Universidade de Aveiro Departamento de Química
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Emanuelle Lima Pache de Faria

Extração de compostos de valor acrescentado de biomassa utilizando solventes alternativos

Extraction of value-added compounds from biomass using alternative solvents

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica do Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora do Departamento de Química, CICECO, da Universidade de Aveiro, e coorientação do Professor Doutor Armando Jorge Domingues Silvestre, Professor Catedrático do Departamento de Química, CICECO, da Universidade de Aveiro.

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“Todas as vitórias ocultam uma abdicação”. (Simone de Beauvoir)

o júri

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palavras-chave

Compostos de valor acrescentado, biomassa, líquidos iónicos, solventes eutéticos, hidrotropia, micelas, extração, água, recuperação.

Resumo

O principal objetivo do presente trabalho consistiu no estudo de soluções aquosas de líquidos iónicos (LIs) e solventes eutécticos profundos (SEPs) como solventes alternativos para extrair compostos de valor acrescentado de biomassa e resíduos associados. Esses compostos incluem ácidos triterpénicos (ATs; ácidos ursólico, oleanólico e betulínico), uma lactona sesquiterpénica (cinaropicrina) e um composto fenólico (ácido siríngico), enquanto que as amostras de biomassa investigadas compreendem folhas de oliveira, cascas de maçã e pêra, e folhas de cardo.

O interesse nestes compostos naturais deve-se às suas propriedades biológicas, com aplicações relevantes nas indústrias alimentar, cosmética e farmacêutica. O trabalho realizado foi focado no desenvolvimento de estratégias de extração/recuperação mais sustentáveis e económicas do que as comumente utilizadas. Para este fim, também se procurou uma melhor compreensão dos mecanismos de solubilização (por efeito hidrotrópico ou através da formação de micelas).

Neste trabalho foi demonstrada a elevada capacidade de soluções aquosas com LIs tensoativos para extrair ATs, atingindo rendimentos de até 2,5 m/m% com folhas de oliveira e de até 2,6 m/m% com cascas de maçã, que são superiores aos obtidos com solventes orgânicos convencionais em condições similares. Demonstrou-se um aumento de 8 ordens de grandeza na solubilidade do ácido ursólico em solução aquosa de LI quando comparado com a sua solubilidade em água. As soluções aquosas de LIs tensoativos também demonstraram atuar como solventes promissores para extrair cinaropicrina de folhas do cardo, com rendimentos de até 3,73 m/m%. Utilizou-se água como anti-solvente, permitindo recuperar 65% de cinaropicrina por precipitação. Por outro lado, demonstrou-se que soluções aquosas de LIs com características de hidrótropos permitem extrair ácido siríngico da casca de pêra, levando a rendimentos de até 2,06 m/m% pela reutilização do solvente e de até 2,22 m/m% por reutilização da biomassa. Face a estes aumentos no rendimento de extração, propôs-se um processo de extração em contracorrente, no qual o solvente e a biomassa são reutilizados em modo contínuo. Devido à dependência da solubilidade do ácido siríngico em função da concentração de LI em solução aquosa, onde se verificou um aumento de solubilidade de até 84 vezes face à água pura, demostrou-se ser possível recuperar 77% de ácido siríngico adicionando água como anti-solvente.

Para além das soluções aquosas de LIs, foram também estudadas soluções aquosas de SEPs na extração de cinaropicrina de folhas do cardo, com rendimentos de extração de até 6,20 m/m%. Estes resultados são superiores aos obtidos com os SEPs puros e com solventes orgânicos tradicionais. Novamente, a adição de água como anti-solvente permitiu recuperar 73,6 m/m% de cinaropicrina.

Com ambos os tipos de solventes, nomeadamente soluções aquosas de LIs e SEPs, demonstrou-se ser possível aumentar os rendimentos de extração dos compostos estudados quando comparados com a utilização de solventes orgânicos voláteis e que estes compostos podem ser recuperados pela adição de água, permitindo a reciclagem de LIs e SEPs.

Keywords

Value-added compounds, biomass, ionic liquids, eutectic solvents, hydrotropy, micelles, extraction, water, recovery.

Abstract

The main objective of the present work was to investigate the potential of aqueous solutions of ionic liquids (ILs) and deep eutectic solvents (DES) as alternative solvents to extract value-added compounds from biomass and related waste. These compounds include triterpenic acids (TTAs; ursolic, oleanolic and betulinic acids), a sesquiterpene lactone (cynaropicrin) and a phenolic compound (syringic acid), whereas the biomass samples investigated correspond to olive tree leaves, apple and pear peels, and cardo leaves.

The interest on the described natural compounds is related to their wide variety of biological properties, with relevant applications in the food, cosmetic and pharmaceutical industries. The developed work was focused on developing more sustainable and cost-effective extraction/recovery strategies than those commonly used. To this end, a better understanding on the solubilisation mechanisms (by hydrotropic or micelle-based effects) was also searched.

It was demonstrated the enhanced capacity of aqueous solutions comprising surface-active ILs to extract TTAs, achieving yields up to 2.5 wt.% from olive tree leaves and up to 2.6 wt.% from apple peels, which are higher to those obtained with conventional organic solvents under similar conditions. An increase of 8 orders of magnitude in the solubility of ursolic acid in aqueous solutions of IL was verified when compared to pure water. Aqueous solutions of surface-active ILs were also demonstrated to be promising solvents to extract cynaropicrin from cardo leaves, leading to extraction yields up to 3.7 wt.% under the best identified conditions. Water was added as an anti-solvent, leading to the precipitation and recovery of 65 wt.% of cynaropicrin. Aqueous solutions of hydrotrope-based ILs were applied to extract of syringic acid from *Rocha* pear peels, leading to extraction yields up to 2.1 wt.% by reusing the solvent and up to 2.2 wt.% by reusing the biomass. These improvements in the extraction yield allowed to propose an extraction continuous process operating in countercurrent, in which the solvent and biomass are reused in a continuous mode. Taking advantage of the syringic acid solubility dependence with the IL concentration in aqueous solutions, where an enhancement up to 84-fold was obtained, water was again added as an anti-solvent allowing to recover 77 wt.% of syringic acid.

In addition to the ILs aqueous solutions investigated, DES aqueous solutions were finally investigated for the extraction of cynaropicrin from cardo leaves, with extraction yields up to 6.2 wt%. These results are higher than those obtained with pure DES and traditional organic solvents. As carried out in the previous works, the water addition as an anti-solvent allowed a recovery yield of 73.6 wt.% of cynaropicrin.

With both types of solvents, namely ILs and DES aqueous solutions, it is possible to increase the extraction yields of most studied biocompounds when compared to the yields obtained with volatile organic solvents and that the target compounds can be recovered by water addition, allowing the ILs and DES recycling.

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wt. %	weight fraction percentage	K_{OW}	Octanol-water partition coefficient
[i]	Concentration of component i	pK_a	Acidic dissociation constant
R^2	Correlation coefficient	T	Temperature
S/S_0	Solubility (mol.L^{-1}) of the compound in each IL aqueous solution to that in pure water	t	Time
$R_{S/L}$ and S/L ratio	Weight of dried biomass *per* weight of solvent	Y_i%	Recovery yield of component i

List of acronyms

Ionic liquids

$[C_2mim]Cl$	1-ethyl-3-methylimidazolium chloride
$[C_2C_1im][CH_3CO_2]$	1-ethyl-3-methylimidazolium acetate
$[C_4mim]Cl$ and $[C_4C_1im]Cl$	1-butyl-3-methylimidazolium chloride
$[C_4mim][N(CN)_2]$ and $[C_4C_1im][N(CN)_2]$	1-butyl-3-methylimidazolium dicyanamide
$[C_4C_1im][SCN]$	1-butyl-3-methylimidazolium thiocyanate
$[C_4C_1im][CH_3CO_2]$ and $[C_4C_1im][Ac]$	1-butyl-3-methylimidazolium acetate
$[C_4C_1im][CH_3SO_4]$	1-butyl-3-methylimidazolium methylsulfate
$[C_4C_1im][TOS]$	1-butyl-3-methylimidazolium tosylate
$[C_4C_1im][HSO_4]$	1-butyl-3-methylimidazolium hydrogensulfate
$[C_4C_1im][C_2H_5SO_4]$	1-butyl-3-methylimidazolium ethylsulphate
$[C_4C_1im][C_8H_{17}SO_4]$	1-butyl-3-methylimidazolium octylsulphate
$[C_6mim]Cl$	1-hexyl-3-methylimidazolium chloride
$[C_8mim]Cl$ and $[C_8C_1im]Cl$	1-octyl-3-methylimidazolium chloride
$[C_{10}mim]Cl$ and $[C_{10}C_1im]Cl$	1-decyl-3-methylimidazolium chloride
$[C_{12}mim]Cl$ and $[C_{12}C_1im]Cl$	1-dodecyl-3-methylimidazolium chloride
$[C_{14}mim]Cl$ and $[C_{14}C_1im]Cl$	1-tetradecyl-3-methylimidazolium chloride
$[C_{16}mim]Cl$ and $[C_{16}C_1im]Cl$	1-hexadecyl-3-methyimidazolium Chloride
$[C_{18}mim]Cl$ and $[C_{18}C_1im]Cl$	1-octadecyl-3-methylimidazolium chloride
$[C_{12}mim]Br$	1-dodecyl-3-methylimidazolium bromide
$[C_{12}mim]I$	1-dodecyl-3-methylimiazolium iodide
$[C_{12}mim][OMs]$	1-dodecyl-3-methylimidazolium mesylate
$[C_{12}mim][TOS]$	1-dodecyl-3-methylimidazolium tosylate
$[C_{12}mim][Me_2PO_4]$	1-dodecyl-3-methylimidazolium dimethylphosphate
$[C_{12}mim][Ac]$	1-dodecyl-3-methylimidazolium acetate
$[C_{12}bet]Cl$	2-(dodecyloxy)-N,N,N-trimethyl-2-oxoethanaminium chloride
$[C_{12}COmim]Cl$	3-(2-(dodecyloxy)-2-oxoethyl)-1-methylimidazolium chloride
$[C_4C_1pyr]Cl$, $[C_4C_1pyrr]Cl$ and $[C_4mpyr]Cl$	1-butyl-1-methylpyrrolidinium chloride
$[C_4mpy]Cl$ and $[C_4C_1py]Cl$	1-butyl-3-methylpyridinium chloride
$[C_4mpip]Cl$ and $[C_4C_1pip]Cl$	1-butyl-1-methylpiperidinium chloride
$[C_4mpy][N(CN)_2]$ and $[C_4C_1py][N(CN)_2]$	1-butyl-1-methylpyridinium dicyanamide
$[N_{4444}]Cl$	tetrabutylammonium chloride
$[P_{4444}]Cl$	tetrabutylphosphonium chloride
$[P_{444,14}]Cl$	tributyltetradecylphosphonium chloride
$[P_{i(444)1}][TOS]$	Triisobutyl(methyl)phosphonium tosylate
[Ch][Ac]	cholinium acetate
[Ch][But]	cholinium butanoate
[Ch][Hex]	cholinium hexanoate

[Ch][C_8CO_2] and [Ch][Oct]	cholinium octanoate
[Ch][$C_{10}CO_2$] and [Ch][Dec]	cholinium decanoate
[Ch][$C_{12}CO_2$]	cholinium dodecanoate
[N_{2222}]Cl	tetraethylammonium chloride
[N_{3333}]Cl	tetrapropylammonium chloride
[N_{3333}]Br	tetrapropylammonium bromide
[N_{4444}]Cl	tetrabutylammonium chloride
[N_{4444}]Br	tetrabutylammonium bromide

Common surfactants

CTAB	cetyltrimethylammonium bromide	CTAC	cetyltrimethylammonium chloride
SDS	sodium dodecylsulfate	CPC	cetylpyridinium chloride
SDBS	sodium dodecylbenzenesulfonate	Genapol C-100	genapol

List of abbreviations

IL	Ionic liquid	SLE	Solid−liquid extraction
FAO	United Nations Food and Agriculture Organization	MAE	Microwave-assisted extraction
HPLC	High performance liquid chromatography	RSM	Response surface methodology
DSC	Differential scanning calorimetry	NMR	Nuclear magnetic resonance
FDA	Food and drug administration	SEM	Scanning electron microscope
DAD	Diode array detector	UAE	Ultrasound assisted extraction
OA	Oleanolic acid	TTAs	Triterpenic acids
BA	Betulinic acid	TFA	Trifluoroacetic acid
UA	Ursolic acid	STD	Standard deviation
CMC	Critical micelle concentration	MW	Microwave heating
ASE	Accelerated solvent extraction	NADES	Natural deep eutectic solvent
DES	Deep eutectic solvent	ANOVA	Analysis of variance
MHC	Hydrotropic concentration	HBA	Hydrogen bond acceptor
RSM	Response surface methodology	HBD	Hydrogen bond donor

1. INTRODUCTION

1.1. Scopes and Objectives

Biomass and biomass-related wastes are rich in high-value compounds with a wide range of relevant properties, namely antioxidant, anti-inflammatory, radical scavenger and antimicrobial features, among others. Most of these high-value compounds are however extracted using volatile organic solvents and often at high temperatures, turning the research on alternative solvents and on the development of cost-effective extraction techniques a crucial need to decrease the environmental footprint and economic inputs of such processes. Therefore, aiming at developing cost-effective and sustainable extraction and recovery strategies of added-value compounds from biomass and biomass-related wastes, this thesis is devoted to the study of aqueous solutions of ionic liquids (ILs) or deep eutectic solvents (DES) as alternative solvents and on the development of extraction and recovery processes that can be carried out at moderate temperatures.

The added-value compounds investigated include triterpenic acids (oleanolic, betulinic and ursolic acids), a sesquiterpene lactone (cynaropicrin), and a phenolic acid (syringic acid), which were extracted from *O. europaea* leaves (olive tree), *Golden* apple peels, *Cynara cardunculus L.* leaves (cardo) and *Rocha* pear peels, respectively. A significant emphasis has been given to biomass-related wastes. Trying to develop more benign extraction/recovery techniques than those used nowadays, aqueous solutions of ILs and DES have been selected as alternative solvents. In addition to other valuable properties, both classes of solvents display a designer solvents ability, allowing to identify promising of ILs and DES that may lead to improved extraction yields of the several classes of natural compounds investigated. In most of the performed studies, extractions with volatile organic solvents were additionally carried out for comparison purposes, and recovery strategies of the target compounds from the IL- or DES-water solutions have been attempted.

This Thesis is divided in 5 chapters as schematically shown in **Figure 1.1.**

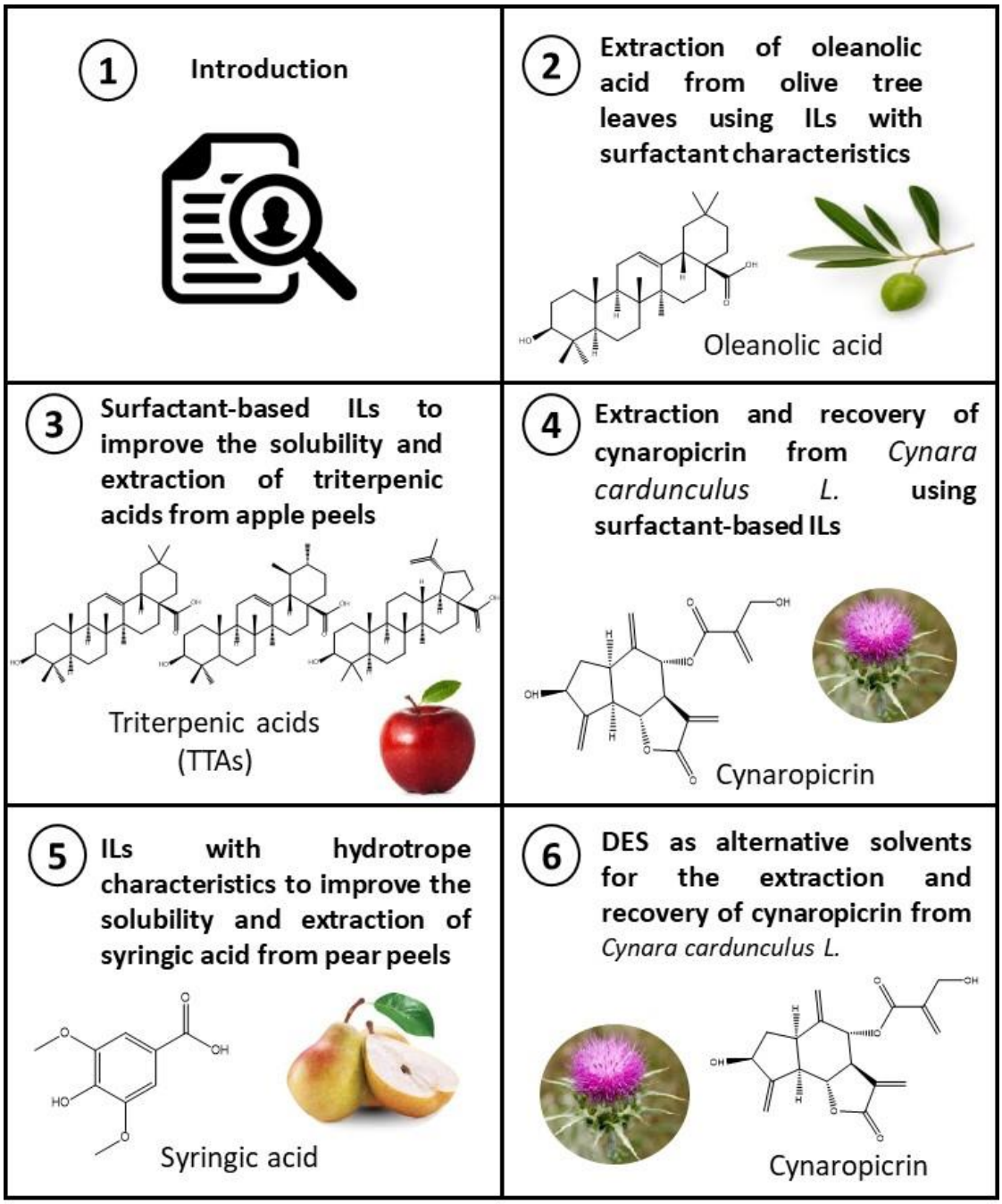

Fig. 1.1. Layout of the current thesis.

After the present chapter which provides a global overview of the background knowledge behind this thesis, the first set of results (**Chapter 2**) comprise the extraction of oleanolic acid (OA) from *O. europaea* leaves using aqueous solutions of ILs with surface-active characteristics as alternative solvents. A number of imidazolium-based ILs with variable alkyl side chain length and side-chain functionalization combined with different anions and different operational conditions were evaluated in order to improve the oleanolic acid extraction yield. Under optimized conditions, up to 2.5 wt.% of OA could be extracted using an aqueous solution of

[C_{14}mim]Cl. Moreover, the results obtained with ILs aqueous solutions were compared with those attained with organic solvents frequently used for the extraction of triterpenic acids (chloroform, ethyl acetate and methanol). Although extraction yields for OA obtained with organic solvents are still slightly higher, it was concluded that IL aqueous solutions are certainly a more environmentally friendly option.

In **Chapter 3**, IL aqueous solutions were investigated to extract a wider range of triterpenic acids (TTAs), namely ursolic, betulinic and oleanolic acids, from apple peels. The solubility of ursolic acid was first studied in aqueous solutions of several IL at different concentrations to identify the most promising solvents to proceed with the extraction from the selected biomass (apple peels). Conventional surfactants were also investigated for comparison purposes. The results obtained revealed a remarkable enhancement in the solubility of ursolic acid in aqueous solutions comprising surface-active ILs (8 orders of magnitude) when compared to pure water. It was found that the total extraction yields of TTAs using aqueous solutions of ILs at moderate conditions overcome the extraction yields obtained with chloroform and acetone at similar conditions.

Aqueous solutions composed of ILs with surface-active (both cationic and anionic) character and conventional surfactants were investigated to extract and recover cynaropicrin from the leaves of cardo, whose results are given in **Chapter 4**. Overall, it is shown that aqueous solutions of cationic surface-active ILs display a better performance for the extraction of cynaropicrin. The recycling of both the cardo leaves and the IL were further investigated to appraise the extraction solvent saturation and with the goal of developing a more sustainable process. Finally, it was demonstrated that 65 wt% of the extracted cynaropicrin can be efficiently recovered by precipitation from the IL aqueous solution through the addition of water as anti-solvent, further allowing the IL recycling.

