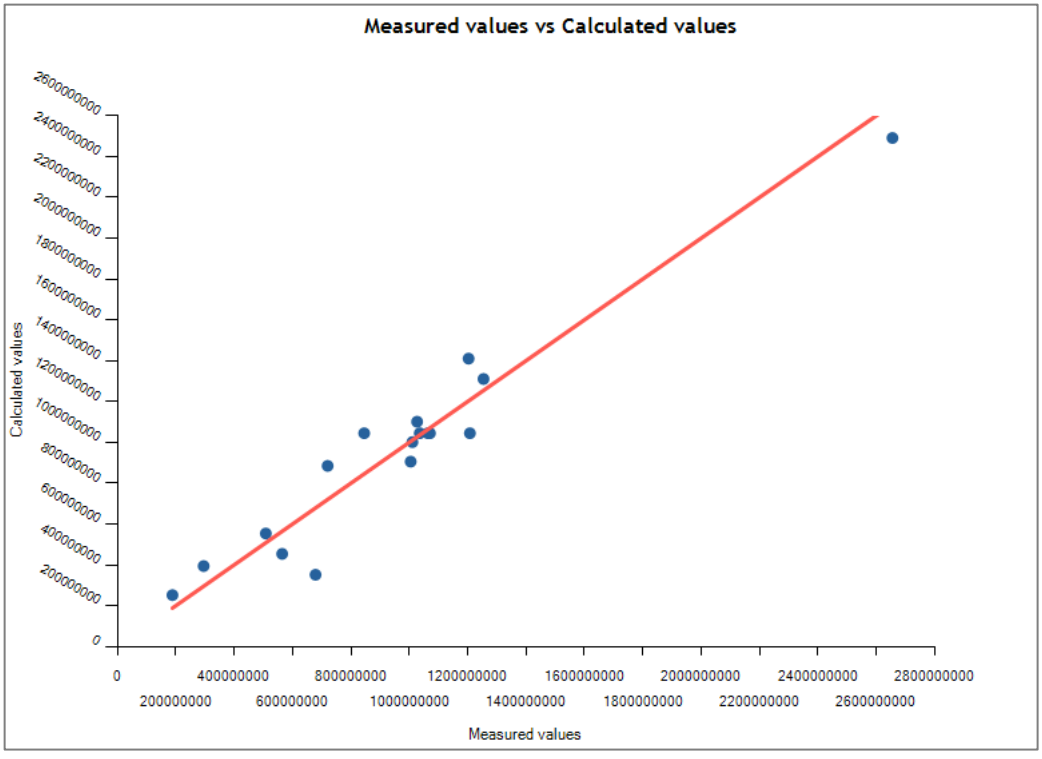


Figure 31: Distribution of measured vs calculated values.

Case	CNPE	R^2 (%)	Adjusted R^2 (%)	Predicted R^2 (%)
1	Only quadratic terms	70.1	52.2	0
2	X_1X_2	70.1	46.9	0
3	**X_1X_3**	**93.2**	**87.9**	**54.2**
4	X_2X_3	71.1	49.6	0
5	$X_1X_2+X_1X_3$	93.2	87.4	49.2
6	$X_1X_3+X_2X_3$	94.8	89.5	38.8
7	$X_1X_2+X_2X_3$	71.7	43.4	0
8	$X_1X_2+X_1X_3+X_2X_3$	94.8	88.1	33.9
9	$X_1X_2X_3$	75.6	56.6	0
10	$X_1X_2+X_1X_3+X_2X_3$ + $X_1X_2X_3$	80.7	74.6	0

Figure 32: variation of R^2. adjusted R^2 and predicted R^2 with the addition of each interaction term.

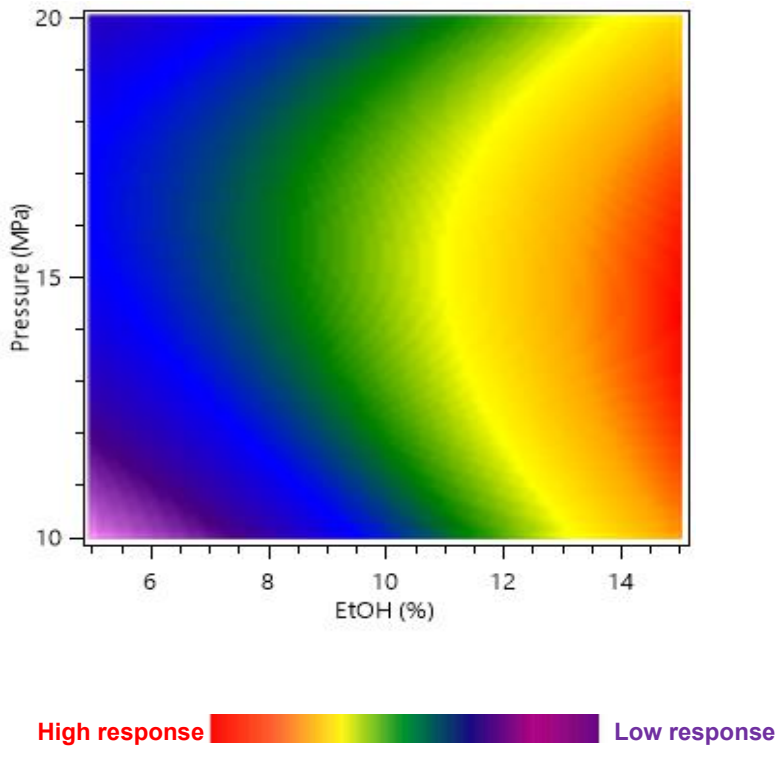


Figure 33: Response surface heat-maps showing the effect of pressure (MPa) and EtOH (%) variation on the total faradiol esters (faradiol myristate and faradiol palmitate) peak area (mAU×min).

Chapter III: Calendula polar extracts (CPE)

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	7	36.096	36.096	36.096	5.1566	34.3206	0.0000		Very significant
X1	1	0.0028125	0.56408	0.56408	0.56408	3.7544	0.0887	3.02%	Limit
X2	1	8.3232	0.22289	0.22289	0.22289	1.4835	0.2579	1.19%	Not significant
X3	1	16.046	4.9439	4.9439	4.9439	32.9049	0.0004	26.5%	Very significant
X1*X1	1	0.58141	0.58141	0.58141	0.58141	3.8697	0.0847	3.12%	Limit
X2*X2	1	0.12076	0.12076	0.12076	0.12076	0.8037	0.3962	0.647%	Not significant
X3*X3	1	4.1311	4.1311	4.1311	4.1311	27.4951	0.0008	22.1%	Very significant
X2*X3	1	6.8906	6.8906	6.8906	6.8906	45.8620	0.0001	36.9%	Very significant
Residuals	8	1.202	1.202	1.202	0.15025			6.44%	
Total	15	37.298							

Figure 34: ANOVA for response surface regression model for narcissin yield (mg/g) in selective CPE.

Y Y^0.2777 Y^0.5 Y^2 — Response studied: Narcissin

Parameters	nb df	S	R^2	R^2 Adjusted	R^2 Predicted
Values	8	0.3876	96.8%	94.0%	71.9%

Narcissin = 4.402 - 0.1134*X1 - 0.09875*X2 - 0.3241*X3 + 0.0009531*X1*X1 + 0.001737*X2*X2 + 0.01016*X3*X3 + 0.01313*X2*X3

Figure 35: : model's R^2. adjusted R^2 and predicted R^2 and equation.

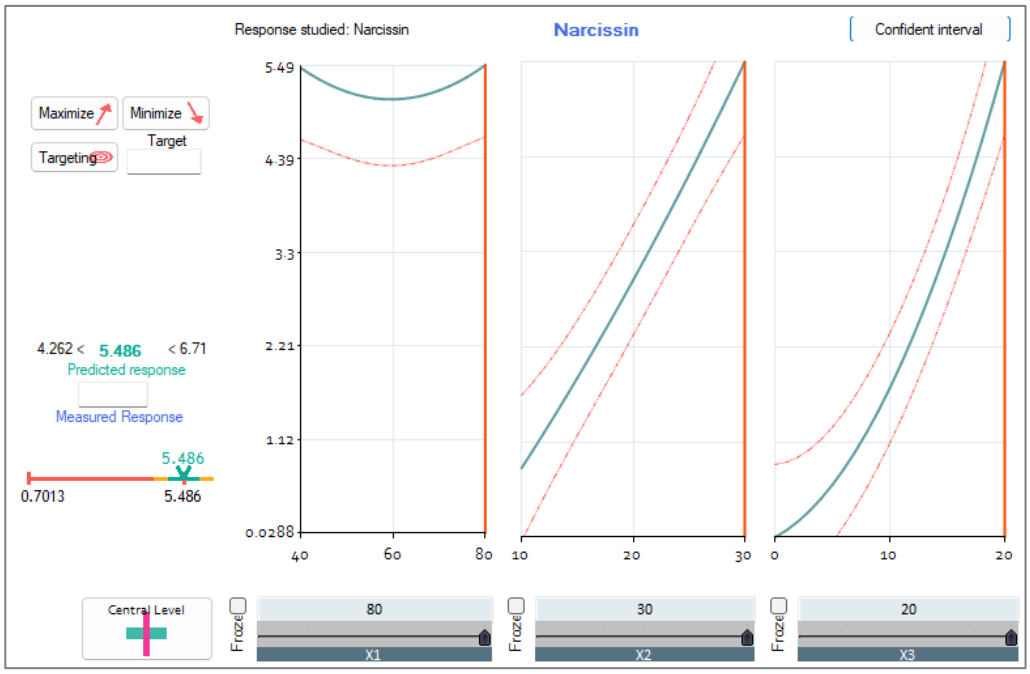


Figure 36: Predicted values for the maximization of the response (narcissin yield mg/g)