Chapter 5 describes the application of ILs aqueous solution to dissolve, extract and recover syringic acid from pear peels. ILs with hydrotropic or surfactant behavior were investigated aiming at better understanding the mechanisms ruling enhanced solubility and extractions of added-value compounds from biomass. The best results were achieved with ILs that behave like hydrotropes, where an increase of 84-fold in the syringic acid solubility was observed when compared with its solubility in pure water. The best IL aqueous solutions were used to extract syringic acid from pear peels, for which a response surface methodology was applied to optimize the extraction conditions. The sustainability of the extraction process was further optimized by carrying out several extraction cycles, reusing either the biomass or the IL aqueous solution. After the solvent saturation, water was added as an anti-solvent, allowing to recover 77% of the target compound with high purity.

Chapter 6 comprises the application of deep eutectic solvents (DES), composed of quaternary ammonium salts and organic acids, for the extraction and recovery of cynaropicrin from cardo leaves, allowing the comparison of these solvents with ILs. After selecting the most promising DES, their aqueous solutions were investigated. The sustainability of the extraction process was optimized by carrying out several extraction cycles, reusing either the biomass or the aqueous solutions of DES. Taking advantage of the cynaropicrin solubility limit in aqueous solutions, water was added as an anti-solvent, allowing to recover 73.6 wt.% of the extracted cynaropicrin. This work demonstrates the potential of aqueous solutions of DES for the extraction of added-value compounds from biomass and the possible recovery of the target compound and solvents reuse.

In the following sections a brief introduction to the several topics considered in this thesis is given.

1.2. Biomass and bioactive compounds

It is estimated that the use of plant raw materials in agroforestry activities produces around 15 to 30% of by-products or wastes, being their valorization a subject of growing economic interest.[1,2] Some biomass by-products are already valued in many sectors and in applications as diverse as bioconversion to organic matter to be applied in soils, animal feed, used as a substrate to produce fungal and yeast related biomass, and production of fuels.[3] However, before most of these applications, biomass wastes can be valorized if used to previously extract high-value low molecular weight compounds, fitting within an integrated biorefinery perspective.[4] Biorefinery is a promising alternative to obtain new materials, intermediates and chemicals (along with fuels and energy) through an integrated utilization of the incoming biomass and other raw materials in an economically, socially and environmentally sustainable way.[5] Furthermore, there is a large and increasing demand for new sources of natural nutraceutical compounds with beneficial properties to human and animal health, with increased markets and added value,[6] for which the use of biomass wastes should be considered.

Bioactive compounds are frequently secondary metabolites in plants and are classified according to two different criteria: biological activity of the compounds and/or chemical families.[7] According to Pagare et al.,[8] bioactive compounds in plants is subdivided into distinct groups, such as terpenes, phenolics, lipids, alkaloids, among others. However, in this thesis, only two classes have been considered, namely terpenes and phenolic compounds.

Terpenes comprise one of the largest groups of secondary metabolites,[9] being naturally occurring compounds based on five-carbon ($(C_5)_n$) isoprene units. Accordingly, terpenes are

divided into monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) and polyterpenes (C_n).[9,10] Their functions in plants physiology and interactions with biotic and abiotic environments are difficult to overestimate, but are usually related to defence responses.[11] Terpenes are closely associated with the sensory and nutritional quality of plant foods, being generally obtained from lemon grass, balm trees, lavender, eucalyptus, peppermint, roses, rosemary, among many others.[9,11] They have relevant biological properties, such as antibacterial, antineoplastic, anti-inflammatory, anticancer and antioxidant properties.[11,12] In this thesis, the following terpenes have been studied: cynaropicrin, and oleanolic, betulinic and ursolic acids, whose chemical structures are shown in **Figure 1.2**.

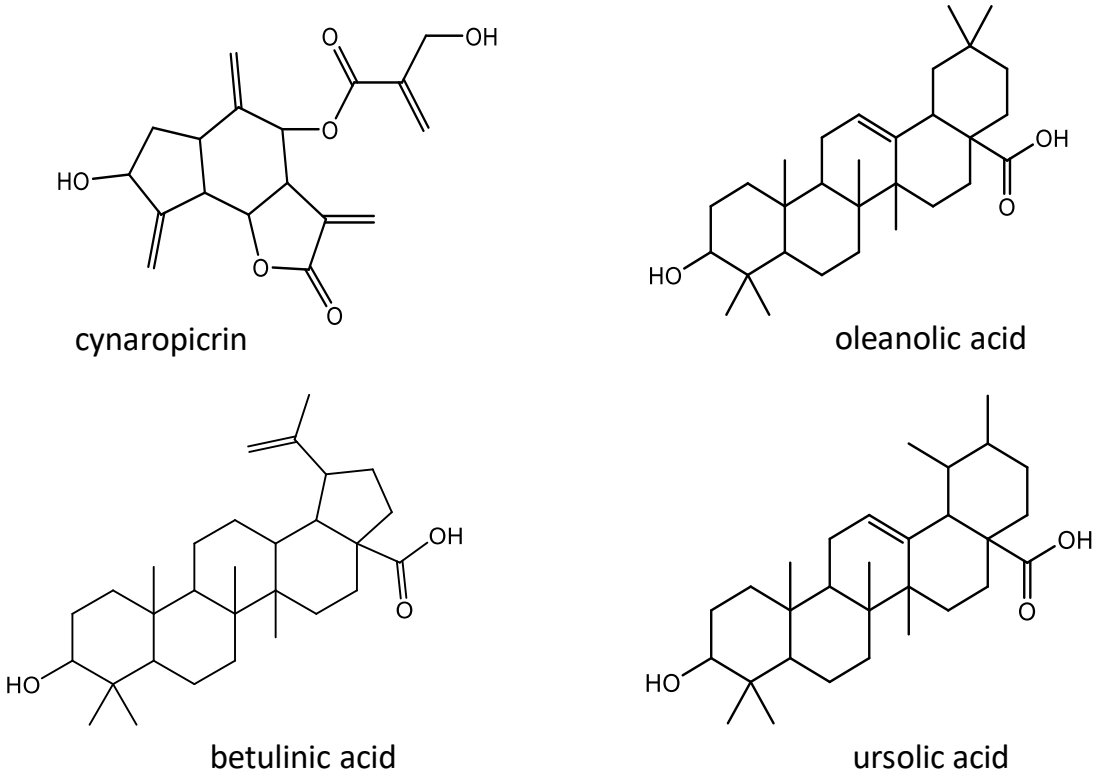

Fig. 1.2. Chemical structures of the terpenes investigated in the current thesis.

Phenolic compounds are a large class of secondary metabolites with a broad range of physiological roles in plants.[13] The basic structural feature of phenolic compounds is an aromatic ring bearing one or more hydroxyl groups.[14] Several classes of phenolics have been categorized on the basis of their skeleton: C_6 (simple phenols, benzoquinones), C_6—C_1 (phenolic acids), C_6—C_2 (acetophenones, phenylacetic acids), C_6—C_3 (hydroxycinnamic acids, coumarins, phenylpropanes, chromones), C_6—C_4 (naphthoquinones), C_6—C_1—C_6 (xanthones), C_6—C_2—C_6 (stilbenes, anthraquinones), C_6—C_3—C_6 (flavonoids, isoflavonoids, neoflavonoids), (C_6—C_3—

$C_6)_{2,3}$ (bi-, triflavonoids), $(C_6—C_3)_2$ (lignans, neolignans), $(C_6—C_3)_n$ (lignins), $(C_6)_n$ (catechol melanins), and $(C_6—C_3—C_6)_n$ (condensed tannins).[15,16]

Phenolic compounds are ubiquitous in all plants and are part of the human diet.[17] Phenolics are partially responsible for some organoleptic properties of plant foods.[17] Phenolic compounds have a strong antioxidant activity, in addition to antibacterial, antifungal, anti-aging, anti-inflammatory and anti-proliferative activities.[17,18] Due to these properties they are used in food, pharmaceutical, and cosmetic applications.[16] Examples of some phenolic compounds include syringic, gallic, ellagic and caffeic acids, eugenol, resveratrol, quercetin, among others (**Figure 1.3)**.

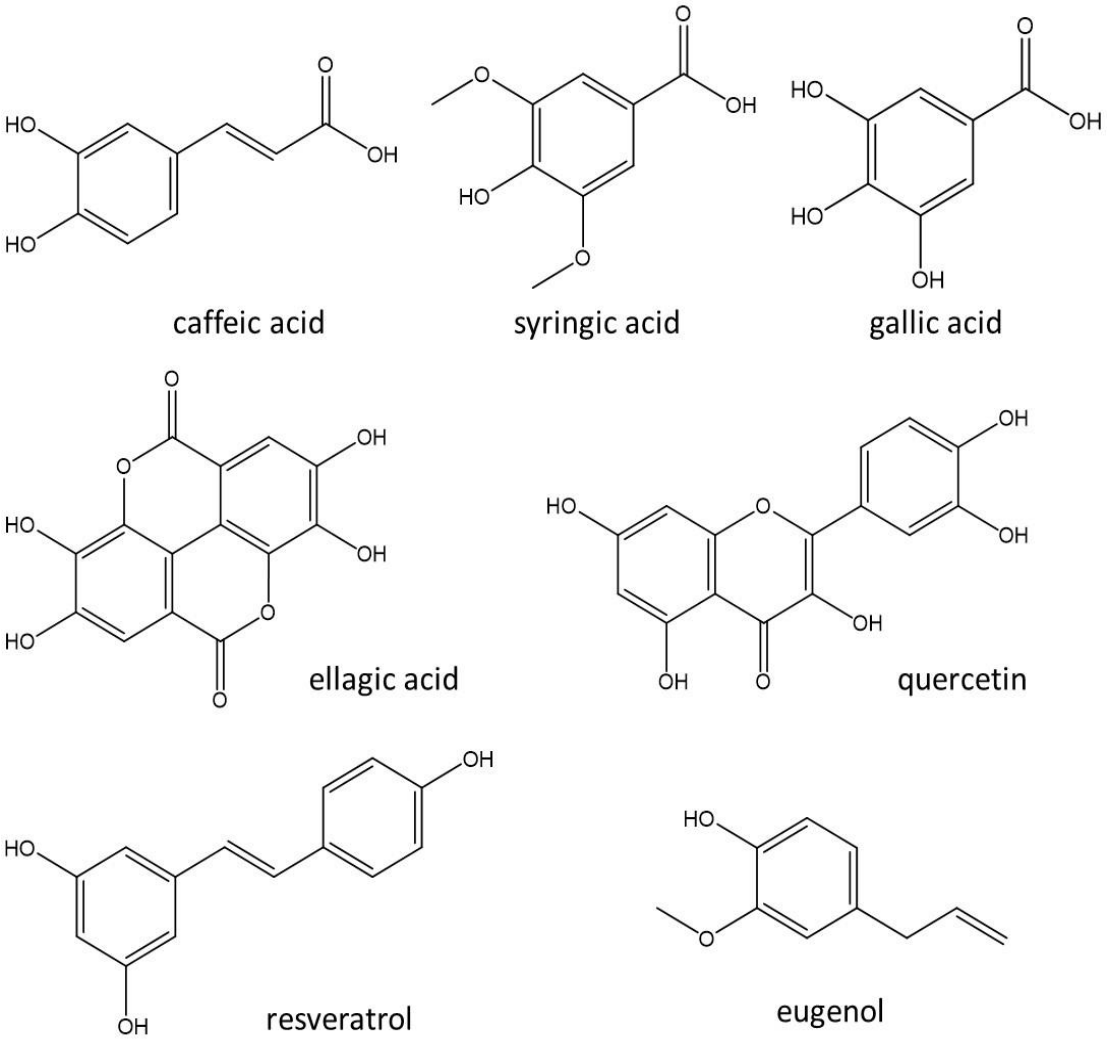

Fig. 1.3. Chemical structures of some phenolic compounds.

1.3. Extraction processes and solvents

Taking into consideration the biological properties of specific plant metabolites, and the growing search for these type of compounds for food, health and cosmetic applications, there is a growing interest on the development of more sustainable and cost-effective extraction processes.[19] These compounds are usually extracted by solid-liquid phase extraction methods, in which the solid biomass is placed in contact with an organic solvent.[20] The solution thus obtained contains the target compounds, and depending on the solvent selectivity and biomass source, many other compounds are present, turning necessary the application of further purification and recovery steps. Several parameters impact the quality of the plant extract or compounds recovered, including the biomass and solvent used, and operational conditions applied to the extraction process, as summarized in **Figure 1.4**.[21]

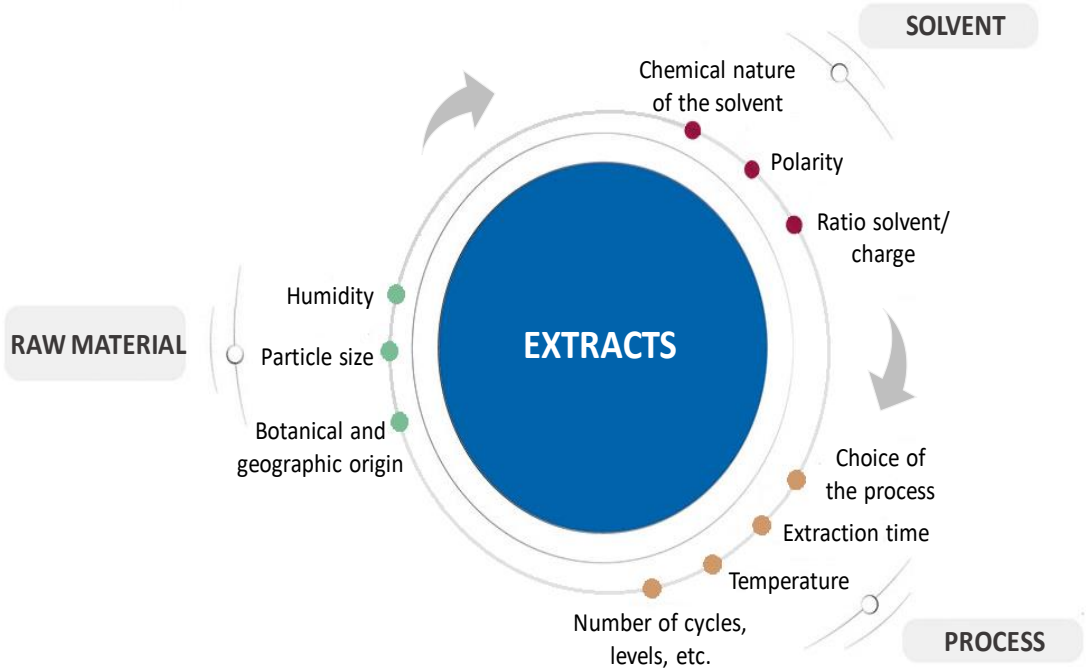

Fig. 1.4. Schematic representation of the parameters influencing the extraction of target natural compounds.

Regarding the raw material, one of the most important parameters is the fact that even within a given plant variety, there is often considerable variation depending on climatic conditions, cultivation practices, geographical origin, etc., and this might affect the efficiency and reproducibility of the extraction process.[21] Moreover, as the target compounds may be highly different in what concerns their polarity and some thermally labile, extraction processes must use broad range polarity solvents and amenable temperatures.[22,23] As far as techniques are

concerned, either those more classical (maceration, infusion, Soxhlet, etc.) or more innovative (microwave- and ultrasound-assisted, etc.) are frequently reported for the extraction of different added-value compounds from biomass.[24,25]

The key aspects in the choice of the extraction solvent include aspects such as selectivity, capability for dissolving the solute, density, viscosity, boiling temperature, volatility, chemical and thermal stabilities, cost, among others.[25] For the conventional extraction of high-value compounds from biomass, various solvents such as ethanol, acetone, dichloromethane, methanol, etc., are commonly used, and applied according to the nature of the target compounds.[26–28] Mixtures of solvents are also used to tailor the solvents polarity in order to reach improved extraction yields. In what concerns the extraction of terpenes and phenolic compounds, **Table 1.1.** reports some examples of bioactive compounds extracted using volatile organic solvents.

Table 1.1. Example of solvents applied in the extraction of terpenes and phenolic compounds from biomass.

Interest compound	**Biomass source**	**Solvent**
Terpenes		
Ursolic, betulinic and oleanolic acids	Myrtaceae species	Ethanol[29]
Ursolic and oleanolic acids	Apple peels of Fuji and Gala	Ethanol[30]
Betulinic acid	Myrtaceae species	Chloroform[31]
Oleanolic acid	Olive fruits	Chloroform[32]
Betulinic acid, cerine, and friedeline	Cork	Dichloromethane[33]
α-, β-, δ- Amyrins	Tomato fruit wax	Chloroform[34]
Cynaropicrin	*Cynara cardunculus L*.	Dichloromethane[35]
Phenolic compounds		
Chlorogenic acid, catechin, caffeic acid, p-coumaric acid and quercetin	Aerial parts of *Potentilla atrosanguinea*	Ethanol/water[36]
Ferulic acid and p-coumaric acid	Rice	Ethanol/water[37]
Caffeic, p-coumaric, ferulic and sinapic acids	Wheat, rye and triticale	Methanol/water[38]
Gallic acid, catechin, vanillic acid, cumaric acid, resveratrol and quercetin	*Vitis vinifera* wastes	Methanol[39]
Chlorogenic acid	Sunflower meal	Acetone/water[40]
Limonene, α-pinene, caryophyllene, α-terpenine, neryl acetate, geraniol	*Inula viscosa*	Dichloromethane[41]
Phenol, guaiacol, cresols, xylenol and syringol	Sugarcane bagasse and palm empty fruit bunch	Dichloromethane[42]

Most of the solvents applied (Table 1.1), are flammable and with high volatility and toxicity.[43] Consequently, the optimization and improvement of processes involving the extraction and recovery of added-value compounds from biomass through the use of appropriate technologies and solvents (ideally with more benign and environmentally-friendly characteristics) is a critical demand. In this context, alternative solvents, such as ionic liquids[44] and deep eutectic solvents,[45] have been largely investigated in this field, as discussed in the next sub-sections.

1.4. Alternative solvents: Ionic Liquids and Deep Eutectic Solvents

Due to increased environmental concerns and requirements to improve processes efficacy, new solvents have been investigated aiming at reducing the environmental burden and associated costs.[43] The search for alternative solvents corresponds to a field that suffered a rapid growth in the last two decades, where ionic liquids (ILS) and deep eutectic solvents (DES) have been largely investigated.[2,46]

1.4.1. Ionic Liquids

Ionic liquids (ILs) are organic salts constituted by large and organic cations and organic or inorganic anions. The low symmetry, weak cation-anion interactions and distribution of charge leads to a decrease in these salts melting points, which may be liquid at or close to room temperature.[47,48] ILs exhibit unique properties, namely negligible vapor pressure, low flammability, high thermal and chemical stabilities, broad liquid temperature range, high ionic conductivity, and a high solvation ability for organic, inorganic and organometallic compounds.[49–51] The ILs physicochemical properties are strongly dependent on their chemical structure, turning possible to tune their properties through the manipulation of the ions that compose them. Accordingly, ILs are usually described as “designer solvents”, allowing to manipulate their extraction capabilities for specific biomass compounds.[47,52,53]

ILs were first reported at the beginning of the 20th century by Walden,[54] when testing new explosive compounds with the aim of replacing nitroglycerin. The author synthesized ethylammonium nitrate, [$EtNH_3$][NO_3], with a melting point of 13-14^{o}C.[48] Later, in 1934, Graenacher[55] filled the first patent for an industrial application regarding the preparation of cellulose solutions using ILs. Despite these early findings, only in the last three decades these compounds have been extensively studied.

Amongst the large range of ILs that can be synthesized, the most commonly studied are nitrogen-based, namely pyrrolidinium-, imidazolium-, piperidinium-, pyridinium-, and tetraalkylammonium-based ILs (**Figure 1.5)**. The cation core can be additionally tailored with

different alkyl side chains and by the addition of different functional groups.[56] The most common IL anions structures (**Figure 1.5)** range from halides to more complex organic structures, such as tosylate and acetate.

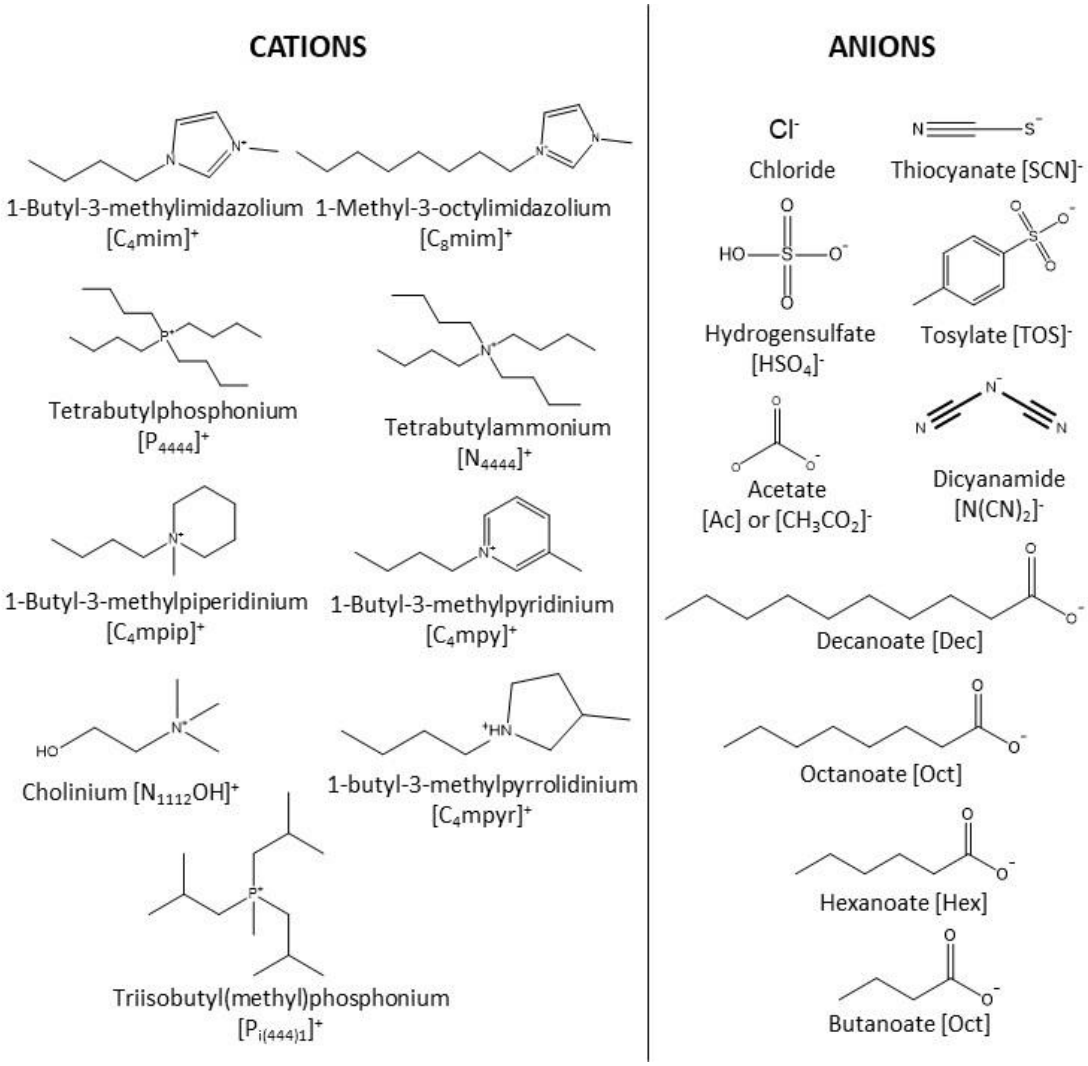

Fig. 1.5. Chemical structures of some common cations and anions in ionic liquids.

Due to their exceptional solvation capacity, as well as to being able to swell or dissolve a wide range of biomass matrices, ILs are promising solvents for the extraction and recovery of added-value compounds from biomass.[57] In addition to pure ILs, which usually exhibit high viscosity and melting points above room temperature, more recently, ILs aqueous solutions have been studied for the extraction/separation of high-value compounds from biomass.[57] ILs aqueous solutions display a lower viscosity, enhancing thus the mass transfer and reducing the energy consumption and, consequently, reducing the process cost.[57] Aqueous solutions of ILs are also granted with a high efficiency and selectivity.[57]

The solubility of biomolecules in ILs aqueous solutions usually goes through a maximum along the IL concentration – a phenomenon that has been attributed to the ILs' ability to act as hydrotropes or to act as surfactants (as shown in **Figure 1.6)**.[58] Surface-active ILs increase the solubility of more hydrophobic compounds through the formation of micelles above the critical micellar concentration (CMC) and, therefore, have been described as enhanced solvents for the extraction of some bioactive hydrophobic compounds, such as piperine, tanshinones, schizandrin, schisantherin A, deoxyschizandrin and γ-schizandrin.[59–61] With these ILs, the existence of an ionic hydrophilic head group and a long hydrophobic tail results in the formation of micelles in water, where the inner part of the micelle can accommodate hydrophobic compounds. On the other hand, hydrotropes are compounds that, at higher concentrations, solubilize sparingly soluble lipophilic compounds in water.[62] Usually, hydrotrope molecules have a shorter hydrophobic tail, leading to a higher solubility in water when compared to surfactants.[58] These substances form micelles with the target solute/compound, defined by a minimal hydrotrope concentration (MHC).[63]

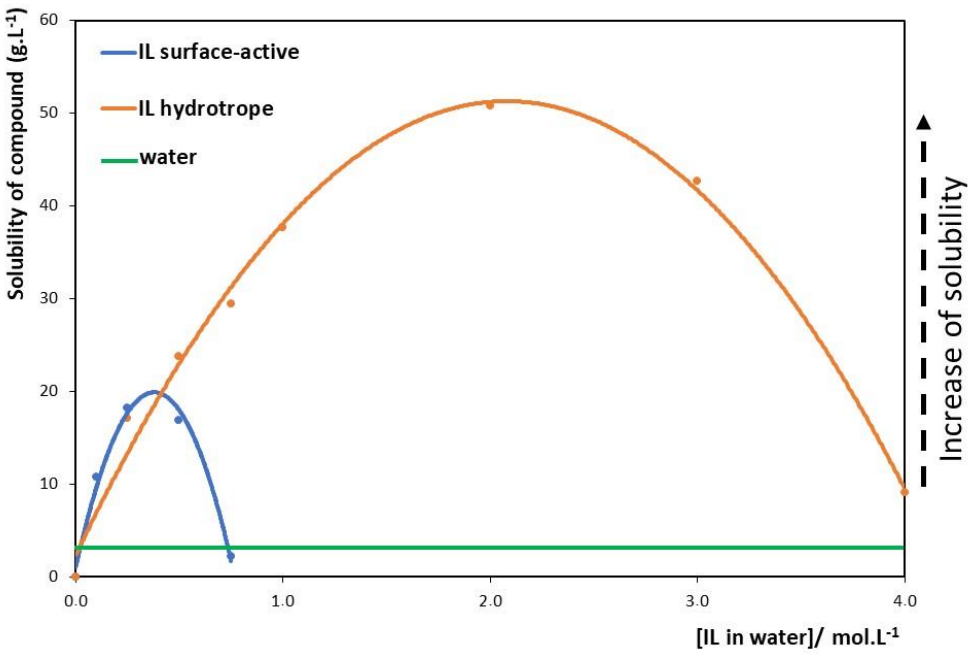

Fig. 1.6. Scheme representing the solubility increase of bioactive compounds using aqueous solution of ionic liquids with surfactant (blue) or hydrotrope characteristics (orange). Solubility in pure water represented by green line.

According to Ventura et al.,[44] more than half of the published articles on the extraction of bioactive compounds from vegetable biomass using ILs are focused on phenolic compounds, terpenoids, flavonoids, anthocyanins, tannins and others.[44,57] In these studies, 1-alkyl-3-

methylimidazolium-based ILs are by far the most widely investigated, mainly combined with the $[BF_4]^-$, Cl^-, and Br^- counterions.[57] Some of these works are overviewed below.

The extraction of hydrophobic bioactive compounds (such as tocopherol, perillyl alcohol, rutin, and ginkgolides) from soybean flour with aqueous solutions of amphiphilic anionic functional long-chain carboxylate ILs, ($[P_{4444}][C_nH_{2n+1}COO]$, n = 7, 9, 11, 13 and 15) was accomplished by Jin et al.[64] Results on the α-tocopherol solubility in ILs aqueous solutions revealed the importance of the length of the anion alkyl chain. Furthermore, the solubility increases with the IL anion alkyl chain length, from *n* = 7 to 11, while for n ≥ 13 the solubility decreases drastically, which can be attributed to a higher viscosity of the solution, hampering mass transfer. Aqueous solutions of ILs led to higher solubilities than those in pure water, pure ILs, and ethanol/water mixtures. Finally, extraction yields from 2 to 12 times higher than those achieved with volatile organic solvents were demonstrated by the authors.[64] Overall, it was concluded that the formation of IL micelles achieved by the use of surface-active ILs allows the incorporation of hydrophobic bioactive compounds inside the micelles, thereby enhancing the solubility and extraction yield.[64]

In the same line, Ressmann and co-workers[59] studied the extraction of piperine from black pepper using aqueous solutions of long alkyl side chain ILs ($[C_n\text{mim}]^+$, with *n* = 10, 12, and 14, combined with different anions. After some optimization tests, the influence of the IL concentration was analyzed, demonstrating that below the ILs CMC only small amounts of piperine (<0.2 wt%) are extracted, whereas a 4.0 wt.% extraction yield was recorded when using IL concentrations higher than the IL CMC. Furthermore, a negligible influence of the IL anion was shown.[59] The authors concluded that hydrophobic nature of piperine ([log(K_{ow}) of piperine = 2.30) requires the creation of hydrophobic cores produced by surface-active ILs to increase its dispersion/solubility in aqueous media.[59] In addition, the authors[59] compared the extraction yields obtained with ILs aqueous solutions with those obtained with conventional organic solvents, such as n-butyl acetate, toluene and methanol, concluding that micelle-based IL solutions are improved solvents over volatile organic ones.[59]

A recent study reported the development and validation of an alternative method for the extraction of sesquiterpenic acids (valerenic and acetoxyvalerenic acids) from the roots of *Valeriana officinalis L.* using aqueous solutions of surface-active ILs.[65] Given the highly hydrophobic nature of these compounds ([log(K_{ow}) of valerenic acid = 5.13] and [log(K_{ow}) of acetoxyvalerenic acid = 4.55]), it was demonstrated that ILs with surfactant behavior comprising long alkyl side chains lead to higher extraction yields. The results obtained showed that the extraction performance of acetoxyvalerenic and valerenic acids is strongly dependent on the ability of ILs to form micelles.[65]

ILs (imidazolium-, pyrrolidinium-, and ammonium-based) aqueous solutions were used by Cláudio et al.[66] and Svinyarov et al.[67] to extract caffeine from *Paullinia cupana* (guaraná seeds) and galantamine from the aerial parts of *L. aestivum*. At the optimal conditions, 1-butyl-3-methylimidazolium chloride was found to be the best IL in both studies, where the role of the aromatic imidazolium cation of the IL was emphasized to provide enhanced extractions.[66,67] The IL concentration effect on the caffeine extraction was studied, and the best results were achieved with higher IL concentrations (2.5 – 3.0 M).[66] Caffeine extraction yields of 9.4 wt.% have been obtained, being these values substantially higher than those obtained by soxhlet extraction using dichloromethane (4.30 wt.%).[66] These results demonstrate that ILs are a class of powerful cationic hydrotropes, able to increase the solubility of biomolecules in water and further contributing to enhanced extraction yields, as confirmed later by showing the hydrotropy effect exerted by ILs.[58,66–68]

Although the recovery of the target compounds and reusability of the solvent are mandatory issues when foreseeing the development of a sustainable and scaled-up extraction-recovery process, few authors studied the possibility of isolating the valuable compounds from the IL-based extract and the IL-based solvent recycling.[44,57] The applied methods mainly comprise back extraction approaches (using ethyl acetate,[69] chloroform,[66] butyl acetate[59] and *n*-hexane[70]) and precipitation of the target compounds using antisolvents.[71–73] Another example of an applied isolation method consists on the evaporation of the compounds or solvents (when applicable), such as essential oils[74] and protic ILs with high volatility.[75] The use of macroporous materials[76] and anion-exchange resins[77] have been also proposed. These materials usually display a high adsorption capacity and selectivity towards the target compound; however, the target compound recovery from these materials usually requires the use of volatile organic solvents.

All the works briefly discussed clearly show the feasibility of using ILs as extraction solvents of bioactive compounds from biomass, opening new possibilities to develop cost-efficient extraction and recovery processes. Nevertheless, given the plethora of possible ILs that can be synthesized, biomass samples and bioactive compounds, further studies on the use of ILs as alternative solvents are still required.

1.4.2. Deep eutectic solvents

Deep eutectic solvents (DES) are defined as mixtures of two or more compounds capable of associating through strong hydrogen bonding interactions deviating from an ideal solid-liquid phase behavior. DES usually comprise a hydrogen-bond acceptor (HBA) and a hydrogen-bond donor (HBD) species. Some examples of these species are given in **Figure 1.7.** When the

compounds that constitute a DES are natural-derived, such as amino acids, sugars, cholinium derivatives, among others, DES are usually described as “natural deep eutectic solvents” (NADES).[78]

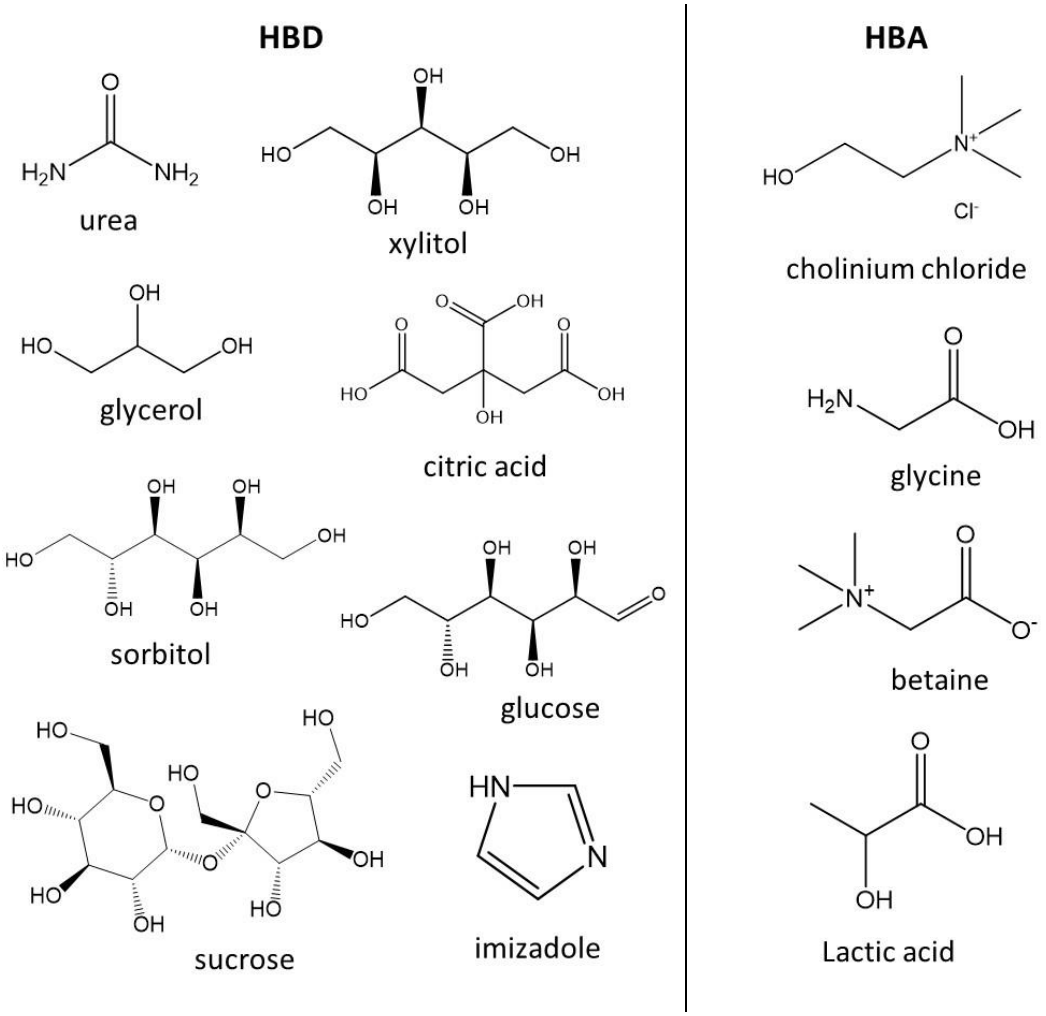

Fig. 1.7. Examples of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) used for DES and NADES preparation.

DES are characterized by a significant depression of the freezing point in respect to the ideal behavior and may be liquid at temperatures close to room temperature.[79] These mixtures are described as designer solvents since they can be tailored to present high solvation ability, low toxicity, and high biodegradability.[79] The most well-known example of a DES is the one composed of cholinium chloride and urea, reported in 2003 by Abbott and co-workers[80] (**Figure 1.8**).

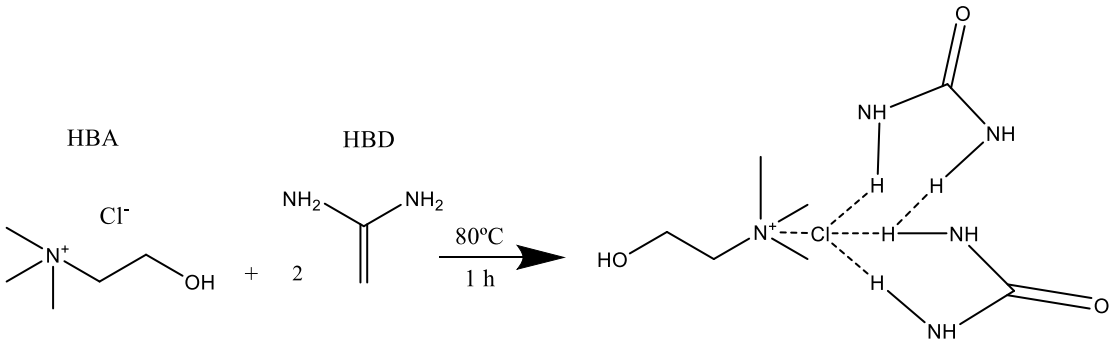

Fig. 1.8. Formation of the DES composed of urea (HBD) and cholinium chloride (HBA).

Together with the rapid screening and characterization of novel DES, their application in a wide range of chemical and biochemical processes has been observed in the past decade.[45] Among these, some studies have focused on the extraction of bioactive compounds from natural sources using DES as alternative solvents.[45] DES have been used in the extraction of phenolic compounds and flavonoids from plants,[81,82] fruits, vegetables and spices,[83] virgin olive oil,[84] among others. Some examples are described below.