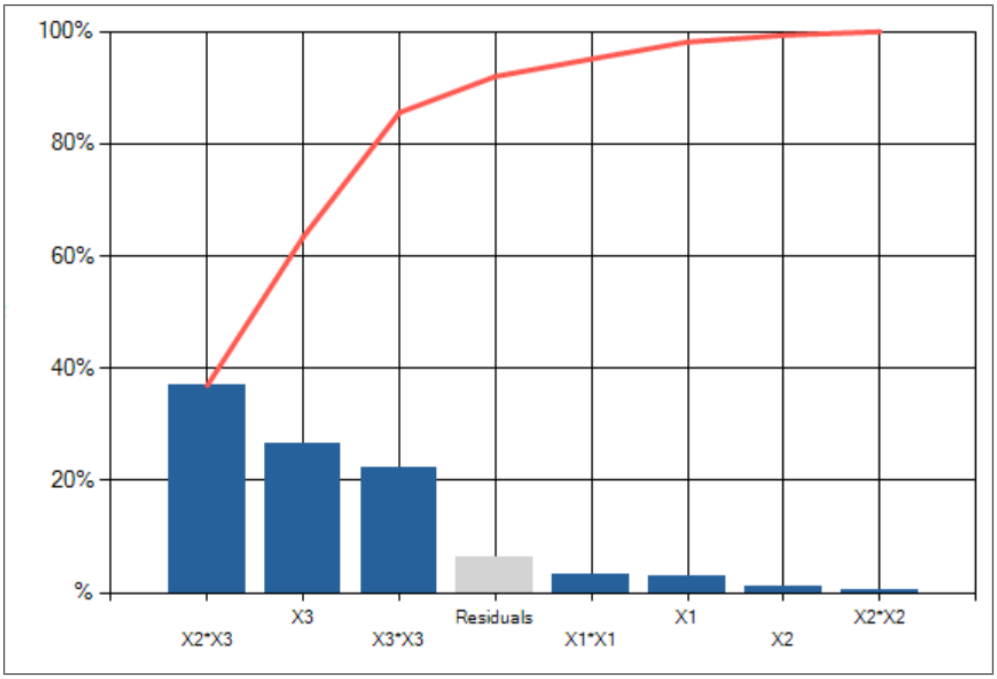


Figure 37: Pareto contributions to variance in standardized ANOVA.

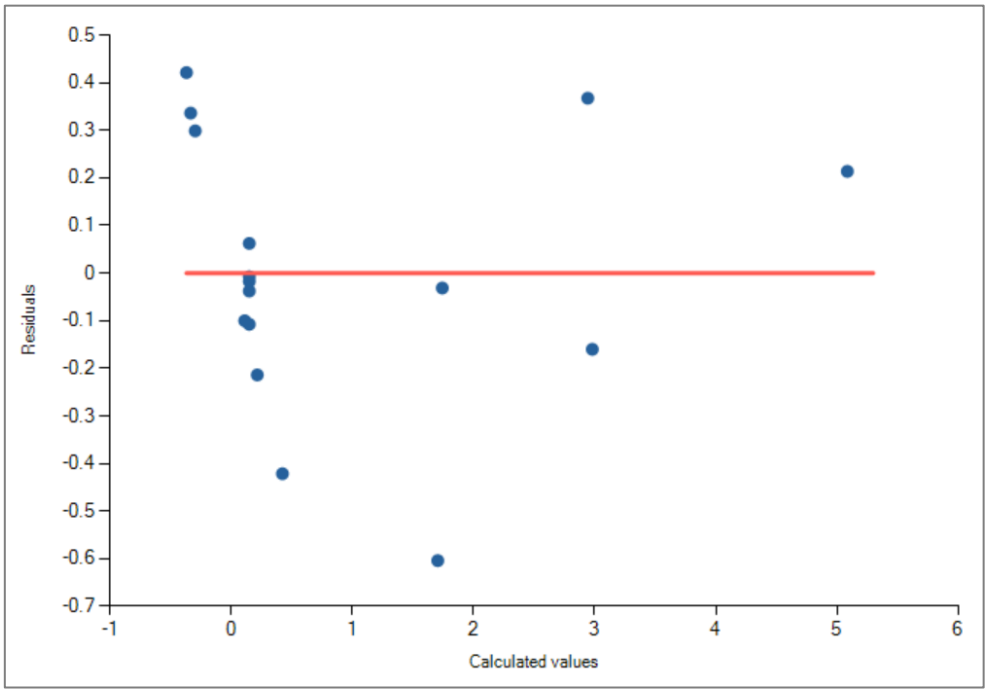


Figure 38: Residuals vs fitted values.

Residuals | Cartography

	X1	X2	X3	Narcissin	Narcissin ~	Error	Res std
1	40	10	10	0	-0.33625	0.33625	0.87
2	80	10	10	0	-0.29875	0.29875	0.77
3	40	30	10	1.1	1.7038	-0.60375	-1.56
4	80	30	10	1.71	1.7412	-0.03125	-0.08
5	40	20	0	0.01	0.11	-0.1	-0.26
6	80	20	0	0.04	0.1475	-0.1075	-0.28
7	40	20	20	3.31	2.9425	0.3675	0.95
8	80	20	20	2.82	2.98	-0.16	-0.41
9	60	10	0	0	0.21375	-0.21375	-0.55
10	60	30	0	0.05	-0.37125	0.42125	1.09
11	60	10	20	0	0.42125	-0.42125	-1.09
12	60	30	20	5.3	5.0863	0.21375	0.55
13	60	20	10	0.11	0.1475	-0.0375	-0.10
14	60	20	10	0.13	0.1475	-0.0175	-0.05
15	60	20	10	0.14	0.1475	-0.0075	-0.02
16	60	20	10	0.21	0.1475	0.0625	0.16

Very high residual ==> This is probably an outlier
high residual (greater than 2σ) ==> check this run
Low residual (less than 2σ) ==> normal situation

Figure 39: experimental data (Y=grey column) vs predicted values (Y~). deviation and residuals

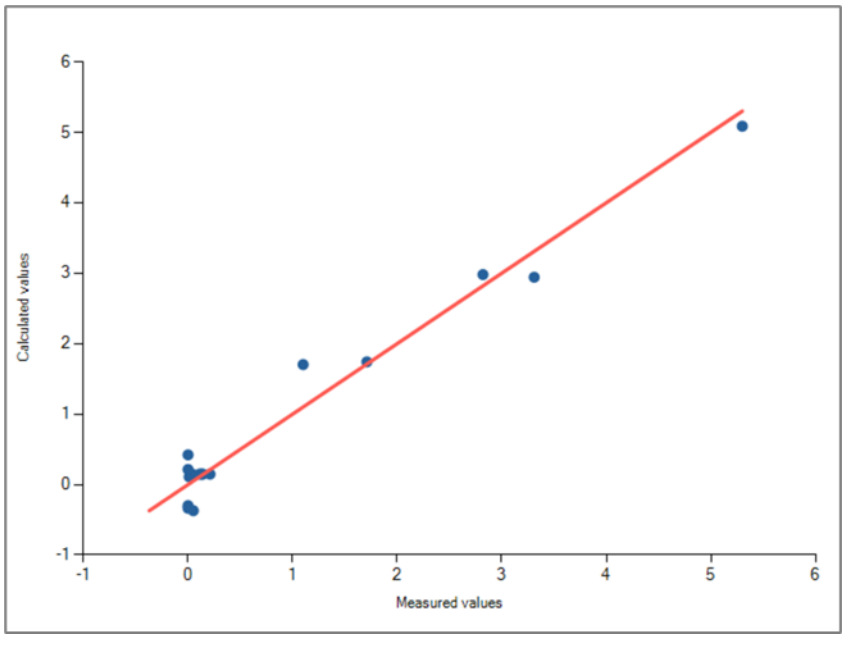


Figure 40: Distribution of measured vs calculated values.

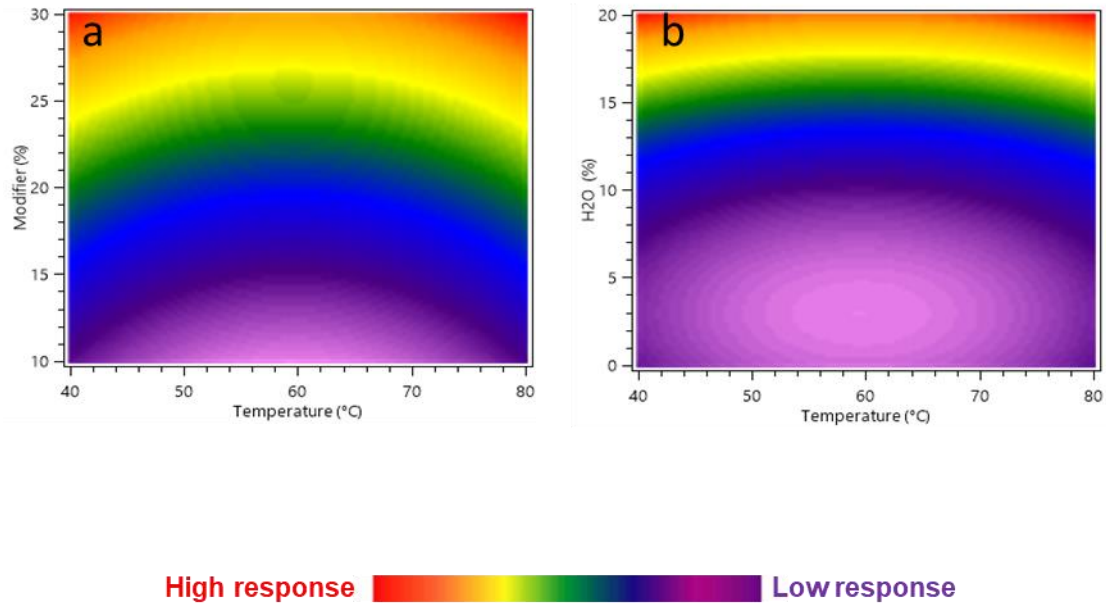


Figure 41: Response surface heat-maps showing the effect of factors variation on narcissin yield.

(a) Modifier (%) and temperature (°C), and (b) H2O (%) et temperature (°C)

Case	CPE	R^2 (%)	Adjusted R^2 (%)	Predicted R^2 (%)
1	Only quadratic terms	78.3	63.8	0
2	X_1X_2	78.6	59.8	0
3	X_1X_3	78.5	59.7	0
4	**X_2X_3**	**96.8**	**94**	**71.9**
5	$X_1X_2+X_1X_3$	78.7	54.4	0
6	$X_1X_3+X_2X_3$	97.0	93.6	57.2
7	$X_1X_2+X_2X_3$	97.0	93.5	68.9
8	$X_1X_2+X_1X_3+X_2X_3$	97.2	93	54.3
9	$X_1X_2X_3$	89.5	80.4	24.6
10	$X_1X_2+X_1X_3+X_2X_{3+}$ $X_1X_2X_3$	64.3	-	0

Figure 42: variation of R^2. adjusted R^2 and predicted R^2 with the addition of each interaction term.

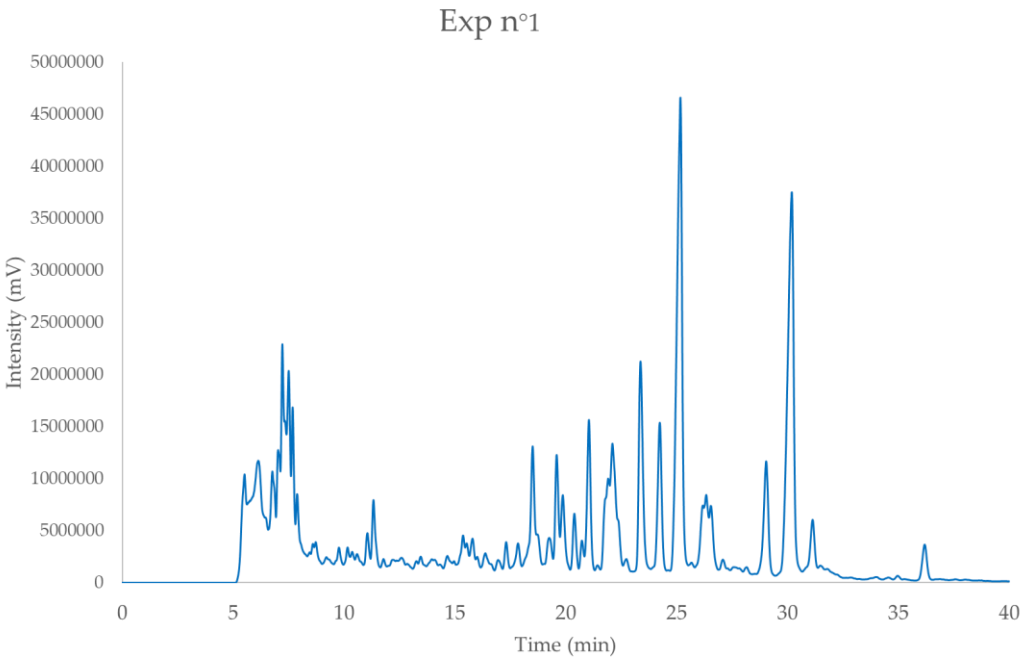


Figure 43: UHE-LP/SFC-ELSD chromatogram, exp n°1 (15°C, 10% MeOH:ACN 50:50 v:v).

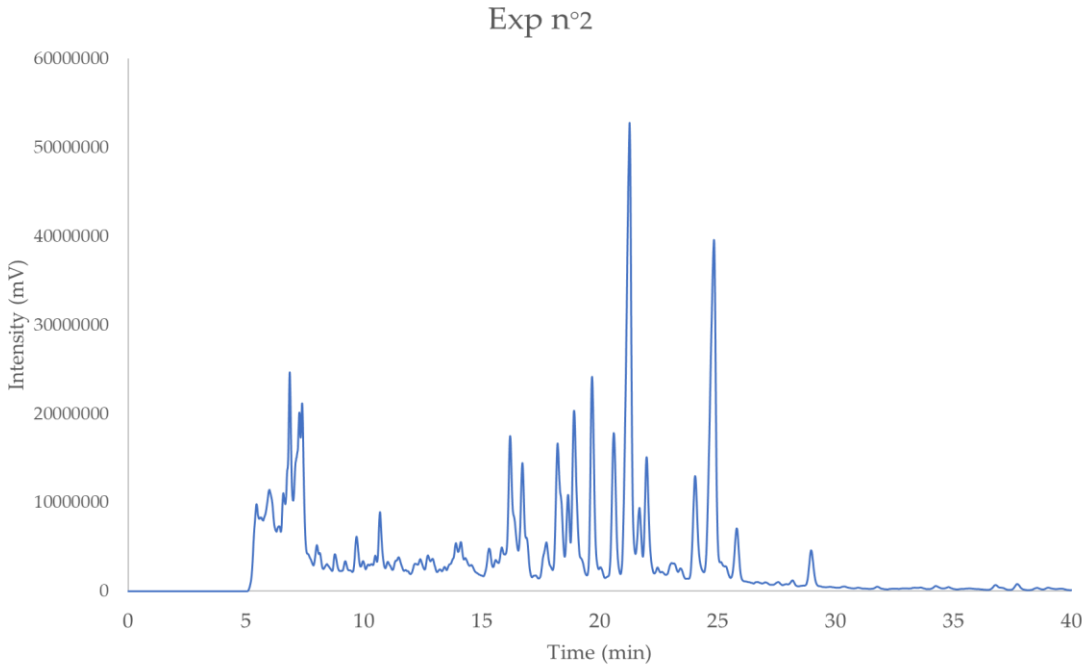


Figure 44: UHE-LP/SFC-ELSD chromatogram, exp n°2 (25°C, 10% MeOH:ACN 50:50 v:v).

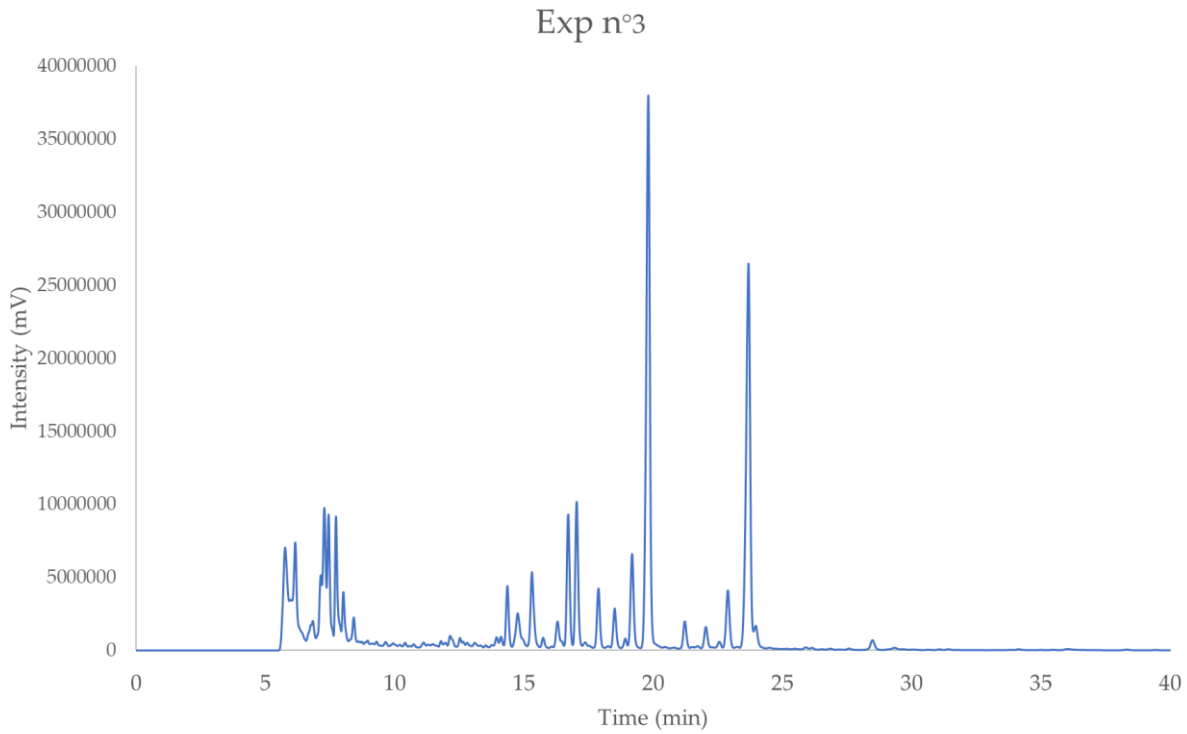


Figure 45: UHE-LP/SFC-ELSD chromatogram, exp n°3 (15°C, 30% MeOH:ACN 50:50 v:v).