In 2013, NADES were investigated for the extraction of phenolic compounds from Safflower.[81] NADES tested included the lactic acid:glucose, proline:malic acid, sucrose:cholinium chloride, glucose:cholinium chloride, sorbitol:cholinium chloride, 1,2-propanediol:cholinium chloride, and fructose:glucose:sucrose. The extraction capacity of NADES was correlated with their polarity and viscosity.[81] PCH showed to have the lowest polarity among all tested NADES and showed the lowest efficiency to extract polar compounds, but higher efficiency for non-polar compounds. Thus, the polarity of NADES has to be considered as an important property affecting its extraction efficiency.[81] Compared with conventional solvents (water and ethanol), the high viscosity of NADES is an important drawback, but it can be decreased by diluting the DES in water. Accordingly, the water content in NADES proved to have an important effect on the extraction yield of phenolic compounds.[81] Nevertheless, it must be noted that excessive amounts of water can result in the suppression of the interactions occurring between the constituents of the DES, and DES will no longer exist. Overall, the authors showed a recovery yield up to 90% for polar compounds (such as cartormin) and 84% for less polar compounds (such as carthamin).[81]

Bajkacz and co-workers,[83] in 2017, reported the use of NADES for the extraction of flavonoids (rutin, hesperidin, neohesperidin, naringenin, naringin, quercetin, hesperetin, and chrysin) from fruits (cranberry, fruits of *Lycium barbarum* L., grape, plum, and orange peels), vegetables (onion and broccoli), and spices (mustard, rosemary, and black pepper). Several NADES based on cholinium chloride, acetylcholinium chloride, cholinium tartrate, betaine, and carnitine with

different compositions were tested. In addition, the water content and different liquid/solid ratio were studied. The extraction yields achieved with NADES were compared with those obtained with water and methanol, demonstrating that the NADES approach improves the extraction yield of the target compounds. An efficient recovery of flavonoids (higher than 70%) was achieved using a 30% acetylcholinium chloride:lactic acid (3:1) aqueous solution at 60 °C, for 30 min and at 1400 rpm.[83]

Garcia et al.[84] applied DES to extract secoiridoid derivatives from virgin olive oil. Different DES composed of cholinium chloride and sugars, alcohols, organic acids, and urea, as well as a mixture of three sugar, were used. The extraction yields were compared with those obtained with an 80% (v/v) methanol/water mixture. The two most abundant secoiridoid derivatives in olive oil, namely oleacein and oleocanthal, extracted with [Ch]Cl:xylitol and [Ch]Cl:1,2-propanediol, showed an increase of 20 – 33% and 67.9 – 68.3% in the extraction yield when compared to the conventional solvent. These results suggest that DES are an efficient alternative solvent for the extraction of bioactive compounds from virgin olive oil.

More recently, Liu et al.[82] reported the extraction of narirutin, naringin, hesperidin and neohesperidin from *Aurantii Fructus*. A series of DES were prepared and investigated by mixing cholinium chloride or betaine with different hydrogen-bond donors. To improve the extraction yield, several parameters such as HBA:HBD ratio, water content, temperature, biomass:solvent ratio and extraction time were investigated. Betaine:ethanediol was found to be the most suitable extraction solvent, combined with the following optimized conditions: 40% of water in the DES betaine:ethanediol (1:4); 60°C; 30 min; biomass:solvent ratio of 1:100 g/mL.[82] The extraction yields of narirutin, naringin, hesperidin, and neohesperidin were 8.39 ± 0.61, 83.98 ± 1.92, 3.03 ± 0.35 and 35.94 ± 0.63 mg/g, respectively, which are higher than those obtained with methanol as extraction solvent (5.5 ± 0.48, 64.23 ± 1.51, 2.16 ± 0.15 and 30.14 ± 0.62 mg/g).[82]

All the works briefly discussed clearly show the feasibility of using DES as extraction solvents of bioactive compounds from biomass and opens new possibilities to design cost-efficient extraction and recovery processes. However, given the multitude of possible DES and biomass, there is still a need for further investigations on the use of DES as alternative solvents.

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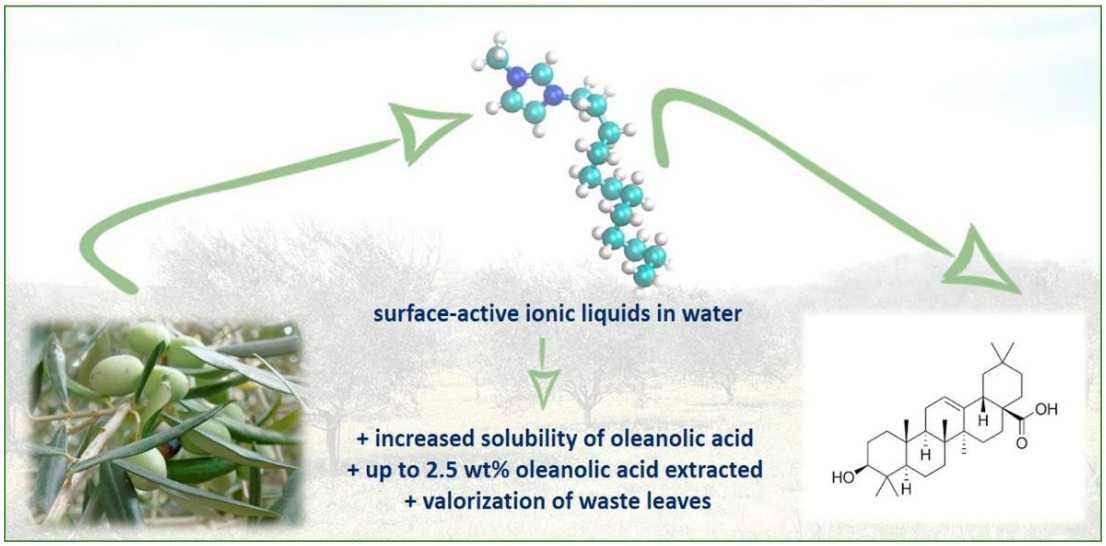
2. VALORIZATION OF OLIVE TREE LEAVES: EXTRACTION OF OLEANOLIC ACID USING AQUEOUS SOLUTIONS OF SURFACE-ACTIVE IONIC LIQUIDS

Chapter based on the published article:

A.F. Cláudio, A. Cognignia, E.L.P. de Faria, A.J.D. Silvestre, R. Zirbsc, M.G. Freire and K. Bica. Valorization of olive tree leaves: Extraction of oleanolic acid using aqueous solutions of surface-active ionic liquids. *Sep. Purif. Technol.* **2018**, 204, pp. 30-37.

Contributions: M.G.F. and K.B. conceived and directed this work. A.F.C., A.C. and E.L.P.F. acquired the experimental data. A.F.C., A.J.D.S., R.Z., M.G.F. and K.B. interpreted the experimental data. The manuscript was mainly written by A.F.C., M.G.F. and K.B. with contributions from the remaining authors.



Abstract

The global olive oil industry annually generates approximately 750,000–1,500,000 tons of *Olea europaea* leaves as waste that are typically burned for energy production. Yet, this agricultural by-product is a rich source of oleanolic acid, a high value triterpenic acid with outstanding pharmaceutical and nutraceutical activities. The present study focuses on the extraction of oleanolic acid from dried *O. europaea* leaves using aqueous solutions of surface-active ionic liquids as alternative solvents. A number of imidazolium-based ionic liquids with variable chain length, different anions and optional side-chain functionalization was synthesized and employed in the extraction of oleanolic acid. Ionic liquids with long alkyl chains remarkably enhance the solubility of oleanolic acid in water, thus being able to compete with the solubilities afforded by molecular organic solvents, such as chloroform. Consequently, they are suitable alternatives for

the solid-liquid extraction of triterpenic acids from natural matrices and provide improved extraction yields of up to 2.5 wt% of oleanolic acid extracted from olive tree leaves.

Introduction

Biomass has emerged as key source of a wide variety of fine chemicals.[1] This perspective is in straight line with the emerging biorefinery concept, which postulates the integrated exploitation of agroforest biomass as a source of chemicals, materials, fuels and energy.[2] In this context, the development of environmentally friendly strategies for the recovery of high-value compounds from biomass by-products prior to their further valorization, typically carried out by burning, is of crucial importance.

Triterpenic acids are a typical example of such high-value compounds that can be extracted from agricultural by-products prior to burning, while adding substantial economic value to biorefinery-based processes.[3] Triterpenic acids, such as oleanolic, betulinic and ursolic acids, are secondary plant metabolites typically found in barks, leaves or peels, with potential pharmaceutical and nutraceutical applications.[4] A number of studies demonstrated that triterpenic acids have potent antimicrobial, antitumor, hepatoprotective, anti-inflammatory, cytotoxic, anti-allergic and anti-HIV activities.[4-6] Oleanolic acid (**Figure 2.1**), in particular, is a key component of olive pomace, and a major contributor to the health promoting effect of the human Mediterranean diet.[7] Consequently, an increasing number of studies addressed the molecular mechanisms of action of plant triterpenes, but also their occurrence and improved isolation from various plant materials.[3,8] Due to their beneficial properties and interest for several industries there is considerable interest in identifying novel sources of triterpenic acids to guarantee the increasing world-wide demand;[9] it should be remarked that the commercial value of oleanolic acid, depending on its purity, can reach 1200 €/g.[10]

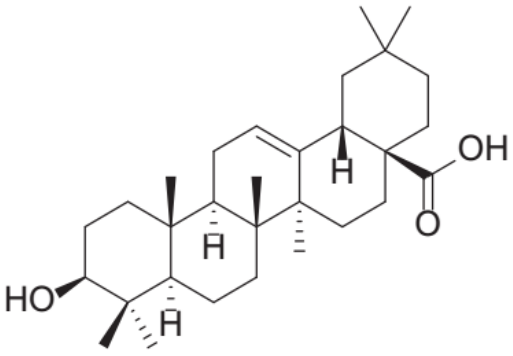


Fig. 2.1 Chemical structure of oleanolic acid.

While the leaves, fruits or bark of many plants are reported as potential sources of this compound, oleanolic acid can be also found in agroforestry waste streams, such as in the leaves of *O. europaea*, the common European olive tree.[11] These by-products are abundantly produced as a result of the activities of olive oil industries, which generate large amounts of wastes currently burned for energy production.[3] Taking the total world production of olive oil in 2012 into account, 750,000–1500,000 tons of leaves are discarded on an annual base. Assuming a maximum content of up to 3.1 wt% of oleanolic acid, the main triterpenic acid present in the leaves of *O. europaea*, this corresponds to large amounts of this high-value compound that could be potentially extracted[12,13] contributing to the integrated valorization of the olive oil value chain.

Still considering the biorefinery concept, and in addition to the use of agroforestry by-products, one of the main challenges for the efficient exploitation of high-value compounds involves the replacement of conventional extraction and purification systems, usually carried out with volatile and often hazardous organic solvents in multistep procedures, by more efficient and environmentally friendly alternatives.[14] Among new prospective extractive solvents for value-added products, ionic liquids (ILs) have gained considerable attention.[15] Unlike molecular solvents, they present outstanding properties due to their ionic nature, namely negligible vapor pressure and high solvation ability. Since the physicochemical properties of ionic liquids are strongly dependent on their ionic nature and composition, the possibility of tailoring them is an important advantage for manipulating their extraction abilities and selectivities.[16,17] As a result, the ionic liquid-assisted targeted extraction of high value natural products from biomass became a rapidly growing field of research in the past years, and numerous studies demonstrated improved extraction yield and selectivity compared to conventional solvents.[18-20] More recently, the possibility of using aqueous solutions of ionic liquids instead of their pure forms, prompted an increased interest due to substantial improvement in extraction efficiency and cost reduction. Moreover, advanced technologies for natural product extraction such as subcritical water extraction can be further boosted by the addition of ionic liquids, as recently demonstrated on the extraction of phenolics or carrageenan from brown or red seaweed.[21,22] Ionic liquid aqueous solutions promote extraction through the formation of hydrotropes, enhancing the solubility of more hydrophilic compounds,[23-26] or as surface-active compounds, increasing the solubility/dispersion of hydrophobic substances in aqueous media.[27-29]

Here, we investigate the extraction of oleanolic acid from *O. europaea* leaves, an industrial waste stream from olive oil industry, relying on aqueous solutions of surface-active ionic liquids, and discuss the impact of ionic liquid chemical structure, extraction method and operational conditions such as microwave irradiation on the extraction yield.

Materials and methods

Commercially available reagents and solvents were used as received from Sigma Aldrich, unless otherwise specified. Doubly-distilled deionized water was obtained from a Millipore Milli-Q water purification system (Millipore, USA). All ionic liquids were dried for at least 24–48 h at 50 °C and 0.01 mbar before use and were stored under argon.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 400 at 400 and 100 MHz, respectively, using the solvent peak as reference. *J* values are given in Hz. ^{13}C NMR spectra were run in proton-decoupled mode.

For the quantification of oleanolic acid, HPLC analysis was performed relying on reversed phase C18-column set-up via the following methods:

Method A: A GILSON HPLC unit coupled to an oven with manual injector and equipped with an analytical C18 reversed-phase column (250 × 4.60 mm, Kinetex 5 µm C18 100 A) from Phenomenex was used. The column temperature was set to 30 °C. The mobile phase consisted of 87 (v/v) % of methanol, 13 (v/v) % of water + 0.1 (v/v) % of trifluoroacetic acid (TFA). Separations were conducted in isocratic mode, at a flow rate of 1 mL/min. Detection was done at a wavelength of 210 nm. Data acquisition and evaluation were performed using the Jasco-Borwin 1.21 software and based on a previously established calibration curve ($R^2 > 0.9998$).

Method B: A Jasco HPLC unit equipped with a Maisch Reprosil 5 µm C18 100 column (250 × 4.60 mm) and security guard pre-column was used. The column temperature was set to 30 °C. The mobile phase consisted of 87 (v/v) % of methanol, 13 (v/v) % of water + 0.1 (v/v) % of trifluoroacetic acid (TFA) at a flow of 1 mL/min. Detection was done at a wavelength of 210 nm. Data evaluation was based on a previously established calibration curve ($R^2 > 0.9993$) prepared using 1-methylcyclohexene in MeOH as internal standard.

Microwave-assisted extractions were performed on a BIOTAGE Initiator^{TM} sixty microwave unit. The reported times are hold times.

SEM pictures were taken with a FEI Inspect F50 at 15 kV. All samples were coated with a 3.5 nm thick gold-layer using a Leica Cool Sputter Coater EM SCD005.

Olive tree leaves were collected in Aveiro, Portugal and pre-dried at 25°C for 5 d. The dried leaves were milled to a particle size > 1 mm using a Retsch ZM 100 cryo mill.

Synthesis of ionic liquids

The 1-alkyl-3-methylimidazolium halide ionic liquids, namely [C_6mim]Cl, [C_8mim]Cl, [C_{10}mim]Cl, [C_{12}mim]Cl, [C_{12}mim]Br, [C_{12}mim]I, [C_{14}mim]Cl, [C_{16}mim]Cl and [C_{18}mim]Cl, were prepared from freshly distilled N-methylimidazol and the corresponding alkyl halide, according to literature.[30,31] Solid surface-active ionic liquids, [$C_n$mim]X with n ≥ 12, were repeatedly crystallized from tetrahydrofuran or ethyl acetate until colourless crystals were obtained. Sulfonate- and phosphonate-based surface-active ionic liquids were synthesized through a two steps procedure involving the alkylation of 1-dodecylimidazole, which was previously synthesized following a procedure reported in literature and distilled before use.[32] 1-Dodecylimidazole was reacted with the corresponding methyl ester to yield 1-dodecyl-3-methylimidazolium mesylate ([C_{12}mim][OMs]), 1-dodecyl-3-methylimidazolium tosylate ([C_{12}mim][OTs]), and 1-dodecyl-3-methylimidazolium dimethylphosphate ([C_{12}mim][Me_2PO_4]). Analytical data were in accordance with literature and details can be found in previous work.[30,31,33,34] In case of 1-dodecyl-3-methylimidazolium acetate ([C_{12}mim][Ac]), the procedure given by Ferguson et al.[35] was adapted, which includes the neutralization of the corresponding hydroxide ionic liquid with acetic acid to obtain the acetate counterion. Analytical data were in accordance with literature and details can be found in our previous work.[26] The synthesis of ionic liquids with ester-functionalization, namely [C_{12}bet]Cl and [C_{12}COmim]Cl, relied on the pre-formation of dodecyl 2-chloroacetate that was further reacted with trimethyl amine or methylimidazole according to literature protocols.[36,37]

3-(2-(Dodecyloxy)-2-oxoethyl)-1-methylimidazolium chloride ([C_{12}COmim]Cl): 1-Methylimidazol (1.19 g, 14.49 mmol) and dodecyl 2-chloroacetate (3.81 g, 14.49 mmol) were stirred at ambient temperature for 24 h. The obtained solid was crystallized from THF, collected via filtration and washed with anhydrous THF and diethyl ether. After drying in vacuum, a colourless solid was yielded in 81% (4.1 g). ^{1}H NMR (200 MHz, $CDCl_3$): δ_H = 0.86 (t, J = 6.36, 3 H, -CH_2-CH_3), 1.18–1.36 (m, 18 H, -O-CH_2-CH_2-$(CH_2)_9$-CH_3), 1.64 (quint, J = 6.55, 2 H, -O-CH_2-CH_2-$(CH_2)_9$-CH_3), 4.06 (s, 3 H, N-CH_3), 4.16 (t, J = 6.84, 2 H, -O-CH_2-CH_2-$(CH_2)_9$-CH_3), 5.47 (s, 2 H, N-CH_2-COO-), 7.40 (s, 1H, H-4), 7.51 (s, 1H, H-5), 10.58 (s, 1H, H-2).

2-(Dodecyloxy)-N,N,N-trimethyl-2-oxoethanaminium chloride ([C_{12}bet]Cl): Dodecyl 2-chloroacetate (5.39 g, 20.51 mmol) was dissolved in 15 mL anhydrous THF. A solution of trimethylamine in THF (102.5 mmol) was added dropwise at room temperature. After stirring overnight, the precipitate was collected via filtration and washed with anhydrous THF and

diethyl ether. After drying in vacuo (2×10^{-2} mbar) overnight, [C_{12}bet]Cl was obtained as colorless crystals in 88% yield. ^{1}H NMR ($CDCl_3$): δ= 0.81 (3H, t, J = 6.95, -$C_{11}H_{22}$-CH_3), 1.19 (18H, m, -C_2H_4-C_9H_{18}-CH_3), 1.58 (2H, t, J = 6.74, -CH_2-CH_2-$C_{10}H_{21}$), 3.60 (9H, s, N-$(CH_3)_3$), 4.10 (2H, t, J = 6.95, -CH_2-$C_{11}H_{23}$), 5.01 (2H, s, Cl-CH_2-CO).

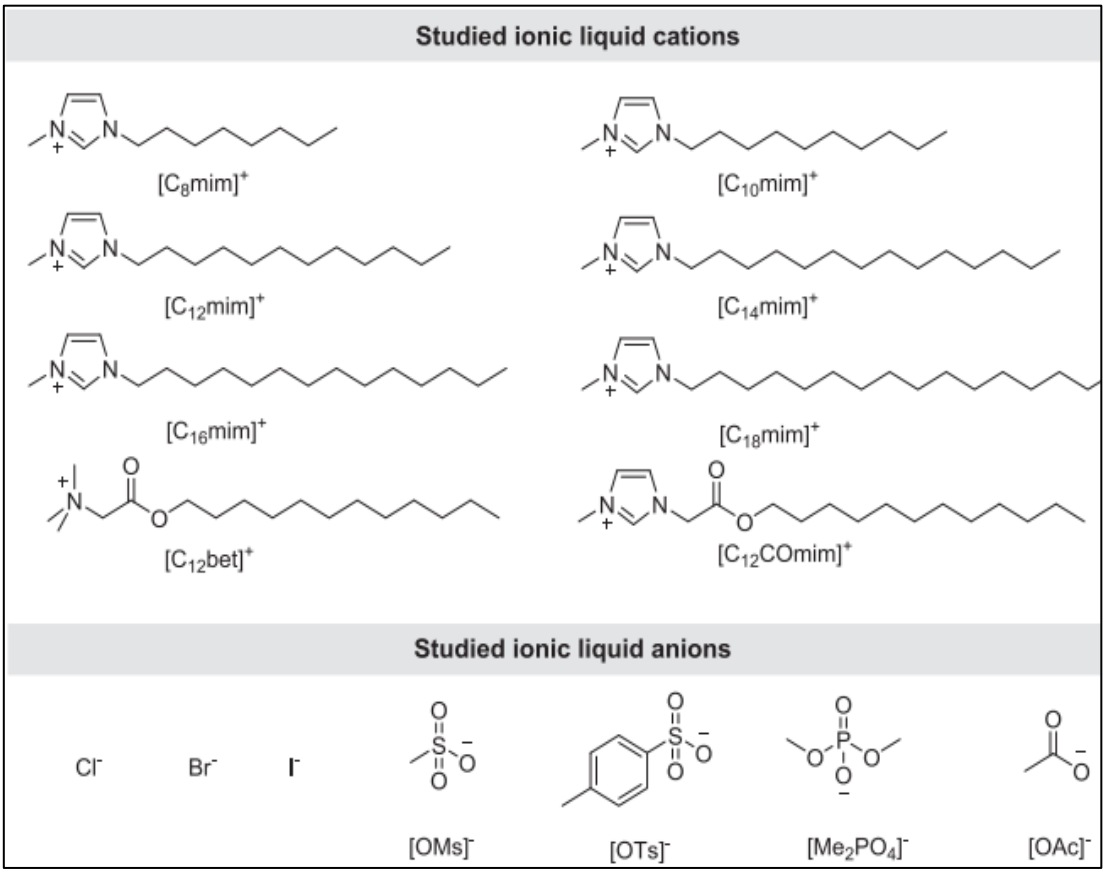


Fig. 2.2. Chemical structure and abbreviation of the ILs cations and anions used.