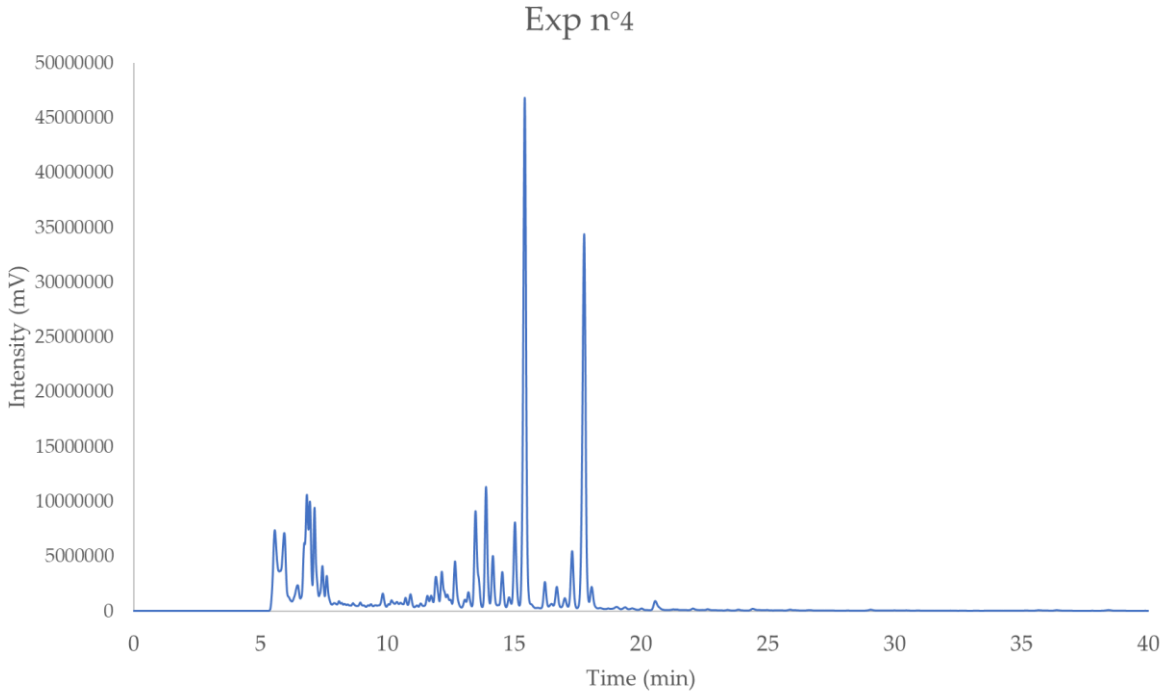


Figure 46: UHE-LP/SFC-ELSD chromatogram, exp n°4 (25°C, 30% MeOH:ACN 50:50 v:v).

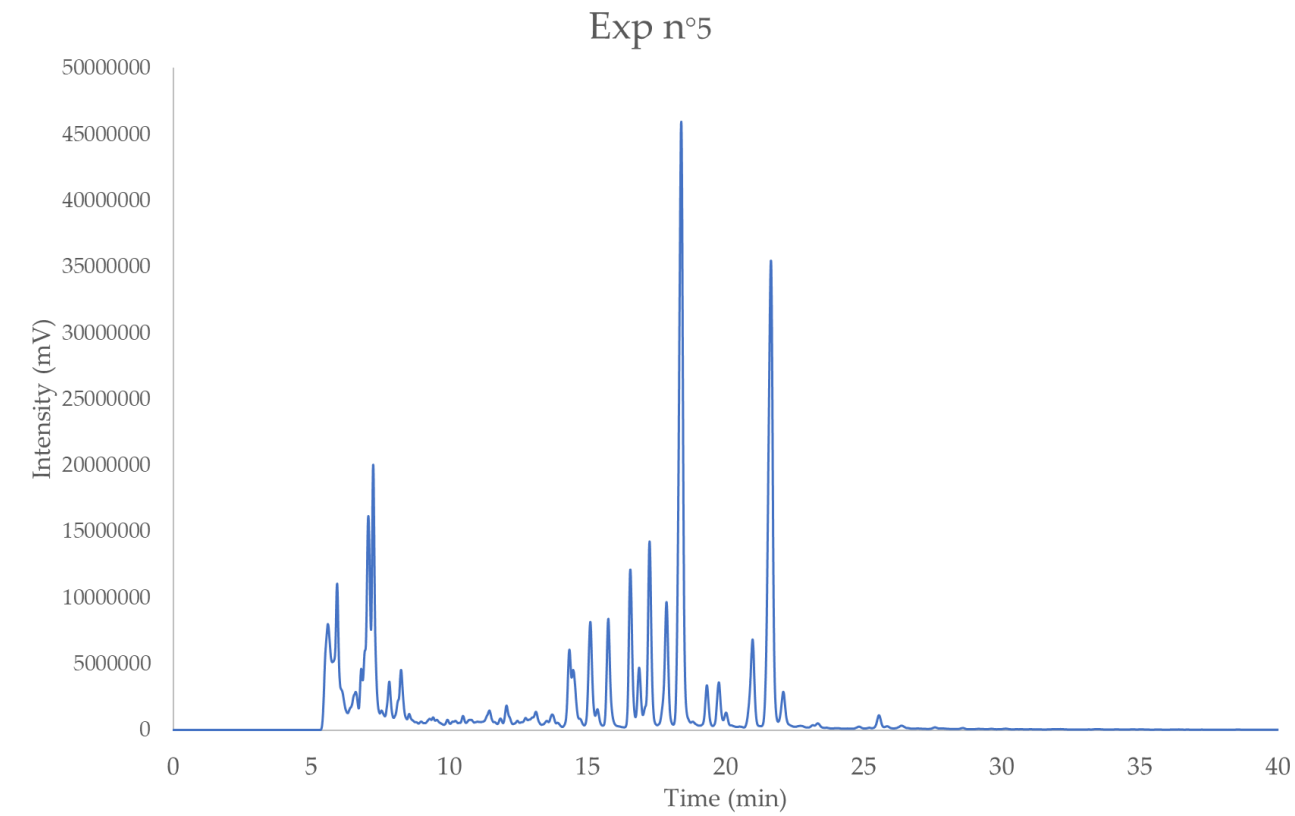


Figure 47: UHE-LP/SFC-ELSD chromatogram, exp n°5 (15°C, 20% MeOH:ACN 75:25 v:v).

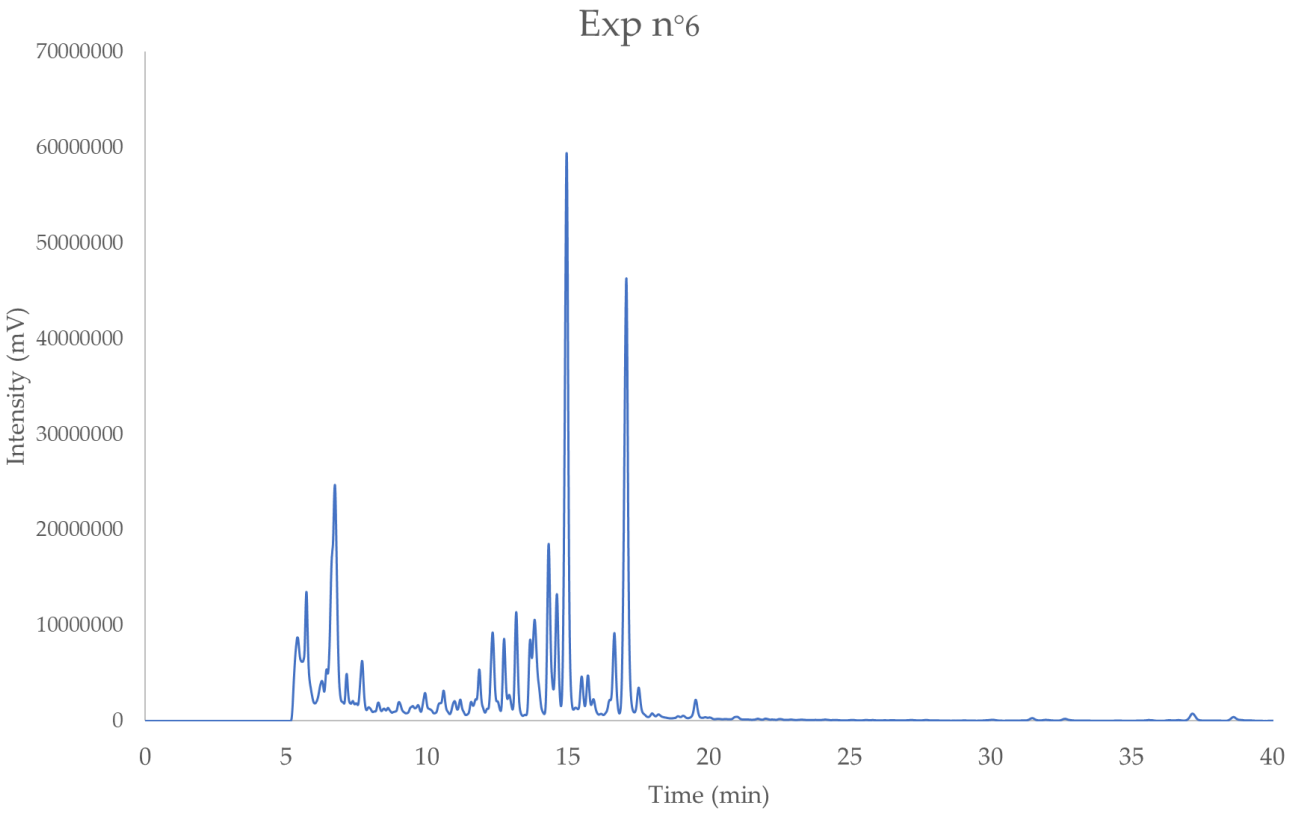


Figure 48: UHE-LP/SFC-ELSD chromatogram, exp n°6 (15°C, 10% MeOH:ACN 50:50 v:v).