Solubility studies

Oleanolic acid was added in excess amount to [C_{12}mim]Cl aqueous solutions (100, 500, 1000 mM) and to pure water. The mixture was then stirred under constant agitation (1000 rpm), at 80 °C ± 0.5 °C for 2 h (preliminary tests on the time required to achieve the equilibrium were carried out). After saturation of the aqueous solutions, and always assuring the presence of a solid phase and thus of oleanolic acid in excess, an aliquot of 200 µL was taken, mixed with 800 µL of methanol, filtered over a 0.2 µm syringe filter, and measured immediately via HPLC (Method A) using a previously established calibration curve. Results given are based on three independent experiments.

Extraction experiments

General extraction process using surface-active ionic liquids: An 8 mL screw-cap vial was charged with olive tree leaves (200 mg) and aqueous ionic liquid solution (1800 mg) in a concentration range varying from 100 to 1000 mM. The extractions were carried out with magnetic stirring (1000 rpm) and at different temperatures within ± 0.5°C and times (25 °C/24 h or 80 °C/2 h).

After the extractions, the suspensions were centrifuged, and the supernatant was filtered using a 0.20 µm syringe filter. An aliquot of 200 µL was taken, mixed with 800 µL of internal standard solution (0.0464 mg 1-methylcyclohexene/mL of methanol) filtered over a 0.2 µm syringe filter and measured immediately via HPLC (Method B). Results given are based on three independent experiments.

General extraction process using ionic liquid under microwave irradiation: A 5 mL microwave vial was charged with olive tree leaves (200 mg) and aqueous ionic liquid solution (1800 mg), sealed with a Teflon septum and heated for 30 min at 80 °C under microwave irradiation (high absorption level) with magnetic stirring.

An aliquot of 200 µL was taken, mixed with 800 µL of internal standard solution (0.0464 mg 1-methylcyclohexene/mL of methanol), filtered over a 0.20 µm syringe filter, and measured immediately via HPLC (Method B). Results given are based on three independent experiments.

Results and Discussion

Based on previous successful results on the extraction of hydrophobic substances from biomass using aqueous solutions of surface-active ionic liquids,[27] herein we focused on the use of 1-alkyl-3-methylimidazolium-based ionic liquids combined with several anions, [$C_n$mim]X. Several studies already addressed the aggregation behavior in terms of critical micelle concentration,[30,31] size and shape of micelles over a broad concentration range[38,39] so that the physico-chemistry nature of 1-alkyl-3-methylimidazolium-based ionic liquids in water is relatively well explored and known. Therefore, we selected a set of N-methylimidazolium-based surface-active ionic liquids with the chloride anion and variable chain length, ranging from n = 8 to 16, for the extraction of oleanolic acid. In case of 1-dodecyl-3-methylimidazolium, the anion was optionally modified to study the impact of different anions, including other halides, sulfates, phosphonates or carboxylates on the extraction of oleanolic acid **(Figure 2.2)**. As the inherent toxicity and biodegradability of long-chain imidazolium salts might restrict their application, we also included in this study a surface-active betaine-derived ionic liquid [C_{12}bet]X with improved biodegradability.[40]

Before addressing the extraction of oleanolic acid from native plant material, and in particular to infer the magnitude of the saturation of the target compound in aqueous solutions of ionic liquids, preliminary studies on the solubility of oleanolic acid in $[C_{12}mim]Cl$ aqueous solutions were made. Pure oleanolic acid was added in excess to pure water and to aqueous ionic liquid solutions at different concentrations and stirred under fixed conditions. Due to the increasing viscosity of ionic liquid solutions at higher concentrations, we decided to work at a fixed temperature of 80°C. The solubility of oleanolic acid in pure water could not be determined as it is below the detection limit of the used analytical equipment. This is in accordance with literature data, were extremely low values for the solubility of rather hydrophobic triterpenic acids in aqueous media are reported.[41,42]

While no value is given for pure water, Jin et al. reported an oleanolic acid solubility of 1.4 ± $1.3 \cdot 10^{-3}$ mg/mL in a 1 N aqueous solution of NaOH at 25 °C.[41] Similarly, Jäger et al.,[42] reported extremely low solubilities of oleanolic and betulinic acids in water (up to $2 \cdot 10^{-5}$ mg/mL).[42] Despite this low solubility in water, the solubility of oleanolic acid in a 100 mM aqueous solution of $[C_{12}mim]Cl$ in water increased up to 2.83 mg/mL **(Table 2.1, entry 3)**. A further increase on the ionic liquid concentration to 500 and 1000 mM drastically improves the solubility of oleanolic acid to a value of 21.10 mg/mL, indicating that aqueous solutions of surface-active ionic liquids lead to a remarkable increase (up to 10^6-fold) on the solubility of oleanolic acid, being thus able to compete with the solubilities afforded by molecular organic solvents.[11]

Table 2.1. Solubility of oleanolic acid in aqueous solutions of the ionic liquids $[C_{12}mim]Cl$ at variable concentrations.

Entry[a]	Concentration $[C_{12}mim]Cl$ (mM)	Solubility (mg/mL)
1	0	n.d.[b]
2	100	2.828 ± 0.016
3	500	11.530 ± 0.039
4	1000	21.097 ± 0.234

[a] Performed with an excess amount of oleanolic acid added in $[C_{12}mim]Cl$ aqueous solutions and in pure water at 80 °C ± 0.5 °C. Results are expressed as average ± standard deviation (STD), n = 3. [b] Below the analytical equipment detection limit.

Encouraged by these initial results of oleanolic acid solubility, we addressed the extraction of oleanolic acid as the main triterpenic acid occurring in the leaves of *O. Europaea* using aqueous solutions of ionic liquids, either via conventional extraction or assisted by microwave heating.

After drying and milling of olive tree leaves to a particle size < 1 mm, we established an extraction procedure as outlined in **Figure 2.3.**

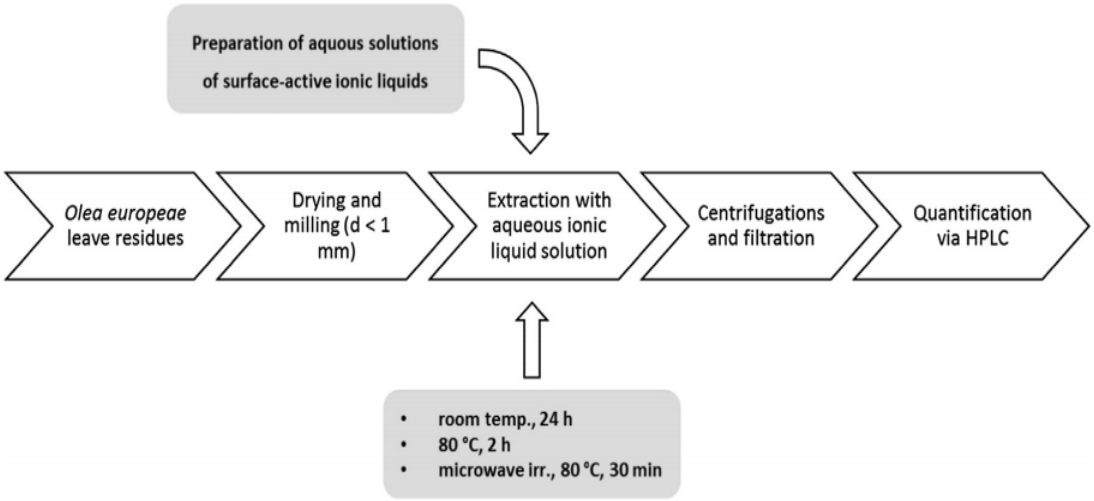


Fig. 2.3. Flow-scheme for the ionic liquid assisted extraction of oleanolic acid from *O. europaea* leaves using aqueous solutions.

The pre-processed olive tree leaves were suspended in the IL aqueous solutions at a fixed solid/liquid ratio of 1:10 (w:w). After extraction, the biomass was separated via centrifugation at 750 rpm. After filtration of the supernatant using a 0.2 µm syringe filter, the extract was diluted with methanol and directly analyzed via HPLC. Based on our previous experience in the analysis of pentacyclic triterpenes in the presence of ionic liquids, we relied on a reversed phase C-18 set-up for the quantification of oleanolic acid.[43,44] To prolong the column life time, sample obtained from biomass extractions were strictly measured on a reversed phase column equipped with protecting guard precolumn. The high polarity of alkylmethylimidazolium-based ionic liquids compared to triterpenes found in olive tree leaves allowed for a directly quantification of oleanolic acid in the crude extract as the ionic liquid was eluted at very short retention times of 2 min. Oleanolic acid was typically eluted at considerable higher retention times of approx. 15.5 min, and peak assignment was verified via standard addition of an authentic sample (see supplementary information).

The oleanolic acid extraction yield is expressed as weight percent (wt%) of extracted oleanolic acid per weight of pre-dried biomass. Initial studies were again performed with aqueous solutions of the surface-active ionic liquid [C_{12}mim]Cl over a concentration range of 100–1000 mM, which is well above the critical micelle concentration (CMC) of the respective ionic liquid. As for the solubility studies, a strong influence of ionic liquid concentration was observed, with an increased ionic liquid concentration resulting in strongly improved extraction

yields **(Figure 2.4)**. This is in good accordance with the data obtained in solubility studies, and points to an aggregate-mediated extraction mechanism.

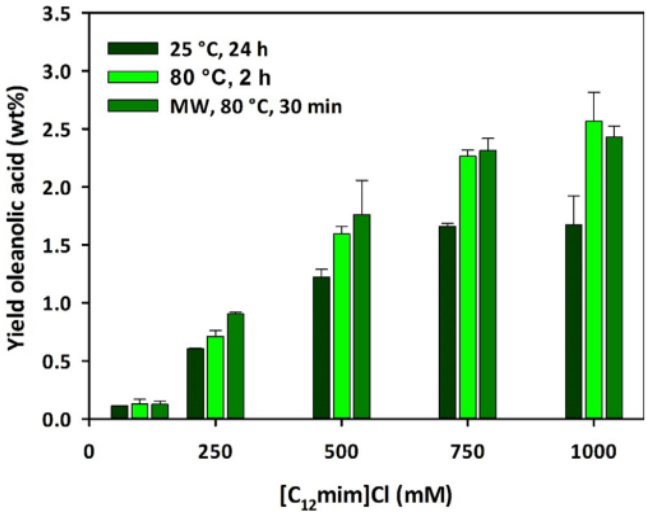


Fig. 2.4. Oleanolic acid extraction yield from olive tree leaves with aqueous solutions of [C_{12}mim]Cl and at different conditions (constant condition: S/L ratio = 1:10; d < 1 mm). MW: microwave heating.

These results show that significantly high amounts of oleanolic acid could be extracted with conventional heating at 80 °C, with an extraction time to 2 h. These strong improvements in extraction yield can be explained by an increased solubility of oleanolic acid in the solvent, but also by the decrease of the viscosity of the aqueous solution at higher temperatures. As microwave-assisted extraction was already suggested as a useful method for the extraction of oleanolic acid and ursolic acid from plant materials, such as traditional Chinese herbs[45] we further investigated the influence of microwave irradiation on the extraction yield. Microwave-assisted extraction at 80 °C could further reduce the required extraction time, and up to 2 wt% of oleanolic acid could be obtained after 30 min. Independent of the chosen extraction parameters the concentration dependency was maintained, and the highest extraction yields were obtained with a 1000 mM of [C_{12}mim]Cl in water. Since oleanolic acid can suffer speciation as a function of the pH (pKa = 4.74),[46] we further investigated the effect of pH over the extraction yields obtained, mainly to address if the target compound could be better extracted in its neutral or charged forms. A change of the pH value of the aqueous solution of ionic liquid from 3.13 to 9.6 did not influence the extraction yield (the results shown in the appendix - Fig. S2.1), meaning that electrostatic interactions between the charged oleanolic acid and ionic liquids do not play a major role on the extraction mechanism. We did not observe any trends when varying the pH

value at a fixed ionic liquid concentration of 500 mM [C_{12}mim]Cl, and similar yields were obtained.

After optimizing extraction time and temperature, we addressed the impact of the ionic liquid structure. **Figure 2.5** shows the effect of the ionic liquid cation alkyl side chain length on oleanolic acid extraction yield.

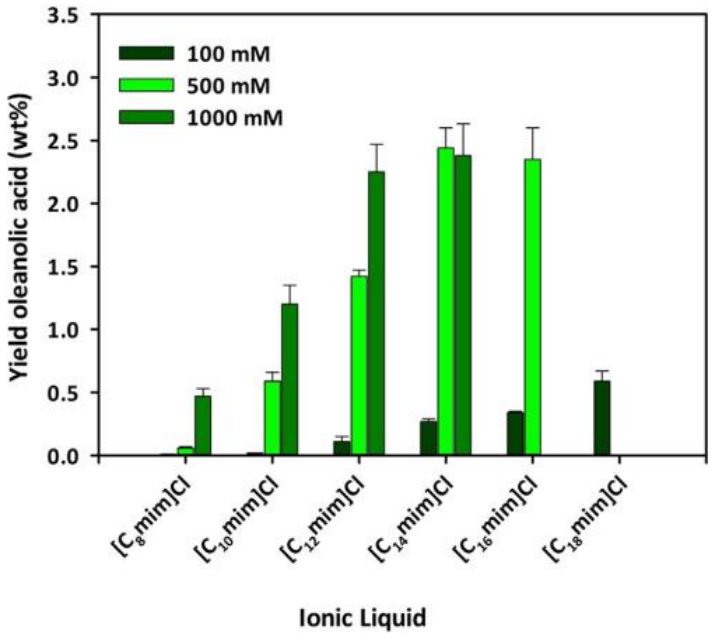


Fig. 2.5. Oleanolic acid extraction yield from olive tree leaves with different ILs, namely [$C_n$mim]Cl (n = 8, 10, 12, 14, 16, 18), at different concentrations and fixed conditions (T = 80°C, t = 2 h, S/L ratio = 1:10, d < 1 mm).

In general, there is an increase on the oleanolic acid extraction yield with the increase of the ionic liquid cation alkyl side chain length. However, from [C_{12}mim]Cl to [C_{16}mim]Cl, particularly at higher concentrations, no major differences on the extraction yields are observed. Furthermore, an increase of the ionic liquid concentration enhances the oleanolic acid extraction yield in the studied cases. However, some viscosity problems were found with long alkyl side chain ionic liquids at higher concentrations. In case of 1-alkyl-3-methylimidazolium-based ionic liquids, [$C_n$mim]Cl with n = 16 and 18, solutions at a concentration of 500 mM and/or 1000 mM became unstirrable after the addition of biomass at the given conditions; consequently, these results are missing in **Figure 2.5**. Best yields were obtained with the surface-active ionic liquid 1-methyl-3-tetradecylimidazolium chloride, and up to 2.5 wt% of oleanolic acid could be extracted using a 500 mM aqueous solution.

The impact of the ionic liquid anion and of surface-active ionic liquids with improved biodegradability was further investigated at a fixed concentration of 500 mM **(Figure 2.6)**. The

ionic liquid anion also influences the oleanolic acid extraction. In particular, acetate- and phosphate-based ionic liquids appear as promising candidates, whereas lower yields were found for surface-active ionic liquids with less hydrated anions, such as iodide or tosylate. This effect might be based on a different association of oleanolic acid to the cationic head groups in surface-active ionic liquids with different degrees of counterion binding,[47] but also on strong hydrogen-bonding interactions established between oleanolic acid and anions with high hydrogen-bond basicity[48] although more investigations would be required to prove this.

For a better comparison with current state of art, the results obtained with aqueous solutions of ionic liquids were compared with those obtained with organic solvents frequently used for the extraction of triterpenic acids, namely chloroform, ethyl acetate and methanol, under the same experimental conditions[49,50] as depicted in **Figure 2.6**. Although extraction yields for oleanolic acid obtained with toxic chloroform are still slightly higher, it should be pointed out that aqueous solutions of ionic liquids are certainly a more environmentally-friendly option and can outperform the usually employed organic solvents, namely methanol, ethyl acetate and n-hexane while requiring considerably lower amount of ionic liquid as it would be the case with pure ionic liquids as extractants. It is also worthwhile to notice that the sidechain functionalized ionic liquids, and in particular the betaine derivative [C_{12}bet]Cl, are able to outperform methanol, ethyl acetate and n-hexane and can compete with unfunctionalized imidazolium-based ionic liquids, thereby providing an attractive alternative and solution to the toxicity and biodegradability problems that are associated with long-chain imidazolium salts.

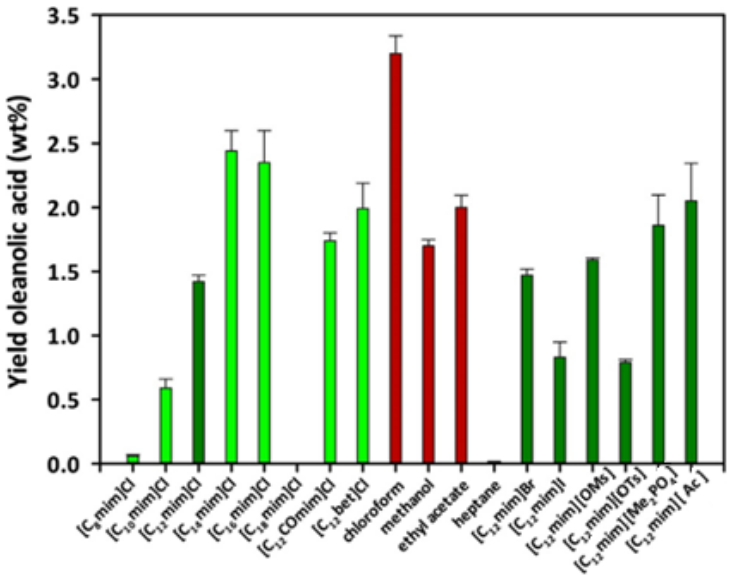


Fig. 2.6. Oleanolic acid extraction yield from olive tree leaves with several solvents at fixed conditions (T = 80 °C, [IL] = 500 mM, t = 2 h, S/L ratio = 1:10, d < 1 mm).