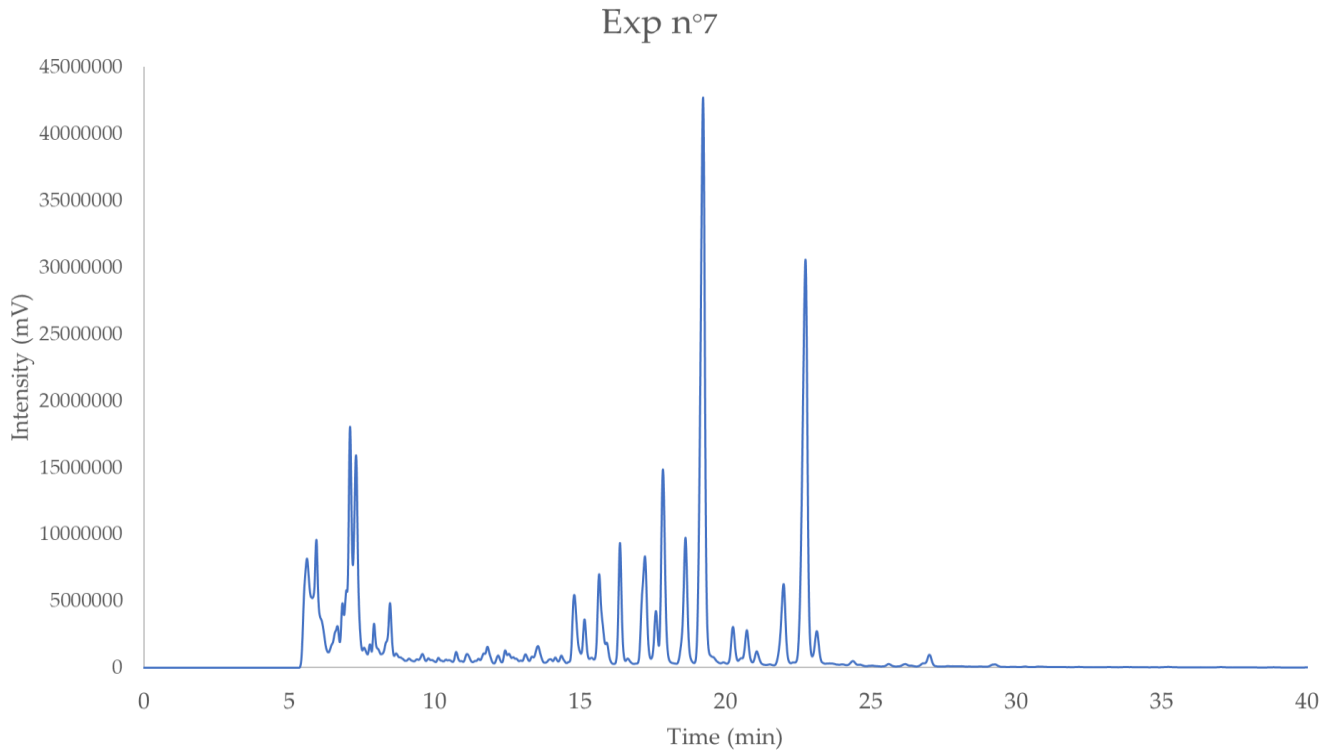


Figure 49: UHE-LP/SFC-ELSD chromatogram, exp n°7 (15°C, 0% MeOH:ACN 75:25 v:v).

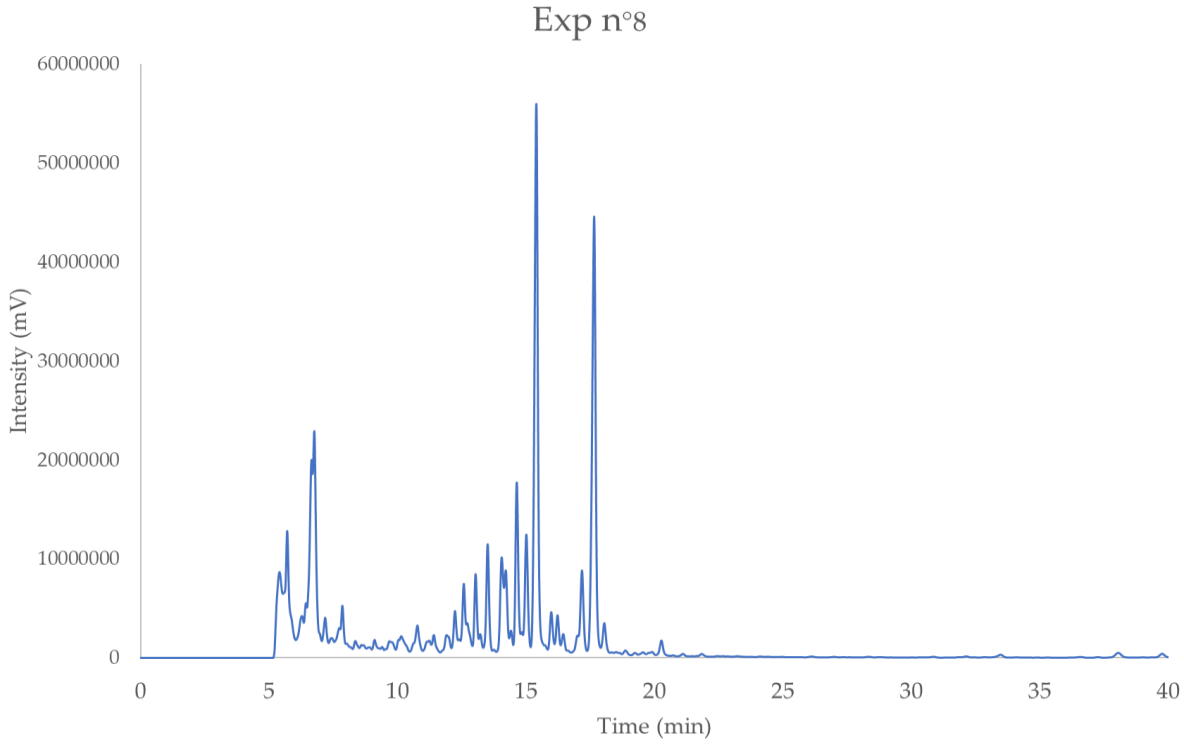


Figure 50: UHE-LP/SFC-ELSD chromatogram, exp n°8 (25°C, 20% MeOH:ACN 75:25 v:v).

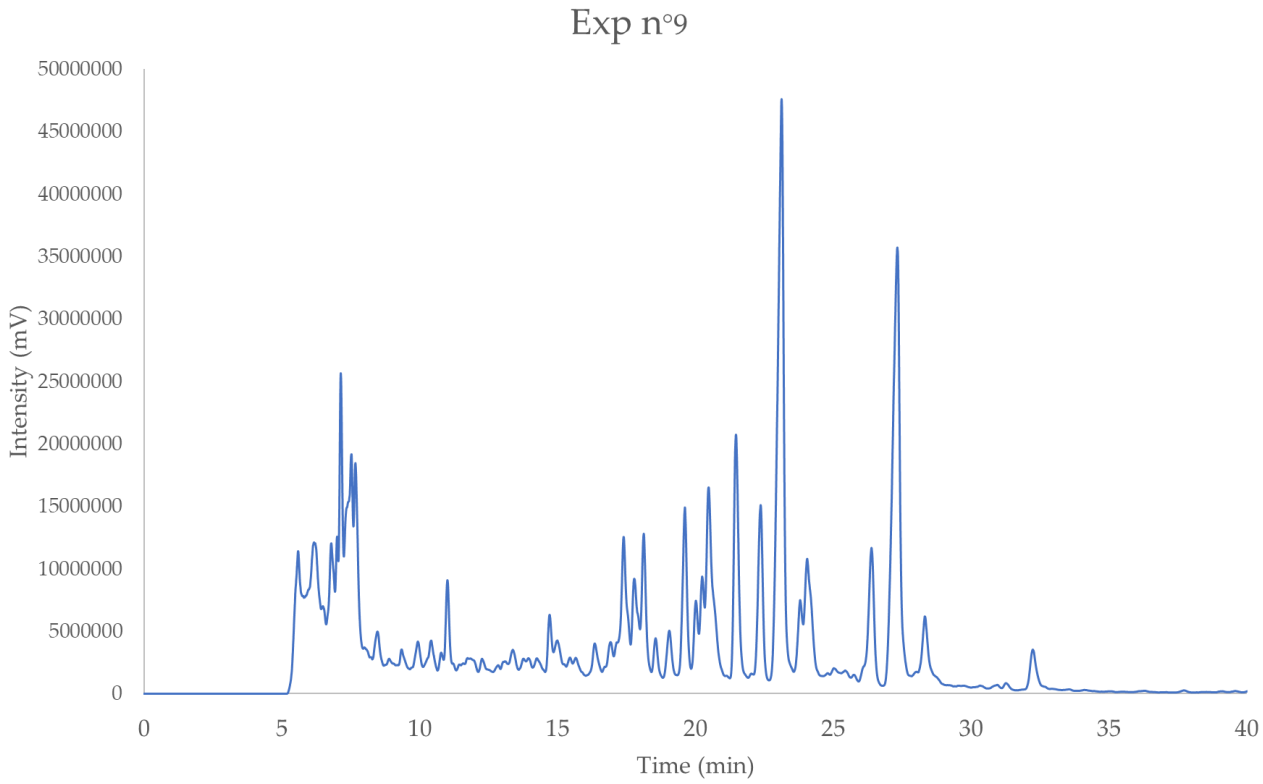


Figure 51: UHE-LP/SFC-ELSD chromatogram, exp n°9 (20°C, 10% MeOH:ACN 75:25 v:v).