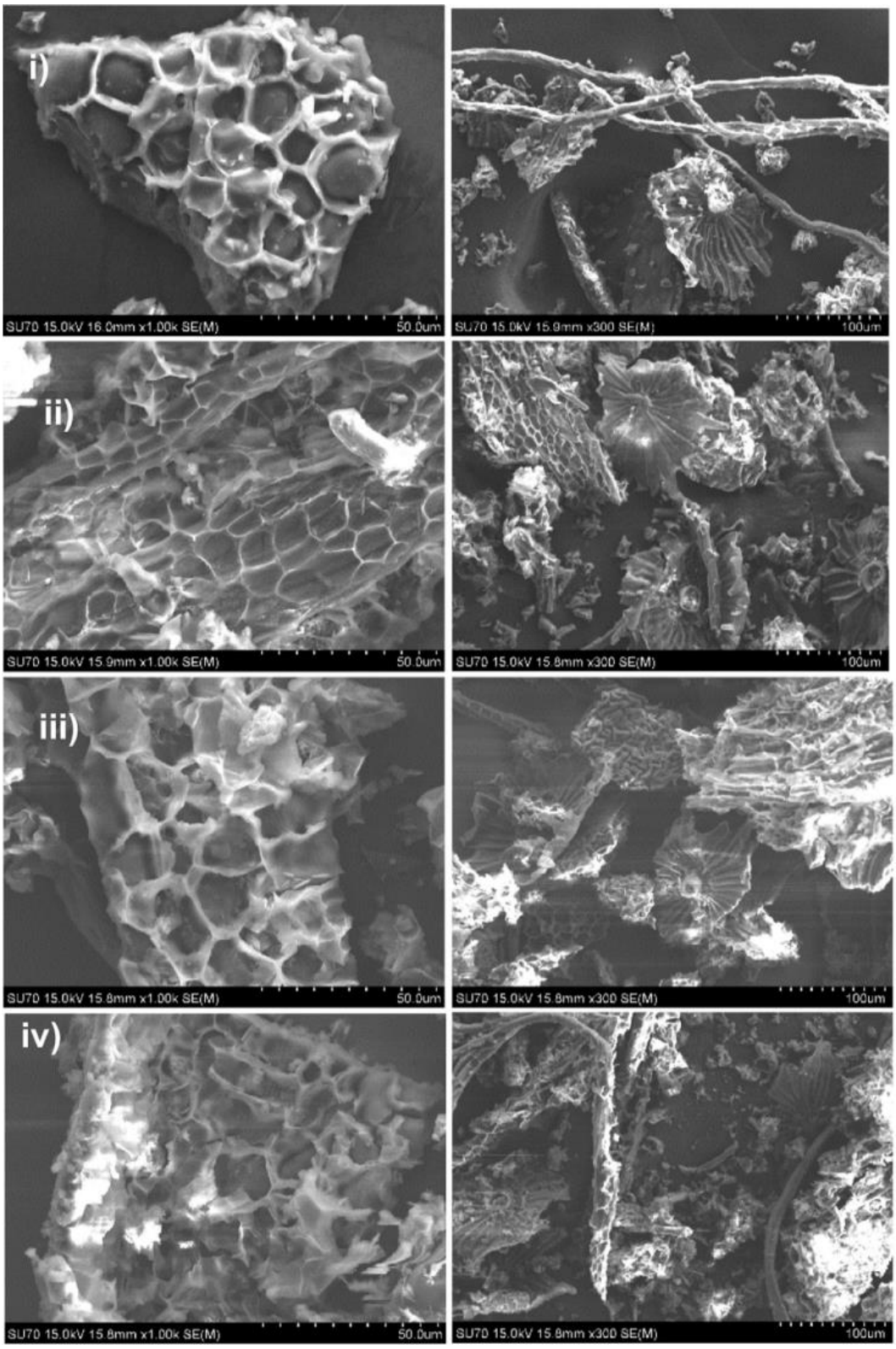


Fig. 2.7. SEM images of the olive leaves samples (i) without extraction; after extraction (T = 80°C, t = 2 h, S/L ratio = 1:10, d < 1 mm) with (ii) water, (iii) an aqueous solution of $[C_{12}mim]Cl$ at 500 mM and (iv) chloroform.

Finally, we investigated the residual biomass (after the extraction step) via scanning electron microscopy **(see Figure 2.7)**. Pictures were taken before and after extraction with water, ionic liquid solution or the conventional solvent chloroform, and compared. In general, small influence or modifications were found in the morphology of the biopolymeric matrix, independently of the extraction being performed with pure water, chloroform or ionic liquid solutions at 500 mM. This is in contrast to previous experiments, were a complete or partial deconstruction and dissolution of biomass was observed, particularly when pure ionic liquids were used.[51,52] It seems that the use of aqueous solutions of ionic liquids can avoid the lignocellulosic matrix dissolution while still allowing the extraction of target compounds, which from the perspective of the recovery of the target compounds, in this case oleanolic acid, is an advantage given the lower complexity of the resulting extract.

Conclusions

In this work, we investigated the extraction of the high-value oleanolic acid from olive tree leaves, an agricultural by-product from olive oil production, using aqueous solutions of surface-active ionic liquids. Initial studies on the solubility of oleanolic acid showed that the extremely low solubility of triterpenic acids in water can be drastically increased (up to 10^6 orders of magnitude) by the addition of surface-active ionic liquids, here confirmed with [C_{12}mim]Cl, pointing to an aggregation-mediated solubilization phenomenon. After the solubility tests, aqueous solutions of various ionic liquids with surfactant activity were investigated for the extraction of oleanolic acid from olive tree leaves. The results obtained clearly confirm that aqueous solutions of ionic liquids are suitable alternatives for solid-liquid extractions of triterpenic acids from biomass by-products. An increase in the ionic liquid concentration from 500 to 1000 mM and an increase in the side chain length of the surface-active ionic liquid to chain length, ≥C_{12}, resulted in higher extraction yields, although both effects lead to an increased viscosity of the aqueous solution not feasible to act as an adequate solvent. Variation of extraction parameters and ionic liquid structure allowed identifying optional extraction conditions. Under optimized conditions, namely 80°C for 2 h, and with MW irradiation for 30 min up to 2.5 wt% oleanolic acid could be extracted using a 500 mM solution of [C_{14}mim]Cl at 80°C. Eventually, this protocol provides an excellent strategy for the valorization of waste olive tree leaves prior to the burning for energy production, demonstrating that aqueous solutions of surface-active ionic liquids can be used as alternatives over conventional organic solvents.

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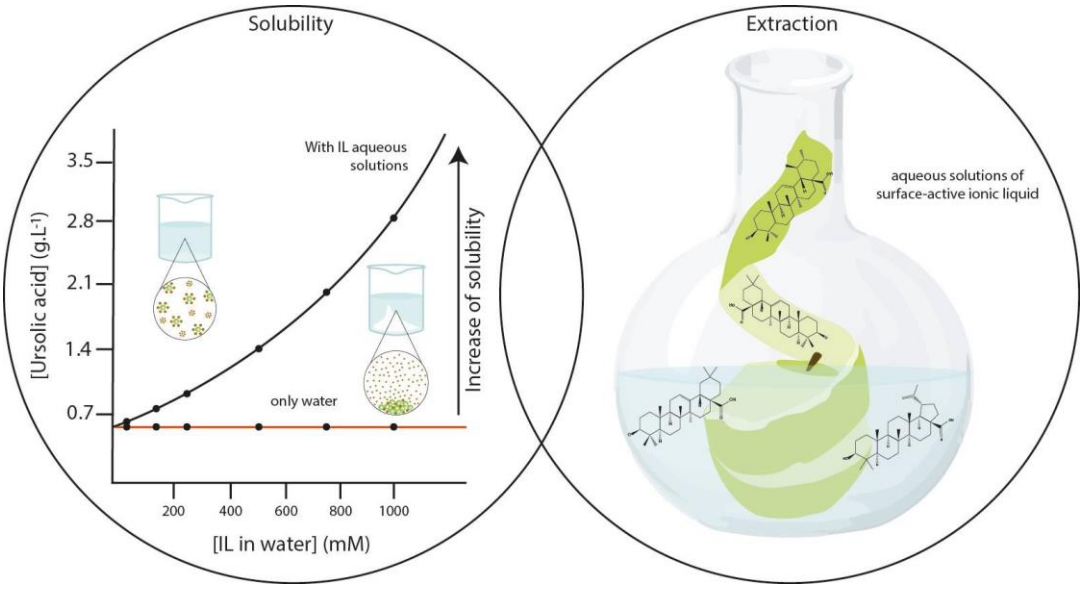
3. Aqueous Solutions of Surface-Active Ionic Liquids: Remarkable Alternative Solvents To Improve the Solubility of Triterpenic Acids and Their Extraction from Biomass

Chapter based on the published article:

E.L.P. Faria, S.V. Shabudin, A.F. Cláudio, M. Válega, F.M.J. Domingues, C.S.R. Freire, A.J.D. Silvestre and M.G. Freire. Aqueous solutions of surface-active ionic liquids: remarkable alternative solvents to improve the solubility of triterpenic acids and their extraction from biomass. *ACS Sustainable Chem. Eng.* **2017**, 5, pp. 7344-7351.

Contributions: A.J.D.S. and M.G.F. conceived and directed this work. E.L.P.F. and S.V.S. acquired the experimental data. E.L.P.F., A.F.C., F.M.J.D., A.J.D.S. and M.G.F. interpreted the experimental data. The manuscript was mainly written by E.L.P.F., A.J.D.S and M.G.F.



Abstract

Triterpenic acids (TTAs) are well known for their relevant biological properties and have been facing an increasing interest for nutraceutical and pharmaceutical applications. To overcome the concerns associated to the commonly used volatile organic solvents for their extraction from biomass, here we investigate the potential of aqueous solutions of ionic liquids (ILs) as alternative solvents. The solubility of ursolic acid (UA) was firstly determined in several aqueous solutions of ILs (hydrotropes or surface-active) at 30°C to appraise the dissolution phenomenon. Conventional surfactants were also investigated for comparison purposes. The collected data reveal a remarkable enhancement in the solubility of UA (8 orders of magnitude) in surface-active ILs aqueous solutions when compared to pure water. Afterwards, the potential of these ILs aqueous solutions was confirmed by their use in the extraction of TTAs from apple peels. Total extractions yield of TTAs of 2.62 wt.% were obtained using aqueous solutions of surface-

active ILs at moderate conditions, overwhelming the extraction yields of 2.48 wt.% obtained with chloroform and 1.37 wt.% with acetone using similar conditions.

Introduction

It is well known that there is a strong link between the consumption of fruits and vegetables and improved human health.[1,2] Some compounds present in fruits and vegetables have high potential to modulate many processes involved in the development of some diseases and degenerative disorders, including cancer,[3] cardiovascular disorders,[4] and diabetes.[5] Amongst the vast plethora of bioactive natural compounds with potential to improve human health, are flavonoids, phenolic acids, carotenoids, tocopherols, alkaloids, lignans, tannins, triterpenoids, among others.[6,7]

Triterpenoids are a vast class of C-30 terpenic compounds, which can be classified into different groups depending on their carbon backbone, including, for instance, lupane, oleanane and ursane-based compounds.[8] Triterpenoids are widely distributed in medicinal and edible plants,[9] and are part of the regular human diet due to their relevant health benefits.[10] In the past few years, there has been a growing trend on the incorporation of triterpenoids-rich extracts in new functional foods, cosmetics, healthcare products and drugs.[9-11] To turn possible the application of these novel products, it is however required to have abundant natural sources of triterpenoids, as well as safe and cost-effective extraction techniques. Agro-food industry by-products are an obvious resource to tackle this challenge, e.g. fruit peels which are rich in triterpenoids.[12] Apples are rich in triterpenoids, and particularly in triterpenic acids (TTAs), such as ursolic, oleanolic and betulinic acids **(Figure 3.1)**.[13] In general, the occurrence of ursolic acid in apple peels is well documented;[14] yet, there is a growing list of other triterpenoids that are also present and still need to be fully characterized.[10,15] According to the United Nations Food and Agriculture Organization (FAO) database (2013), the global fruit production in 2013 was of 610 million tons, among which apples comprised 81 million tons, i.e., 13% of the total fruit production.[16] Moreover, during the industrial processing of apples for the preparation of juices, jams, etc., large amounts of residues are generated, such as apple peels, being these a promising raw-material to obtain triterpenoids-rich extracts.

To obtain extracts rich in TTAs from apple peels, several extraction methods and solvents have been used, including extractions with ethanol (yields ranging between 0.01 and 1.3 wt.%),[17] with chloroform (yield of 0.7 wt.%),[18] and accelerated solvent extraction (ASE) with ethyl acetate (yields ranging between 0.2 and 2.1 wt.%).[9] All these studies have been carried out with volatile, and some hazardous, organic solvents. Taking into account the envisaged application of TTA-rich extracts in nutraceutical and pharmaceutical products, there is a crucial demand to use safer

and more biocompatible solvents, and to develop cost-effective extraction processes. Amongst the possible solvents, water appears as the greener and safer solvent overall; however, TTAs display negligible solubility in water.[19] Therefore, aqueous solutions of ionic liquids (ILs) can be envisioned as promising solvents if an increase in the solubility of TTAs and further extraction ability from biomass are verified.

ILs are organic salts with melting temperatures below 100°C, typically composed of a large organic cation and an inorganic or organic anion.[20] Their chemical nature is responsible for a number of unique properties, such as negligible volatility and nonflammability at ambient conditions, and high thermal and chemical stabilities.[21,22] Furthermore, physicochemical properties of ILs can be modulated by an adequate selection and combination of their ions, allowing the design of ILs with target properties.[20,23] Due to these features, ILs display a good solvation capacity for a wide range of solutes and are well known as potential substitutes of conventional organic solvents for the extraction of bioactive compounds from biomass.[24,25] Despite the potential of pure ILs in the extraction of bioactive components from biomass **i**t was recently demonstrated that ILs aqueous solutions display a tremendous potential in this domain, in which they can act either as hydrotropes or as surface-active agents, by promoting an increase in the solubility of bioactive compounds in aqueous media and by favouring their extraction from raw materials.[26-28] In addition, the use of ILs aqueous solutions leads to foremost advantages since the IL consumption is reduced, and the viscosity of the extraction solvent is decreased, leading to enhancements in mass transfer phenomena and to a decrease in the energy consumption.[25] Furthermore, ILs aqueous solutions also shown to be advantageous since they are more selective to target compounds while avoiding the dissolution of the biomass lignocellulosic part.[25]

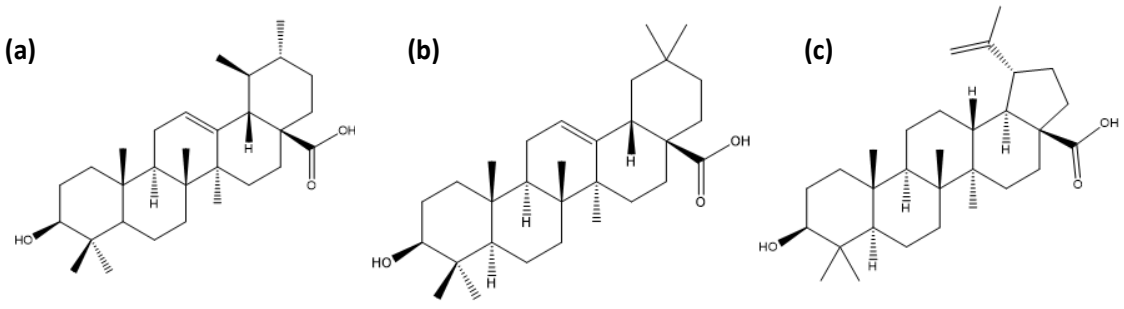


Fig. 3.1. Chemical structure of some triterpenic acids presents in apple peels: (a) ursolic, (b) oleanolic and (c) betulinic acids.

TTAs (**Figure 3.1**) are aliphatic polycyclic structures with a low solubility in water.[19] Aiming at finding alternative water-rich solvents, in this work, we investigated the potential of ILs aqueous solutions to increase the solubility of TTAs, using ursolic acid (UA) as a representative compound of this class, and further applied the most promising IL aqueous solutions in the extraction of TTAs (ursolic, oleanolic and betulinic acids) from green apple peels. For comparative purposes, the solubility and extraction of TTAs using common surfactants aqueous solutions and organic solvents, respectively, were also addressed. To the best of our knowledge, no attempts have been previously reported in the literature on the use of ILs aqueous solutions for improving the solubility and extraction of TTAs from biomass.

Materials and methods

Materials

To infer the molecular structure characteristics which enhance the solubility of TTAs in aqueous media, a large array of ILs was investigated (chemical structures shown in **Figure 3.2**): 1-ethyl-3-methylimidazolium acetate, $[C_2C_1im][CH_3CO_2]$; 1-butyl-3-methylimidazolium ethylsulphate, $[C_4C_1im][C_2H_5SO_4]$; 1-butyl-3-methylimidazolium octylsulphate, $[C_4C_1im][C_8H_{17}SO_4]$; 1-butyl-3-methylimidazolium chloride, $[C_4C_1im]Cl$; 1-butyl-3-methylimidazolium dicyanamide, $[C_4C_1im][N(CN)_2]$; 1-butyl-3-methylimidazolium thiocyanate, $[C_4C_1im][SCN]$; 1-butyl-3-methylimidazolium methylsulfate, $[C_4C_1im][CH_3SO_4]$; 1-butyl-3-methylimidazolium tosylate, $[C_4C_1im][TOS]$; 1-butyl-1-methylpyrrolidinium chloride, $[C_4C_1pyr]Cl$; 1-butyl-1-methylpyridinium dicyanamide $[C_4C_1py][N(CN)_2]$, 1-methyl-3-octylimidazolium chloride, $[C_8C_1im]Cl$; 1-decyl-3-methylimidazolium chloride, $[C_{10}C_1im]Cl$; 1-dodecyl-3-methylimidazolium chloride, $[C_{12}C_1im]Cl$; 1-tetradecyl-3-methylimidazolium chloride, $[C_{14}C_1im]Cl$; 1-hexadecyl-3-methylimidazolium chloride, $[C_{16}C_1im]Cl$; 1-octadecyl-3-methylimidazolium chloride, $[C_{18}C_1im]Cl$; and tributyltetradecylphosphonium chloride, $[P_{444,14}]Cl$. The imidazolium-, pyridinium-, and pyrrolidinium-based ILs were purchased from Iolitec. The phosphonium-based IL was kindly offered by Cytec Industries Inc. All ILs used have a purity higher than 98 wt.%, according to the information provided by suppliers.

In addition to ILs, conventional surfactants were also studied for comparison purposes (chemical structures depicted in **Figure 3.2**), namely sodium dodecylsulphate (SDS) from Alfa Aesar, sodium dodecylbenzenesulfonate (SDBS) from Sigma Aldrich, and hexadecyltrimethylammonium bromide (CTAB) from Fluka. All conventional surfactants have a purity higher than 99 wt.%.

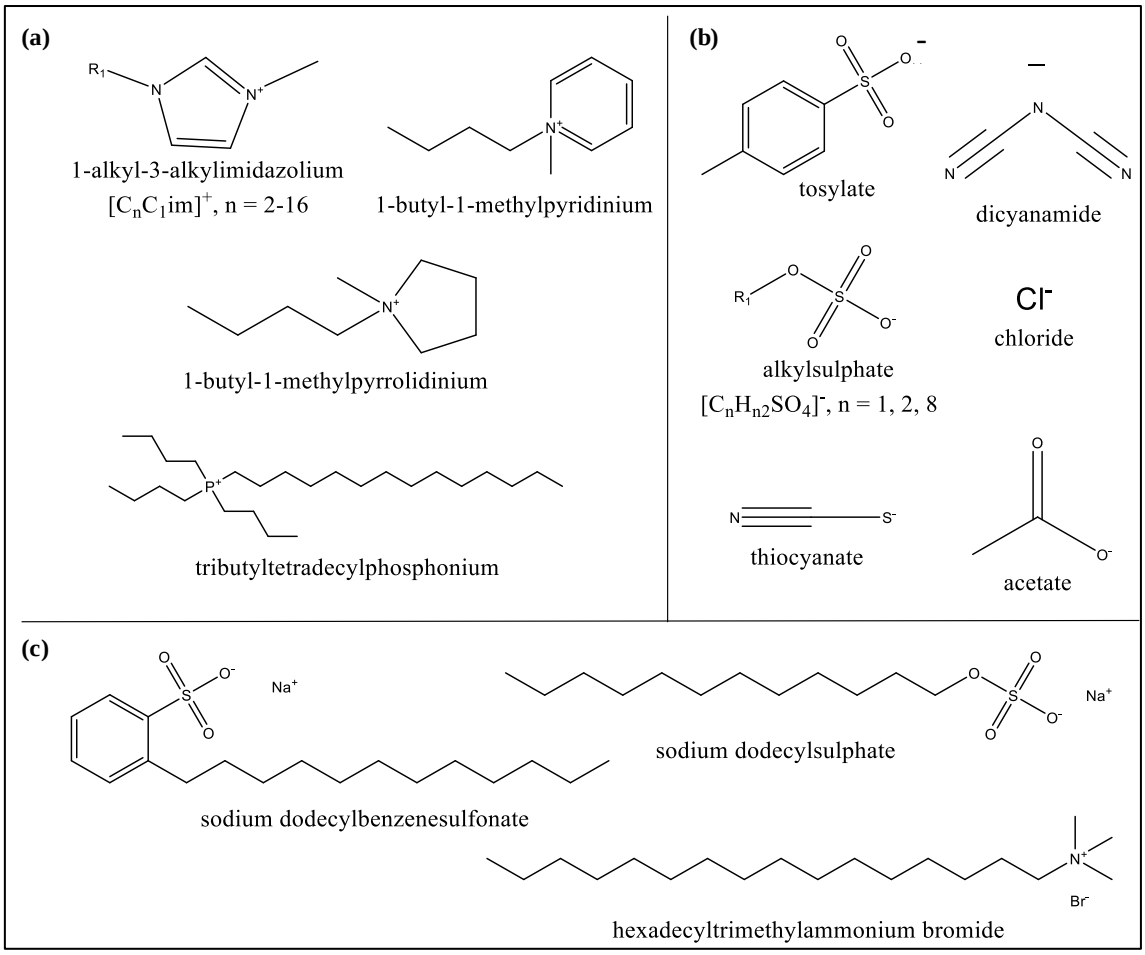


Fig. 3.2. Chemical structures of (a) cations and (b) anions composing the ILs and (c) conventional surfactants used.

Ursolic acid (UA), oleanolic acid (OA) and betulinic acid (BA) standards, with a purity higher than 98 wt.%, were acquired from Sigma. The solvents used for the extraction of TTAs in addition to aqueous solutions of ILs and surfactants, included distilled water, acetone and chloroform (purity ≥ 99.99 wt.%) from VWR chemicals. The mobile phase used in the HPLC analysis was composed of methanol (purity ≥ 99.99 wt.%) from VWR Chemicals, and ultra-pure water (purity ≥ 99.99 wt.%) from Merck, both HPLC grade.

Solubility of ursolic acid in aqueous solutions of ILs

While there is a large diversity of apple cultivars available for consumption and, consequently, a broad variation in their bioactive compounds composition,[12] ursolic acid (UA) is the most well documented triterpenic acid present in apple peels.[14] Thus, we have chosen UA as a major representative of the TTAs class for carrying out screening studies of solubility in aqueous solutions of ILs, as well as in aqueous solutions of conventional surfactants for comparison purposes.

Pure UA (solid) was added in excess amounts to aqueous solutions of ILs and conventional surfactants of different concentrations (50, 250, 500, 750 and 1000 mM). Samples were kept under constant agitation using an Eppendorf Thermomixer Comfort equipment at (30±0.5°C). Previously optimized equilibration conditions were established as follows: stirring velocity of 750 rpm, and equilibration time of at least 72 h. At least two independent samples were prepared to determine the average solubility value and respective standard deviation. After saturation of the aqueous solutions, and always assuring the presence of a solid phase and thus of UA in excess, a 200 µL aliquot was taken, diluted with 800 µL of methanol, carefully filtered using a 0.20 µm syringe filter, and subsequently quantified using a GILSON HPLC unit coupled to an oven and with manual injector, using a previously established calibration curve ($R^2 > 0.9990$). Data acquisition and evaluation were performed using the Jasco-Borwin 1.21 software. An analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 µm C18 100 A, from Phenomenex, was used. The mobile phase consisted of 87 (v/v) % of methanol, 13 (v/v) % of water + 0.1 (v/v) % of trifluoroacetic acid (TFA). Separations were conducted in isocratic mode, at a flow rate of 1 $mL.min^{-1}$ and using an injection volume of 10 µL. The wavelength was set at 210 nm. Each sample was analysed at least two times. The column oven and the autosampler were operated at 30°C.

The pH of ILs and surfactants aqueous solutions was determined using a pH meter (Digimed, model DM21), previously calibrated with buffer solutions (pH 7.0 and pH 4.0, Reagent QM) (Appendix – Fig. S3.1).

Due to the amphiphilic character of the ILs studied, and aiming at better understanding the role played by surface-active ILs in the solubility enhancement, the critical micellar concentration (CMC) of the studied ILs was determined by electric conductivity.[29] The conductivity of several aqueous solutions of different concentrations of IL was determined using a Russel RL105 Conductivity Meter at 25°C, by continuous dilution of an IL concentrated solution in water. Each conductivity value was recorded when its fluctuation was less than 1% within 2 min.

Extraction of TTAs from apple peels using aqueous solutions of ILs

Apple peels from Portuguese origin Golden apples were manually removed. The apples peels were dried at 25°C for 2 days, and then their grinding with a commercial coffee grinder was carried out. The samples of ground apple peels were further divided and classified according to the particle size by means of stainless-steel sieves. Samples with a diameter smaller than 1 mm were used.

Weighted amounts (with an uncertainty of 10^{-4} g) of ground apple peels were added to aqueous solutions of ILs with a concentration selected from the UA solubility studies, at a fixed solid-liquid ratio ($R_{S/L}$, weight of dried biomass *per* weight of the IL aqueous solution) of 0.1. The extractions were carried out at different temperatures (25, 50, 80 and 90°C, within $\pm$ 0.5°C), a fixed extraction time (60 minutes) and constant stirring (1000 rpm), in order to appraise the effect of temperature on the extraction yield. A similar procedure was applied in the extractions with conventional organic solvents, in which the IL aqueous solution was replaced by organic solvents, however under reflux due to the lower boiling temperature of organic solvents. At least three independent extractions were carried out for each condition and solvent.

After the extraction step, the overall solution and extract were centrifuged, and the supernatant filtered using a 0.20 µm syringe filter. A 200 µL aliquot was taken, mixed with 800 µL of methanol, filtered over a 0.2 µm syringe filter, and the TTAs content determined by HPLC-DAD at 210 nm. Three major TTAs have been identified in the extracts, namely oleanolic acid (OA), betulinic acid (BA), and ursolic acid (UA), according to the respective standards and retention time values. Previous calibration curves have been established for each TTA (R^2 > 0.9992, 0.9995, 0.9990, for oleanolic, betulinic and ursolic acids, respectively). HPLC-DAD (Shimadzu, model PROMINENCE) analyses were performed using the same column and conditions described previously in the solubility tests. The TTAs extraction yield is expressed as the percentage ratio between the weight of TTAs and the total weight of the dried biomass.

Results and discussion

Solubility of ursolic acid in aqueous solutions of ILs

TTAs **(Figure 3.1)** are almost insoluble in water,[19] and their extraction from biomass is usually carried out with volatile organic solvents.[9,17,18] Aiming at improving the TTAs solubility in aqueous-rich media and to favor their extraction from biomass, we first investigated the potential of ILs aqueous solutions to increase the solubility of TTAs, using ursolic acid (UA) as a representative compound of this class since it is the most well documented triterpenic acid present in apple peels.[14] The solubility of ursolic acid was determined in several aqueous solutions of ILs, as well as aqueous solutions of conventional surfactants for comparison purposes, at concentrations of 50, 150, 250, 500, 750 and 1000 mM.

Although pure ILs have been described as potential solvents for the extraction of value-added compounds from biomass, e.g. betulin from birch bark,[30] aqueous solutions of ILs also display a high potential and additional advantages since they imply lower amounts of IL as solvent,[25] with

additional benefits in terms of solvent toxicity and cost, and reduce the overall viscosity of the extraction media, thus enhancing the mass transfer phenomena and reducing energy consumptions. In summary, whenever possible, ILs aqueous solutions should be the preferred choice.[25] When dealing with compounds with a low solubility in water, such as TTAs, two classes of ILs can be selected to improve their solubility and extraction in aqueous media: ILs that act as hydrotropes[26] or as surfactants.[28] Based on this possibility, both classes of ILs were studied to infer the main IL structural characteristics which rule the solubility and extraction of TTAs. It should be remarked that it was not possible to determine the solubility of ursolic acid in pure water, as found to be below the detection limit of the analytical method used. Still, the water solubility of ursolic acid reported in the literature[19] is 1.02×10^{-7} g.L^{-1}, used as a reference in this work.

In a first approach, ILs that behave as hydrotropes[26,31] were selected, namely [C_4C_1im][$N(CN)_2$], [C_4C_1im][TOS], [C_4C_1im][SCN], [C_4C_1im][$C_2H_5SO_4$], [C_4C_1py][$N(CN)_2$] and [C_4C_1pyrr]Cl, and tested in aqueous solutions in concentrations ranging from 50-1000 mM at 30°C to dissolve ursolic acid. These ILs comprise a butyl chain as the longest alkyl chain at the cation, and do not present a critical micellar concentration (CMC) nor are surface-active. With these ILs, and at different concentrations, the target TTA was not detected by HPLC analysis in any of the aqueous solutions, meaning that the solubility of UA is below the detection limit of the analytical equipment and method used (0.002 g.L^{-1}, as determined by us). Therefore, hydrotropy does not play a significant role in enhancing the solubility of TTAs in aqueous media. Aqueous solutions of hydrotrope ILs seem thus more valuable for enhancing the solubility of moderately hydrophobic compounds, such as phenolic acids, and as previously reported.[26]

Subsequently, surface-active ILs, both cationic and anionic, as well as composed of different cations and anions, were investigated. In particular, different ILs constituted by long alkyl side chains with known surface-active characteristics[28,32] have been studied ([$C_nC_1$im]X with n = 8, 10, 12, 14, 16 and 18 and X = Cl and [$C_8H_{17}SO_4$], and [$P_{444,14}$]Cl). **Figure 3.3** shows the solubility data of UA at 30°C in the different surface-active ILs aqueous solutions in a 50-1000 mM concentration range; detailed data are provided in the Appendix (Table S3.1). It should be remarked that some ILs were exploited up to lower concentrations due to the high viscosity obtained with more concentrated solutions and difficulties encountered in their handling for subsequent quantification. Based on the amphiphilic character of the studied ILs, we also determined their CMC values by conductivity, aiming a better understanding of the role played by the IL in what regards the dissolution mechanism which enhances the solubility of ursolic acid in aqueous media. These results are reported in the Appendix (Table S3.3).

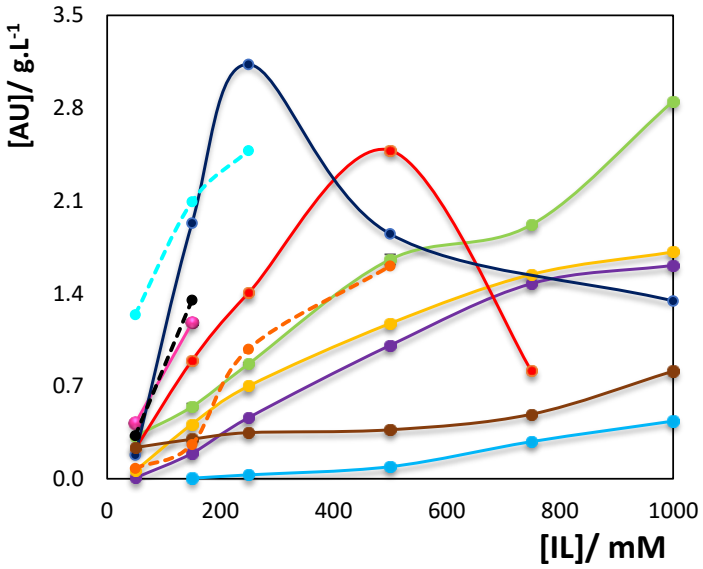


Fig. 3.3. Solubility of UA in aqueous solutions of surface-active ionic liquids and conventional surfactants at different concentrations at 30°C: (●) $[C_8C_1im]Cl$, (●) $[P_{444,14}]Cl$, (●) $[C_{10}C_1im]Cl$, (●) $[C_{12}C_1im]Cl$, (●) $[C_{14}C_1im]Cl$, (●) $[C_{16}C_1im]Cl$, (●) $[C_{18}C_1im]Cl$, (●) $[C_4C_1im][C_8H_{17}SO_4]$, (– –) SDS, (– –) SDBS and (– –) CTAB.

In general, the addition of all the investigated surface-active ILs leads to an increase in the solubility of ursolic acid in aqueous solutions. The pH of almost all ILs aqueous solutions is below the pKa of UA (pKa = 4.90),[33] meaning that UA is being solubilized in its protonated or neutral form. Thus, the gathered solubility results are a main result of the IL chemical structure and respective CMC and not a direct result of the solution pH. The pH detailed data combined with the solubility results are presented in the Appendix (Fig. S3.1).

For most ILs it was found a monotonous increase in the solubility of the TTA along the IL concentration, whereas for $[C_{16}C_1im]Cl$ and $[C_4C_1im][C_8H_{17}SO_4]$, a maximum in the solubility was observed, occurring at 500 and 250 mM, respectively. This behavior is analogous to that observed with hydrotrope-based ILs, although for these the maximum in solubility is observed at higher IL concentrations.[26] As also shown in **Figure 3.3**, an increase in the IL cation alkyl side chain leads to an increased capacity to solubilise UA in aqueous media. It is well known that an increase in the IL alkyl side chain decreases the CMC and promotes the IL aggregation[32] as shown in the Appendix (Table S3.3) with the CMC values determined in this work and respective comparison with literature values – supporting therefore the higher capacity of ILs comprising longer alkyl side chains to solubilize UA. For instance, at 500 mM of IL, the solubility of UA increases in the following order: $[C_{16}C_1im]Cl$ (2.48 $g.L^{-1}$) > $[C_4C_1im][C_8H_{17}SO_4]$ (1.85 $g.L^{-1}$) > $[C_{14}C_1im]Cl$ (1.66 $g.L^{-1}$) > $[C_{12}C_1im]Cl$ (1.17 $g.L^{-1}$) > $[C_{10}C_1im]Cl$ (1.01 $g.L^{-1}$) > $[P_{444,14}]Cl$ (0.37 $g.L^{-1}$) > $[C_8C_1im]Cl$ (0.09 $g.L^{-1}$). This trend follows the CMC values of all ILs investigated. However, an

exception occurs with $[C_4C_1im][C_8H_{17}SO_4]$, since it presents a CMC (43.3 mM) between those displayed by $[C_{10}C_1im]Cl$ (58.7 mM) and $[C_{12}C_1im]Cl$ (15.2 mM), meaning that anionic surfactants may be promising options for enhancing the solubility of UA in aqueous solutions. Although a maximum solubility of ursolic acid would be expected with $[C_{18}C_1im]Cl$, as shown in **Figure 3.3** for lower IL concentrations and according to its lower CMC, only a maximum concentration of 150 mM was used due to the high viscosity of $[C_{18}C_1im]Cl$ aqueous solutions and difficulties in handling such solutions for further quantification. Similar viscosity problems have been described by Ressmann et al.[28] with long alkyl chain ILs for the extraction of piperine from biomass.

When comparing the solubility of UA achieved using an imidazolium-based ($[C_{14}C_1im]Cl$ (1.66 $g.L^{-1}$)) with a phosphonium-based ($[P_{444,14}]Cl$ (0.37 $g.L^{-1}$)) IL at 500 mM, it is clear that the presence of an aromatic ring in the structure of the IL cation is a relevant factor to increase the solubility of ursolic acid. According to these results, the role of the aromatic ring may be highly relevant not only to increase the solubility of UA in aqueous solutions, as demonstrated in **Figure 3.3**, but possibly also will influence the extraction process of TTAs.

Since a significant increase in the solubility of UA in surface-active ILs aqueous solutions was observed, in order to truly confirm the potential of ILs we further compared the results obtained with those gathered with aqueous solutions of conventional surfactants, such as sodium dodecylsulphate (SDS), sodium dodecylbenzenesulfonate (SDBS) and hexadecyltrimethylammonium bromide (CTAB). The results obtained are depicted in **Figure 3.3**. Although conventional surfactants aqueous solutions may lead to some competitive solubility data, these are limited by the lower solubility of conventional surfactants in water (if compared with ILs), and thus are more restricted in their ability to enhance the solubility of UA in aqueous media. A similar trend is expected with non-ionic surfactants, and for this reason this type of surfactants was not investigated in this work. Taking into account the surfactants molecular structures **(Figure 3.1)** it is evident the relevant role of anionic surfactants, which is in agreement with the high performance discussed above with the IL $[C_4C_1im][C_8H_{17}SO_4]$. Moreover, the presence of aromatic rings also appears as a relevant factor toward the enhancement of the solubility of UA in aqueous solutions. Overall, the obtained results emphasize the potential of ILs aqueous solutions to solubilize poorly-water soluble compounds, such as TTAs.

Maximum solubility values of UA of 2.48 and 3.13 $g.L^{-1}$, respectively, have been obtained with the two best ILs identified, namely $[C_{16}C_1im]Cl$ and $[C_4C_1im][C_8H_{17}SO_4]$, that if compared with the solubility of the target compound in pure water (1.02×10^{-7} $g.L^{-1}$),[19] represents an increase in the solubility of UA of 8 orders of magnitude. An increase in the solubility of UA up to 5 orders of magnitude has been obtained with volatile organic solvents, such as tetrahydrofuran,

cyclohexane and ethyl acetate[34] and methanol, ethanol and 2-propanol.[35] This remarkable enhancement in solubility discloses the high potential of ILs aqueous solutions as alternative solvents for the extraction of TTAs from biomass, as shown below.

This remarkable increment in the solubility of UA along the IL concentration (up to 8 orders of magnitude) can be also tackled as a way of recovering the target solutes from the IL-water solvent, by a simple addition of water as an anti-solvent. To test this hypothesis we prepared and aqueous solutions of $[C_4C_1im][C_8H_{17}SO_4]$ at 250 mM containing 2.5 mg/mL of UA. At room temperature (ca. 25°C) we added water under constant agitation to reach a concentration of IL down to 50 mM. During the addition of water, it was macroscopically visible the precipitation of UA. This precipitate was recovered by filtration and washed several times with water at room temperature, aiming at removing any traces of IL present. The precipitate was then dried up to constant weight at 50°C, allowing the recovery of 89% of the initial UA added to the system. The IL can be further recovered by an evaporation step to remove the excess of water and thus recycled and reused. Despite the good performance of ILs to solubilize and extract value-added compounds from biomass, the isolation/purification of the target compounds from the IL-rich medium remains a challenge, mainly because of the inability to apply a simple solvent evaporation step due to the non-volatile nature of the most studied aprotic ILs. Aiming at overcoming this drawback, some strategies have been proposed, including back-extractions with organic solvents, precipitation with anti-solvents, and use of macroporous and ion-exchange resins.[24,25] Herein, taking advantage of the remarkable solubility dependence of TTAs along the IL concentration, water can be used as an appropriate anti-solvent, therefore making use of the greenest anti-solvent overall.

Extraction of triterpenic acids from green apple peels

After the previous screening on the ILs chemical features to enhance the solubility of TTAs in aqueous solutions, we selected $[C_{14}C_1im]Cl$, at 500 mM, to be used as an extraction solvent of TTAs from green apple peels. This IL and concentration were chosen since good solubility data for TTAs have been obtained (well above of what could be extracted from biomass taking into account the apple peels composition)[13] and to work with ILs aqueous solutions of lower viscosity – as previously discussed an increase in the cation alkyl side chain length leads to aqueous solutions of high viscosity. Moreover, it is generally accepted that the decrease on the extraction yields from biomass observed at higher IL concentrations mainly result from the increased solution viscosity which hinders an efficient solvent penetration into the plant tissues.[25,28]

The extraction of TTAs from apple peels was carried out at 25, 50, 80 and 90°C, while keeping the other operational conditions constant, namely a biomass-solvent ratio of 1:10, an IL

concentration of 500 mM and 60 min of extraction time. Three TTAs have been identified by HPLC-DAD, namely ursolic, oleanolic and betulinic acids, according to a wide range of standards tested. The extraction yields of the three identified TTAs at the studied temperatures are shown in **Figure 3.4**.

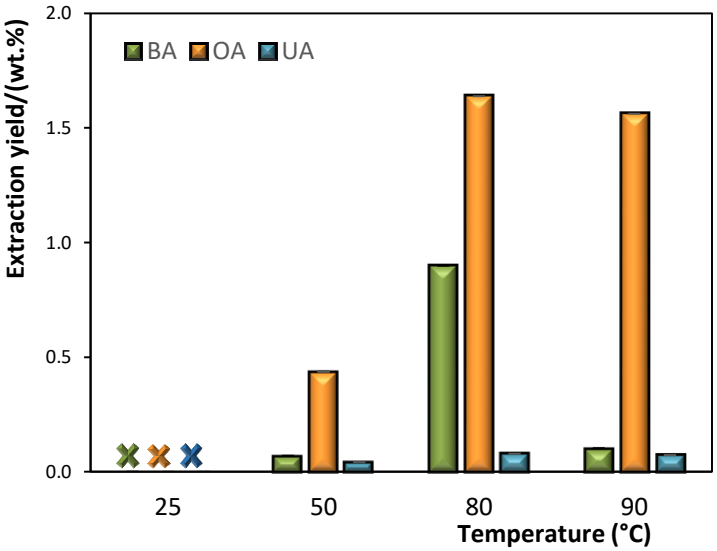


Fig. 3.4. Extraction yields of TTAs (BA (green), OA (yellow) and UA (blue)) from green apple peels with $[C_{14}C_1im]Cl$ at different temperatures and other fixed conditions ([IL] = 500 mM, t = 60 min, S/L ratio = 1:10).