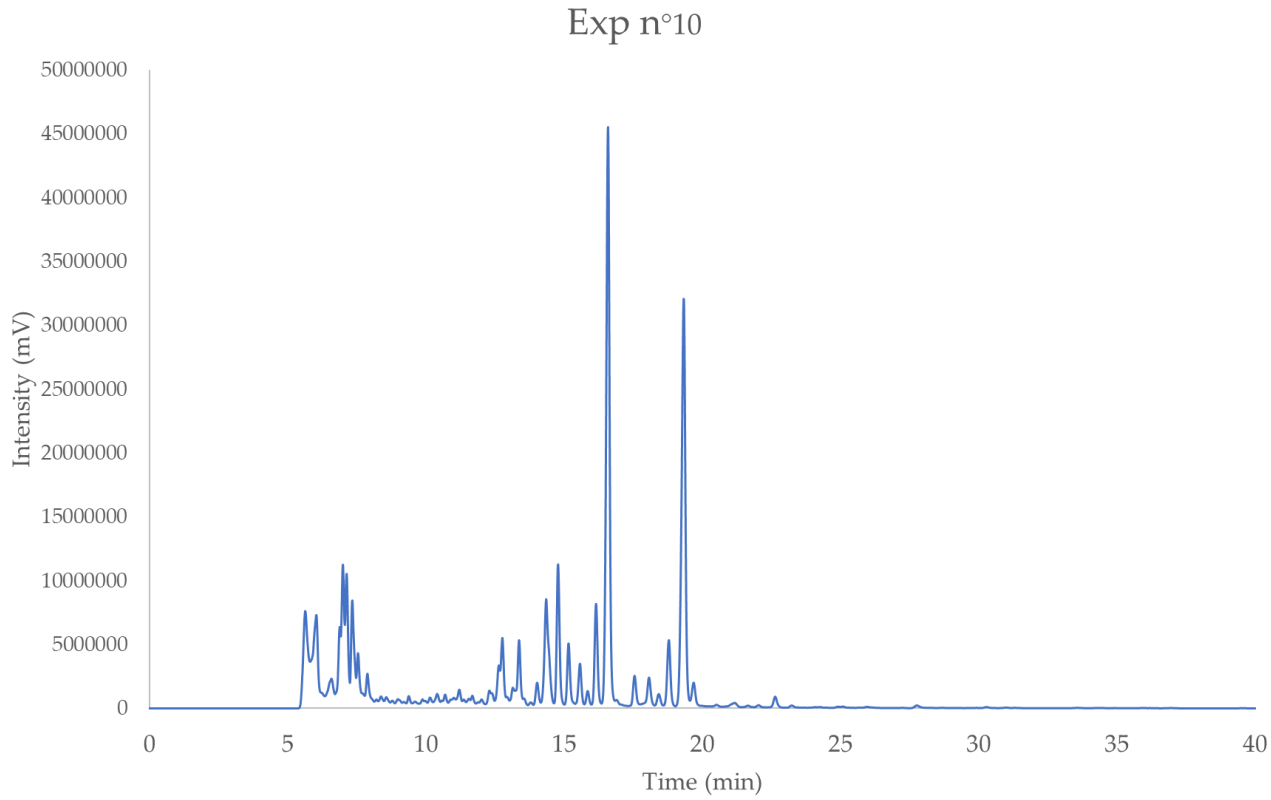


Figure 52: UHE-LP/SFC-ELSD chromatogram, exp n°10 (20°C, 30% MeOH:ACN 75:25 v:v).

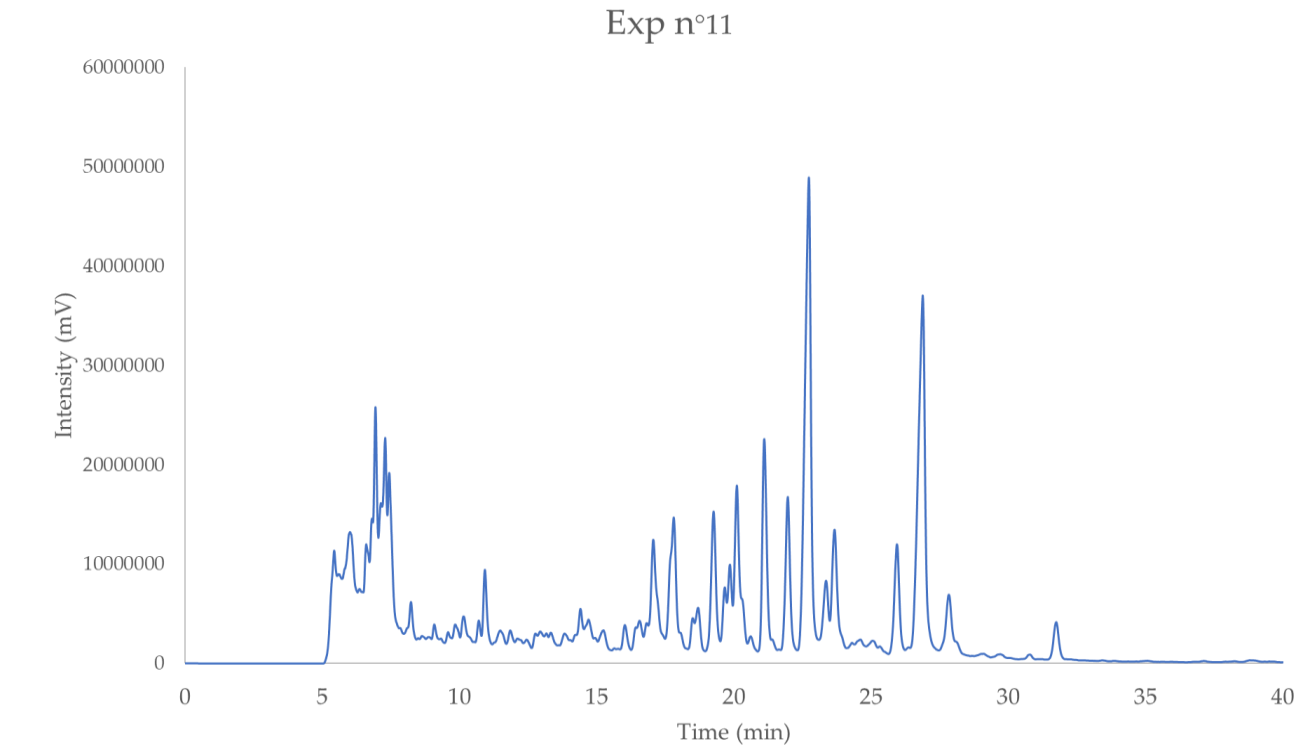


Figure 53: UHE-LP/SFC-ELSD chromatogram, exp n°11 (20°C, 10% MeOH:ACN 75:25 v:v).

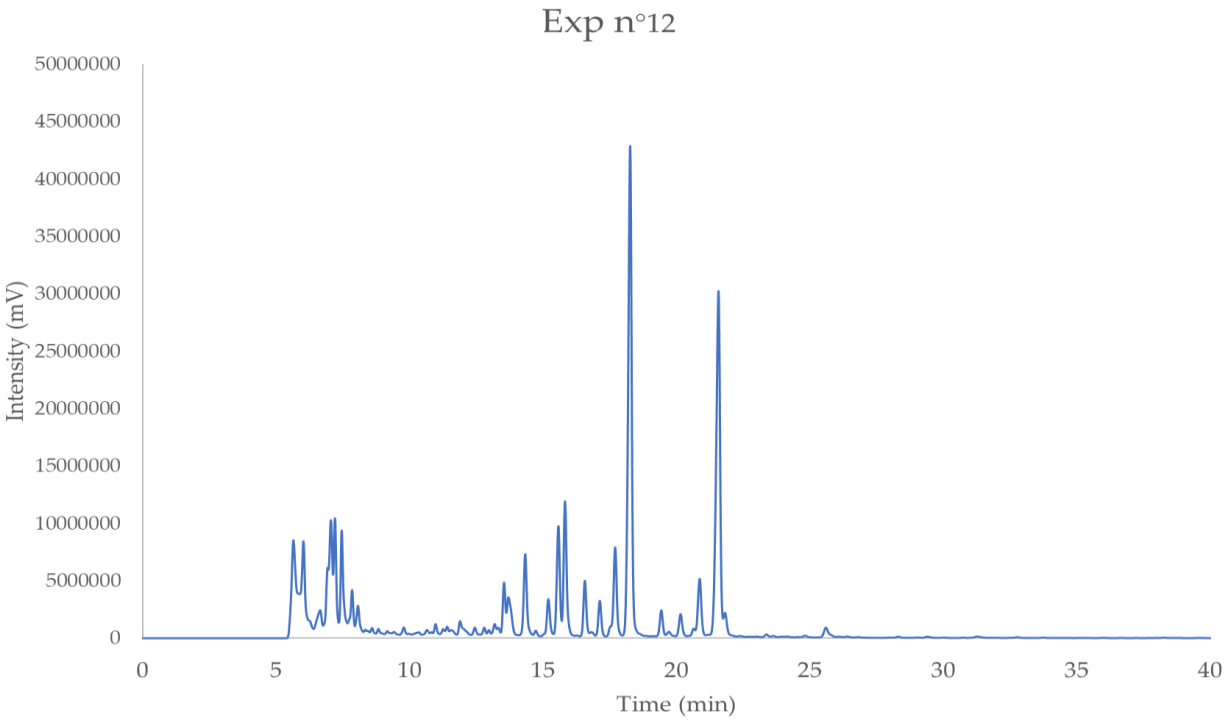


Figure 54: UHE-LP/SFC-ELSD chromatogram, exp n°12 (20°C, 30% MeOH:ACN 75:25 v:v).

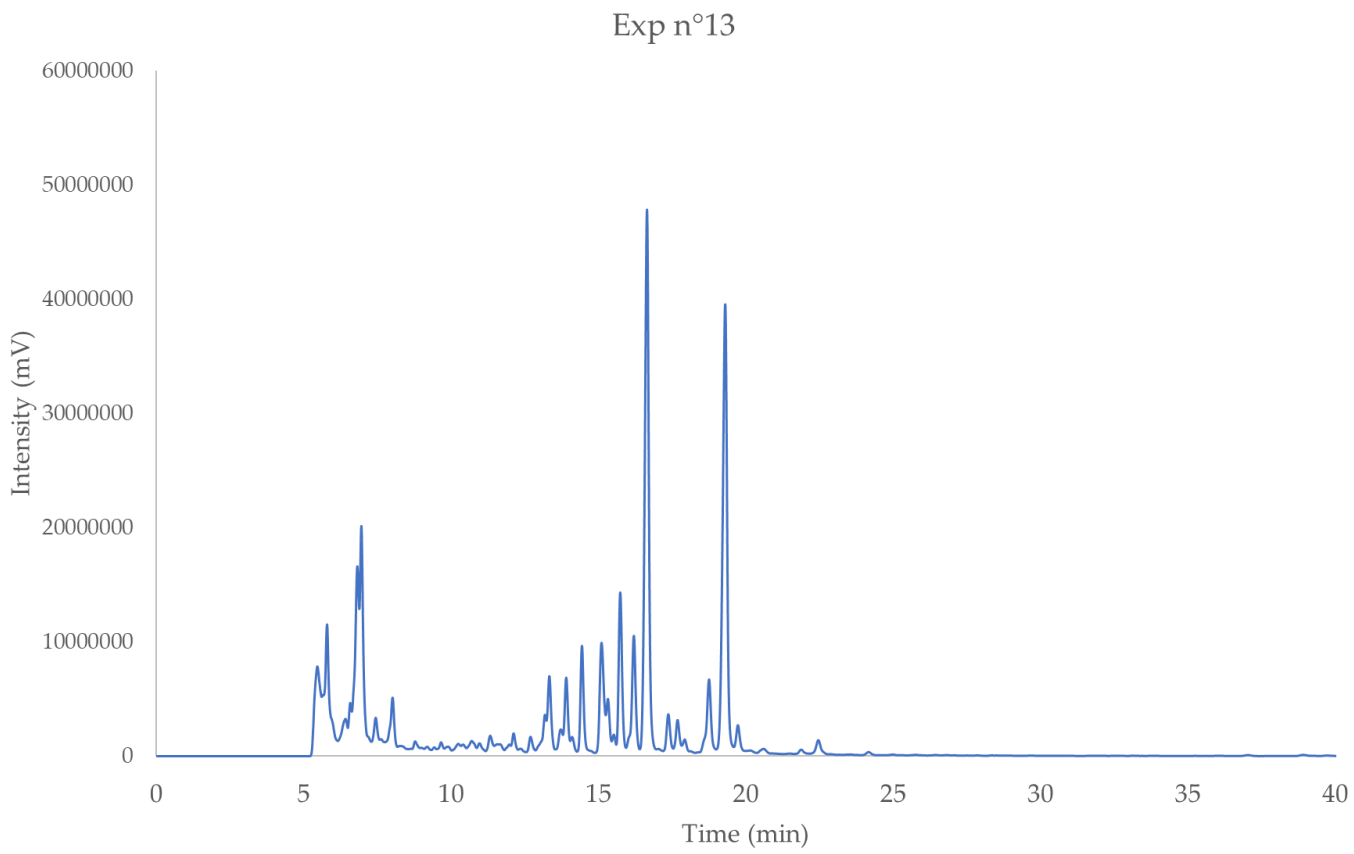


Figure 55: UHE-LP/SFC-ELSD chromatogram, exp n°13 (20°C, 20% MeOH:ACN 50:50 v:v).

Experience N°	Peak area (%)		P1			P2		
	P1	P2	*hp*	*hv*	Minor peak n°	*hp*	*hv*	Minor peak n°
1	21.418	19.989	15.35	1.2	15	6.04	1.07	24
2	19.762	17.177	9.4	4.2	17	12.94	2.14	21
3	30.706	23.971	6.6	0.16	17	1.69	1.29	24
4	30.432	24.744	8.06	0.48	14	2.18	0.82	22
5	27.438	23.624	9.66	0.4	10	2.89	0.62	17
6	26.555	22.598	13.26	1.56	13	3.48	0.9	21
7	28.2	22.262	9.69	0.35	11	2.73	0.85	18
8	26.821	22.852	12.46	1.09	15	3.51	1.02	23
9	19.197	17.662	7.46	1.7	21	11.66	0.64	27
10	29.598	23.733	8.18	0.39	16	2.02	0.61	24
11	20.845	18.559	8.32	2.4	17	11.99	1.36	19
12	29.68	23.44	7.8	0.24	11	2.21	1.71	19
13	27.279	25.184	10.51	0.61	12	2.7	0.8	19
14	27.271	23.697	11.85	0.66	12	2.86	0.7	20
15	26.896	24.021	10.97	0.64	12	2.79	0.67	20
16	28.31	23.78	11.06	0.6	13	2.52	0.7	21
17	26.683	23.851	10.39	0.6	11	2.51	0.63	18
Min	19.197	17.177						
Max	30.706	25.184						

Figure 56: values for d_0 calculation (hv, hp and minor peak number) for P_1 (Faradiol myristate) and P_2 (faradiol palmitate), and percentage of peak area of P_1 and P_2.

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	360.57	360.57	360.57	60.094	145.1019	0.0000		Very significant
Temperature (°C)	1	97.441	4.3151	4.3151	4.3151	10.4192	0.0091	1.37%	Very significant
Modifier (%)	1	126.8	174.52	174.52	174.52	421.3875	0.0000	55.6%	Very significant
ACN (%)	1	2.7261	0.011532	0.011532	0.011532	0.0278	0.8708	0.00367%	Not significant
Temperature (°C)*Temperature (°C)	1	3.3821	1.4176	1.4176	1.4176	3.4230	0.0940	0.451%	Limit
Modifier (%)*Modifier (%)	1	130.18	129.59	129.59	129.59	312.9027	0.0000	41.3%	Very significant
ACN (%)*ACN (%)	1	0.036221	0.036221	0.036221	0.036221	0.0875	0.7735	0.0115%	Not significant
Residuals	10	4.1415	4.1415	4.1415	0.41415			1.32%	
Total	16	364.71							

Figure 57: ANOVA summary of Y1 (LPRT) in BBD model.

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	0.16787	0.16787	0.16787	0.027978	7.0732	0.0038		Very significant
Temperature (°C)	1	0.03645	1.6313E-08	1.6313E-08	1.6313E-08	0.0000	0.9984	1.31E-05%	Not significant
Modifier (%)	1	0.099013	0.052803	0.052803	0.052803	13.3493	0.0044	42.6%	Very significant
ACN (%)	1	1.25E-05	0.00013186	0.00013186	0.00013186	0.0333	0.8588	0.106%	Not significant
Temperature (°C)*Temperature (°C)	1	0.00078464	0.00030421	0.00030421	0.00030421	0.0769	0.7872	0.245%	Not significant
Modifier (%)*Modifier (%)	1	0.031457	0.031141	0.031141	0.031141	7.8728	0.0186	25.1%	Significant
ACN (%)*ACN (%)	1	0.00015158	0.00015158	0.00015158	0.00015158	0.0383	0.8487	0.122%	Not significant
Residuals	10	0.039555	0.039555	0.039555	0.0039555			31.9%	
Total	16	0.20742							

Figure 58: ANOVA summary of Y_2 (d_0 P_1) in BBD model.

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	0.4646	0.4646	0.4646	0.077433	6.2342	0.0060		Very significant
Temperature (°C)	1	0.0162	0.0069774	0.0069774	0.0069774	0.5618	0.4708	3.96%	Not significant
Modifier (%)	1	0.36125	0.0019955	0.0019955	0.0019955	0.1607	0.6970	1.13%	Not significant
ACN (%)	1	0.05445	0.010345	0.010345	0.010345	0.8329	0.3829	5.87%	Not significant
Temperature (°C)*Temperature (°C)	1	0.0060444	0.0052318	0.0052318	0.0052318	0.4212	0.5309	2.97%	Not significant
Modifier (%)*Modifier (%)	1	0.022924	0.023842	0.023842	0.023842	1.9196	0.1960	13.5%	Not significant
ACN (%)*ACN (%)	1	0.0037266	0.0037266	0.0037266	0.0037266	0.3000	0.5959	2.11%	Not significant
Residuals	10	0.12421	0.12421	0.12421	0.012421			70.4%	
Total	16	0.5888							

Figure 59: ANOVA summary of Y_3 (d_0 P_3) in BBD model.

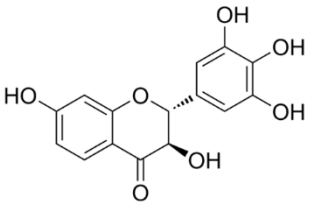
Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	42.279	42.279	42.279	7.0466	6.2636	0.0059		Very significant
Temperature (°C)	1	1.125	0.85746	0.85746	0.85746	0.7622	0.4031	1.37%	Not significant
Modifier (%)	1	18	28.089	28.089	28.089	24.9682	0.0005	44.9%	Very significant
ACN (%)	1	0.125	0.0039809	0.0039809	0.0039809	0.0035	0.9537	0.00636%	Not significant
Temperature (°C)*Temperature (°C)	1	1.6544	1.0526	1.0526	1.0526	0.9357	0.3562	1.68%	Not significant
Modifier (%)*Modifier (%)	1	21.375	21.316	21.316	21.316	18.9474	0.0014	34.1%	Very significant
ACN (%)*ACN (%)	1	-2.1316E-14	-2.1316E-14	-2.1316E-14	-2.1316E-14	0.0000	1.0000	-3.41E-14%	Not significant
Residuals	10	11.25	11.25	11.25	1.125			18%	
Total	16	53.529							

Figure 60: ANOVA summary of Y_4 (TPN) in BBD model.

Source	ddl	Seq SS	Adj SS	ADJ SS std	MS	F	p	Contribution	Conclusion
Regression	6	76.309	76.309	76.309	12.718	5.5904	0.0088		Very significant
Temperature (°C)	1	40.5	2.5489	2.5489	2.5489	1.1204	0.3147	8.53%	Not significant
Modifier (%)	1	28.125	2.1059	2.1059	2.1059	0.9257	0.3587	7.05%	Not significant
ACN (%)	1	6.125	0.8957	0.8957	0.8957	0.3937	0.5444	3%	Not significant
Temperature (°C)*Temperature (°C)	1	1.0588	1.0526	1.0526	1.0526	0.4627	0.5118	3.52%	Not significant
Modifier (%)*Modifier (%)	1	0.23684	0.26316	0.26316	0.26316	0.1157	0.7408	0.881%	Not significant
ACN (%)*ACN (%)	1	0.26316	0.26316	0.26316	0.26316	0.1157	0.7408	0.881%	Not significant
Residuals	10	22.75	22.75	22.75	2.275			76.1%	
Total	16	99.059							

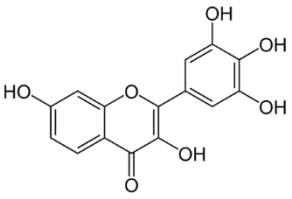
Figure 61: ANOVA summary of Y_5 (TRPN) in BBD model.

Structure of targeted compounds



Dihydrorobinetin (DHR)

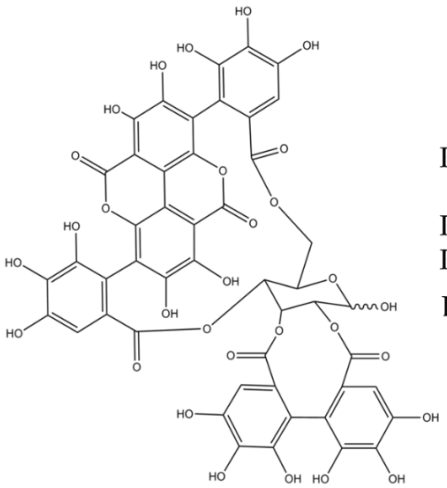
Log P = 1.232



Robinetin (Rob)

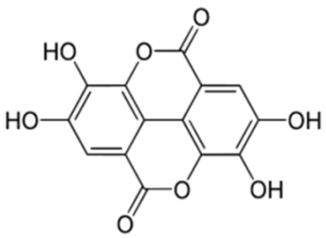
Log P = 2.263

Figure 62: structure of compounds mentioned in chapter II/part I (black locust)



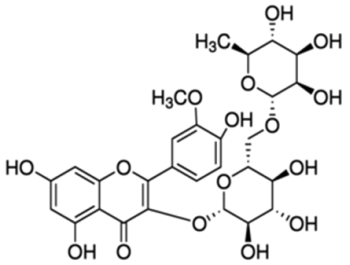
Punicalagins:

PNGα
PNG β
Log P= 1.7



Ellagic acid
Log P= 1.1

Figure 63: structure of compounds mentioned in chapter II/part III (pomegranate)



Isorhamnetin- *3-O*-rutinoside
(Narcissin)
Log P= -1

Figure 64: structure of narcissin mentioned in chapter III (Calendula)

Sirine ATWI GHADDAR

Procédés intégrés d'éco-extraction de composés bioactifs de plantes
Investigation de l'extraction par fluides supercritiques

Résumé :

L'utilisation d'ingrédients naturels d'origine végétale provenant de sources durables est un sujet d'actualité dans l'industrie cosmétique. Les propriétés inhérentes aux plantes (anti-âge, antioxydant, dépigmentation, etc.) peuvent être attribuées à leur composition chimique, en particulier aux métabolites secondaires. Cette thèse vise à développer des méthodes d'extraction verte pour les composés bioactifs polaires et non polaires en utilisant des fluides supercritiques (SFE), à base de dioxyde de carbone (SC-CO2). L'objectif est de réduire la consommation de solvants organiques et les opérations unitaires (filtration, centrifugation, concentration, etc.), tout en augmentant le rendement d'extraction de ces métabolites. Pour cela, des approches de sélection des paramètres d'extraction ont été mises en œuvre au travers de plan d'expériences, pour les molécules d'intérêt polaires ou non-polaires.

Tout d'abord, la SFE a été optimisée pour extraire les composés polaires de plusieurs plantes clés : les feuilles de thé vert, le péricarpe de la grenade et le bois de cœur du robinier (faux-acacia). Des comparaisons avec l'extraction assistée par ultrasons ont montré la pertinence de l'emploi de la SFE. De plus, une méthode d'extraction sélective et séquentielle (S3FE) a été développée et appliquée aux fleurs de calendula (soucis). Celle-ci est riche en composés lipophiles et hydrophiles intéressants pour des applications cosmétiques. Cette méthode a permis de réduire les opérations unitaires grâce à l'extraction dynamique séquentielle et de récupérer deux fractions, l'une riche en triterpénoïdes estérifiés et l'autre en flavonoïdes glycosylés. Enfin, l'application des fonctions de Derringer ont permis d'optimiser la séparation d'isomères de triterpénoïdes estérifiés du calendula par chromatographie en fluide supercritique en utilisant cinq colonnes C18 couplées, améliorant ainsi l'efficacité de la séparation.

Mots clés : ingrédients cosmétiques naturels, extraction sélective, phytochimie, chimie analytique

Integrated eco-extraction of plant bioactive compounds
Investigation of supercritical fluid extraction (SFE)

Summary :

The utilization of natural plant ingredients from sustainably derived sources is a topic of current interest in the cosmetic industry. The inherent properties featured by plants (anti-aging, antioxidant, depigmentation, etc.) can be attributed to their chemical composition, particularly secondary metabolites. This thesis aimed to develop sustainable extraction methods for polar and non-polar bioactive molecules employing supercritical fluids (SFE), and more specifically supercritical carbon dioxide (SC-CO2). The objective is to reduce organic solvents consumption, unit operations (filtration, centrifugation, concentration, etc.), and increase the yield of the metabolites of interest. Statistical tools like experimental design were employed to optimize the extraction while reducing the number of experiments for both polar and non-polar compounds.

Initially, SFE was optimized to extract polar bioactive compounds from several key plants: green tea leaves, pomegranate pericarp, and black locust heartwood. A comparison between SFE and ultrasound assisted extraction was evaluated in each case, demonstrating the benefits of SFE application in terms of yield and solvent consumption. In addition, a selective-sequential supercritical fluid extraction (S3FE) was applied to marigold flowers. This plant is rich in both hydrophilic and lipophilic compounds, interesting for cosmetic application. This allowed to reduce unit operation thanks to sequential dynamic extraction, recovering two fractions one rich in esterified triterpenoids and the second in glycosylated flavonoids. Finally, the application of Derringer functions allowed separation optimization of marigold's esterified triterpenoids isomers using supercritical fluid chromatography with a stationary phase consisting of five coupled C18 core shell columns, enhancing separation efficiency.

Key words: natural cosmetic ingredients, selective extraction, phytochemistry, analytical chemistry

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