Negligible values have been obtained at 25°C (below the analytical equipment detection limit), increasing from 50°C to 80°C, followed by a decrease at 90°C. Therefore, temperature strongly influences the extraction yield of TTAs from biomass. Maximum extraction yields for the three identified TTAs have been obtained at 80°C, namely 0.079 wt.% for UA, 0.90 wt.% for BA and 1.64 wt.% for OA.

The increase in the extraction yield observed from 25 to 80°C may result both from a solubility increment and from the solvent viscosity decrease at higher temperatures, thereby increasing both the solution and the target compounds diffusion. The decrease in the extraction yields observed at higher temperatures may result from the co-dissolution of plant polysaccharides that turns the aqueous media into a gel, turning the recovery of TTAs highly difficult, as discussed in other works.[36,37] Based on the results obtained, and amongst the temperatures range investigated, 80°C is the best temperature for the extraction of TTAs from apple peels using $[C_{14}C_1im]Cl$ aqueous solutions at 500 mM.

The TTAs extraction yield using $[C_{14}C_1im]Cl$ aqueous solutions was then compared with common organic solvents well described in the literature for the extraction of TTAs from

biomass,[18,38] such as acetone and chloroform, under reflux at 80°C. The results obtained are shown in **Figure 3.5**.

$[C_{14}C_1im]Cl$ aqueous solutions are particularly more relevant than the studied volatile and hazardous organic solvents for the extraction of OA. When comparing the total amount of extracted TTAs while envisaging the preparation of TTAs-rich extracts for incorporation in new functional foods, cosmetics, healthcare products and drugs, $[C_{14}C_1im]Cl$ aqueous solutions appear as the most promising solvents, with a total extraction yield of TTAs of 2.62 wt.%, when compared with 2.48 wt.% obtained with chloroform and 1.37 wt.% with acetone under similar conditions.

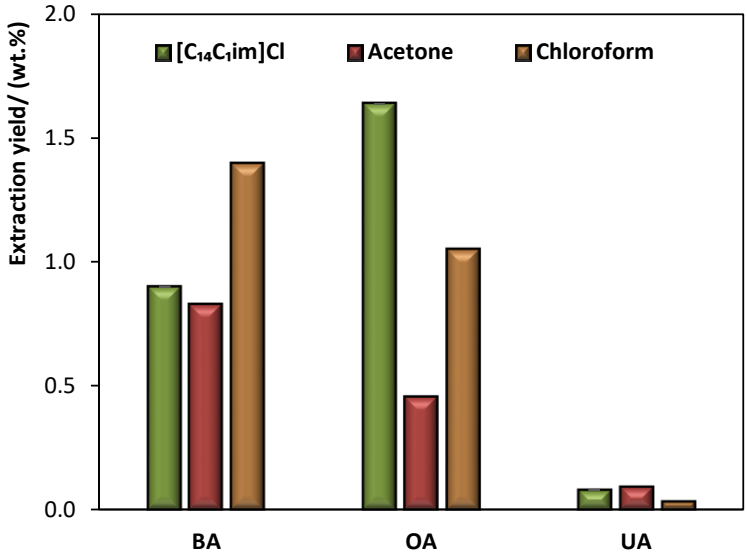


Fig. 3.5. Extraction yields of TTAs (BA, OA and UA) from green apple peel with several solvents at fixed conditions (T = 80°C, [IL] = 500 mM, t = 60 min, S/L ratio = 1:10; organic solvents were used as pure and not as aqueous solutions).

The interest on TTAs has increased over the past few years, as well as the number of extraction studies of these target compounds from a broad range of biomass sources (apple,[15] grape,[36] tomato,[37] olive[38]), while using several extraction methods (microwave,[39] maceration,[40] solid-liquid[36] and supersonication[41]) and different solvents (ethyl acetate,[36] n-hexane,[15] and ethanol[17]). The results obtained in this work have been obtained with aqueous solutions of ILs, instead of the commonly used volatile organic solvents, under moderate temperatures and without using energy-intensive methods. Extraction processes based on aqueous solutions of ILs are thus potential platforms for enhancing the solubility and extraction of triterpenic acids and other value-added compounds present in biomass, performing even better than the pure ILs commonly investigated in the literature,[25] while avoiding the dissolution of the lignocellulosic

fraction and allowing the recovery of richer value-added extracts. Moreover, based on the solubility trends of TTAs along the IL concentration and the previous demonstrated induced-precipitation approach by using water as anti-solvent, it is envisioned the possibility of applying the same procedure to recover TTAs-rich extracts from the IL-water solvent, and for which the IL can be recovered and reused after an evaporation step to remove the excess of water.

Conclusions

In the past years, there has been a growing trend on the incorporation of triterpenoids-rich extracts in new functional foods, cosmetics, healthcare products and drugs. To turn possible the application of these products, it is yet required to have abundant natural sources of triterpenoids, preferentially agro-food industry by-products, as well as to use safer and more cost-effective extraction techniques. Based on these requirements, in this work, we investigated the potential of aqueous solutions of ILs as alternative solvents over the commonly used volatile organic solvents for the extraction of TTAs from biomass. Aiming at identifying the most promising IL aqueous solutions for the extraction of TTAs, we first addressed a comprehensive study based on the solubility of ursolic acid in aqueous solutions of ILs, allowing us to better understand the dissolution phenomenon and the IL chemical structure features which enhance the TTAs solubility and further extraction yield. The collected data reveal that hydrotropy does not play a significant role in the improvement of solubility of TTAs in aqueous media. However, surface-active ILs allow a significant increase in the solubility of ursolic acid in aqueous solutions with an observed enhancement of 8 orders of magnitude when compared with its solubility in pure water. Based on the remarkable increase in the solubility of ursolic acid, a major representative of the TTAs class, aqueous solutions of surface-active ILs were then tested in the extraction of TTAs from apple peels, allowing the simultaneous extraction of betulinic, oleanolic and ursolic acids. A total extraction yield of TTAs of 2.62 wt.% was obtained using the best identified conditions, overwhelming the total extraction yields of 2.48 wt.% obtained with chloroform and 1.37 wt.% with acetone (under the same conditions and determined in this work for comparison purposes). The results obtained clearly confirm that aqueous solutions of ILs are an improved alternative for the extraction of TTAs from biomass, representing a promising alternative over the commonly used volatile organic solvents.

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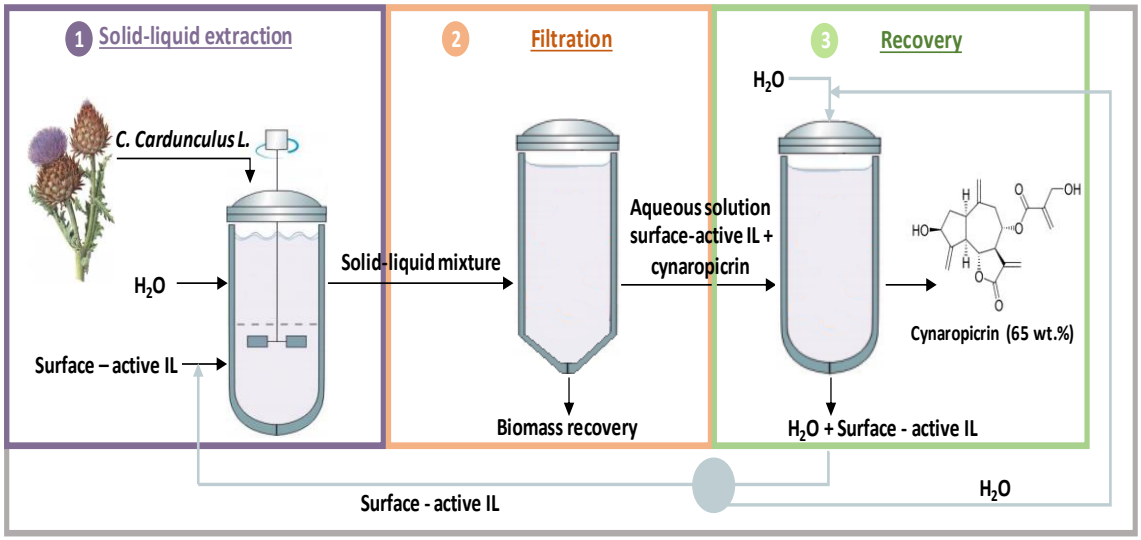
4. EXTRACTION AND RECOVERY PROCESSES FOR CYNAROPICRIN FROM *CYNARA CARDUNCULUS* L. USING AQUEOUS SOLUTIONS OF SURFACE-ACTIVE IONIC LIQUIDS

Chapter based on the published article:

E.L.P. Faria, M.V. Gomes, A.F. Cláudio, C.S.R. Freire, A.J.D. Silvestre and M.G. Freire. Extraction and recovery processes for cynaropicrin from *Cynara cardunculus L.* using aqueous solutions of surface-active ionic liquids. *Biophys. Rev.*, **2018**, 10, pp. 915-925.

Contributions: A.J.D.S. and M.G.F. conceived and directed this work. E.L.P.F. and M.V.G. acquired the experimental data. E.L.P.F., A.F.C, C.S.R.F, A.J.D.S. and M.G.F. interpreted the experimental data. The manuscript was mainly written by E.L.P.F., A.J.D.S and M.G.F. with contributions from the remaining authors.



Abstract

Due to the wide range of relevant biological activities and high commercial value of cynaropicrin, and aiming at developing cost-effective processes, aqueous solutions of ionic liquids (ILs) were investigated for the extraction and recovery of cynaropicrin from the leaves *of Cynara cardunculus L.* Both cationic (1-alkyl-3-methylimidazolium chloride) and anionic (cholinium carboxylate) surface-active ILs were investigated, as well as a wide range of conventional surfactants and molecular organic solvents, allowing us to conclude that aqueous solutions of cationic surface-active ILs display a better performance for the extraction of cynaropicrin. Operational conditions were optimized, leading to a cynaropicrin extraction yield of 3.73 wt%. The reusability of both the biomass and the solvent were further investigated to appraise the extraction media saturation and to achieve a higher cynaropicrin extraction yield (6.47 wt%). Finally, it was demonstrated that 65 wt% of the extracted cynaropicrin can be efficiently recovered by precipitation from the IL aqueous extract through the addition of water as anti-

solvent, allowing us to put forward both the extraction and recovery processes of the target value-added compound from biomass followed by solvent recycling. This approach opens the door to the development of more sustainable processes using aqueous solutions of ILs instead of the volatile organic solvents commonly used in biomass processing.

Introduction

Cynara cardunculus L. (Asteraceae) is a Mediterranean plant species comprising three varieties, namely var. *sylvestris* (L.) *Fiori* (wild cardoon), var. *scolymus* (L.) *Fiori* (globe artichoke), and var. *altilis* (DC) (cultivated cardoon).[1] Among these, cultivated cardoon has been largely explored due to its fleshy stems and leaf petioles. This variety is used in regional dishes in European countries,[2] as a source of aspartic proteinases for milk clotting during ewe cheese manufacturing,[3] and used in traditional medicine.[4] In addition to these more conventional uses, several industrial applications involving cardoon have been also considered, namely in pulp and paper production,[5] power generation and heating,[6] among others.

C. cardunculus species are known for the bitter taste of their leaves, which is mainly due to the presence of guaianolide type sesquiterpenes lactones, among which cynaropicrin **(Figure 4.1)** is the most abundant and accounting for ~80% of the characteristic bitterness of the plant.[7] *C. cardunculus* extracts are of commercial interest due their hepatoprotective effect, and anti-inflammatory, antispasmodic, proapoptotic and antitrypanosomal properties.[8-11] Therefore, more fundamental investigations, particularly regarding the extraction and recovery of bioactive compounds (mainly cynaropicrin) from cardoon, have been explored envisaging their use in nutraceutical formulations.[7,12] Considering the biological activity of cynaropicrin, together with its abundance in *C. cardunculus* leaves (up to 87 g/kg)[7] and its high current commercial value, it is of utmost interest to develop more effective, environmentally-friendly and economically viable extraction and recovery methods. Currently, cynaropicrin is mainly extracted from different types of biomass using volatile and often toxic organic solvents, such as chloroform[13] and dichloromethane,[7] leading to several human risks and safety issues and to a poor environmental performance. Other used methods include the extraction of cynaropicrin with water at elevated temperatures,[14] and with supercritical CO_2[15] or related mixtures making use of co-solvents[16] to improve the extraction yields. These methods have some disadvantages since they require several hours of extraction, they are energetic-consuming, may require the use of more sophisticated equipment and result in low cynaropicrin yields.[13-16] Therefore, in the past decade, a large interest has been devoted to the development of more cost-efficient and sustainable solvents and methods for the extraction of value-added compounds from biomass,

which apart from supercritical CO_2, comprise mainly ionic liquids (ILs) and, more recently, deep eutectic solvents (DES) and their natural counterparts (NADES).[17-19]

Within alternative solvents, ILs are amongst the most studied, particularly for the extraction of bioactive compounds from biomass, as summarized in recent reviews and books on the subject.[19-22] For instance, ILs have been used in the extraction of shikimic acid via dissolution of *Illicium verum*[23] or from *Ginkgo biloba* leaves,[24] in the isolation of tannins from *Acacia catechu* (catechu) and *Terminalia chebula* (myrobolan),[25] in the dissolution of suberin from *Quercus suber* (cork),[26] and in the extraction of artemisinin from *Artemisia annua.*[27,28] In addition to the extraction and dissolution studies, some attempts on the products recovery from the IL solution have also been carried out, by means of back-extraction steps using organic solvents, precipitation with anti-solvents, use of resins, among others.[19] A recent study focused on the use of more benign ILs that could be used together with the biomass rich-extracts, avoiding thus the need of the recovery step, has also been proposed.[29] In summary, given the proven efficiency of ILs as powerful solvents for the extraction of value-added compounds from several biomass sources[19,20] and since their unique properties can be tailored by adequate cation-anion combinations, ILs are "ideally" excellent alternatives to dissolve and/or extract target value-added compounds from specific natural resources.[19]

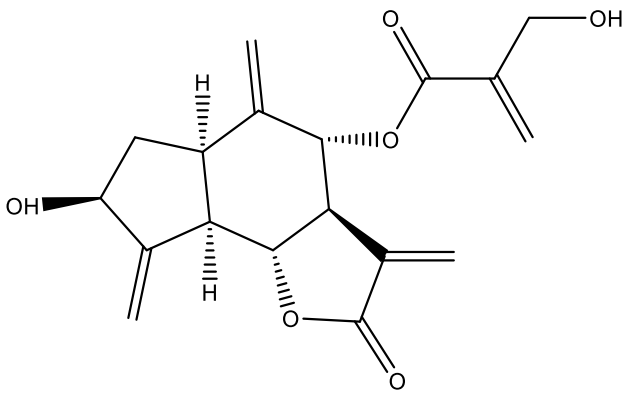


Fig. 4.1. Chemical structure of cynaropicrin.

In addition to pure ILs, recent studies demonstrated that their aqueous solutions or hydrated ILs are more efficient for the extraction of bioactive components from biomass, either by applying ILs with surface-active[30] or hydrotropic behavior.[31] The high efficiency achieved using hydrated ILs has been related with the enhanced solubility of biomolecules in aqueous solutions of ILs when compared with their solubility in the respective pure solvents.[19,20] The concept of hydrated-IL systems has been exploited to improve the biomolecules stability and biological activity, such as enzymes,[32,33] and as extraction media for target compounds from biomass, such

as in the extraction of caffeine from guaraná seeds,[31] in the extraction of piperine from white pepper,[34] in the extraction of 7-hydroxymatairesinol from *Norway spruce* knots,[29] among others.[19] More recently, it has been demonstrated that aqueous solutions of ILs can provide an impressive improvement in the solubility and further extraction of triterpenic acids from apples peel.[35] With aqueous solutions, there is a decrease on the ILs consumption (that are replaced by water – the greenest solvent overall) and on the solvent viscosity, thus enhancing the mass transfer and reducing the energy consumption, resulting in more low-cost and environmentally-friendly processes.[19]

Previous investigations demonstrated that some ILs can form micelles in aqueous solution,[36] being these known as surface-active ILs. This is the case of long-chain 1-alkyl-3-methyl-imidazolium chloride salts, [$C_n$mim]Cl with n = 8–18.[37] However, and despite their potential, only few studies explored the extraction of bioactive compounds from biomass using surface-active ILs, either pure or in aqueous media.[30,38-45] Based on this, we have chosen to investigate aqueous solutions of surface-active ILs for the extraction of cynaropicrin from the leaves of cultivated cardoon, *C. cardunculus L.*

Different families of ILs were evaluated, namely surface-active 1-alkyl-3-methyl-imidazolium chloride and cholinium carboxylate salts, allowing us to explore the potential of both cationic and anionic surfactants. Organic solvents and aqueous solutions of conventional surfactants were also investigated for comparative purposes. After identifying the most promising IL, the process variables for the solid–liquid extraction were optimized, namely the solid–liquid ratio (S/L ratio, weight of dried biomass *per* weight of solvent), the temperature (T), the time of extraction (t) and the concentration of the IL. Finally, based on the cynaropicrin solubility dependence on the IL concentration, its recovery was accomplished using water as an anti-solvent, allowing us to propose both the extraction and recovery processes.

Materials and methods

Materials

Two families of surface-active ILs have been investigated, namely 1-alkyl-3-methylimidazolium chloride (acting as cationic surfactants) and cholinium carboxylate (acting as anionic surfactants) salts, 1-octyl-3-methylimidazolium chloride, [C_8mim]Cl, 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl, 1-dodecyl-3-methylimidazolium chloride, [C_{12}mim]Cl, 1-tetradecyl-3-methylimidazolium chloride, [C_{14}mim]Cl, cholinium octanoate, [Ch][C_8CO_2], cholinium decanoate, [Ch][$C_{10}CO_2$], and cholinium dodecanoate [Ch][$C_{12}CO_2$]. In addition to these, 1-ethyl-

3-methylimidazolium chloride, [C_2mim]Cl, 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl, and 1-butyl-3-methylimidazolium dicyanamide, [C_4mim][$N(CN)_2$], were investigated to confirm the relevance of surface-active ILs and to address the possible potential of hydrotropic ILs[31] to extract the target compound. All imidazolium-based ILs were purchased from Iolitec (99% purity), while the cholinium-based ILs were synthesized by us according to well-known protocols.[46] The three cholinium-based carboxylates investigated were synthetized by the neutralization of [Ch]OH with octanoic, decanoic and dodecanoic acids. The reaction mixtures were stirred overnight at room temperature, under nitrogen atmosphere, and protected from light, resulting in the cholinium carboxylate and water as by-product. Unreacted acids were eliminated by washing steps with ethyl acetate. Ethyl acetate and water were then removed under reduced pressure at 60°C. Finally, the obtained ILs were dried under high vacuum for at least 48 h at 50°C. After this purification step, the purity of all ILs was confirmed by ^{1}H and ^{13}C NMR, shown to be ≥ 98 wt%.

In addition to ILs, aqueous solutions of conventional cationic, anionic and nonionic surfactants were used for comparison purposes, namely cetyltrimethylammonium bromide (CTAB), sodium dodecylsulfate (SDS), cetyltrimethylammonium chloride (CTAC), cetylpyridinium chloride (CPC), sodium dodecylbenzenesulfonate (SDBS), and Genapol C-100. All conventional surfactants were acquired from Fluka with a purity higher than 99.0 wt%. The chemical structures of the ILs and conventional surfactants investigated are shown in **Figure 4.2.**

Several organic solvents, namely acetone and dichloromethane, with a purity of ≥ 99.99 wt%, and *n*-hexane with a purity of 95 wt%, all acquired from Sigma-Aldrich, were also tested as extraction media.

Dried *C. cardunculus L.* leaves, packed under vacuum, with a granulometry between 40-60 mesh, were supplied by the Experimental Center of Agriculture School of the Polytechnic Institute of Beja, Southern Portugal. The cynaropicrin standard was purchased from Extrasynthesis, with a purity ≥ 97.5 wt%.

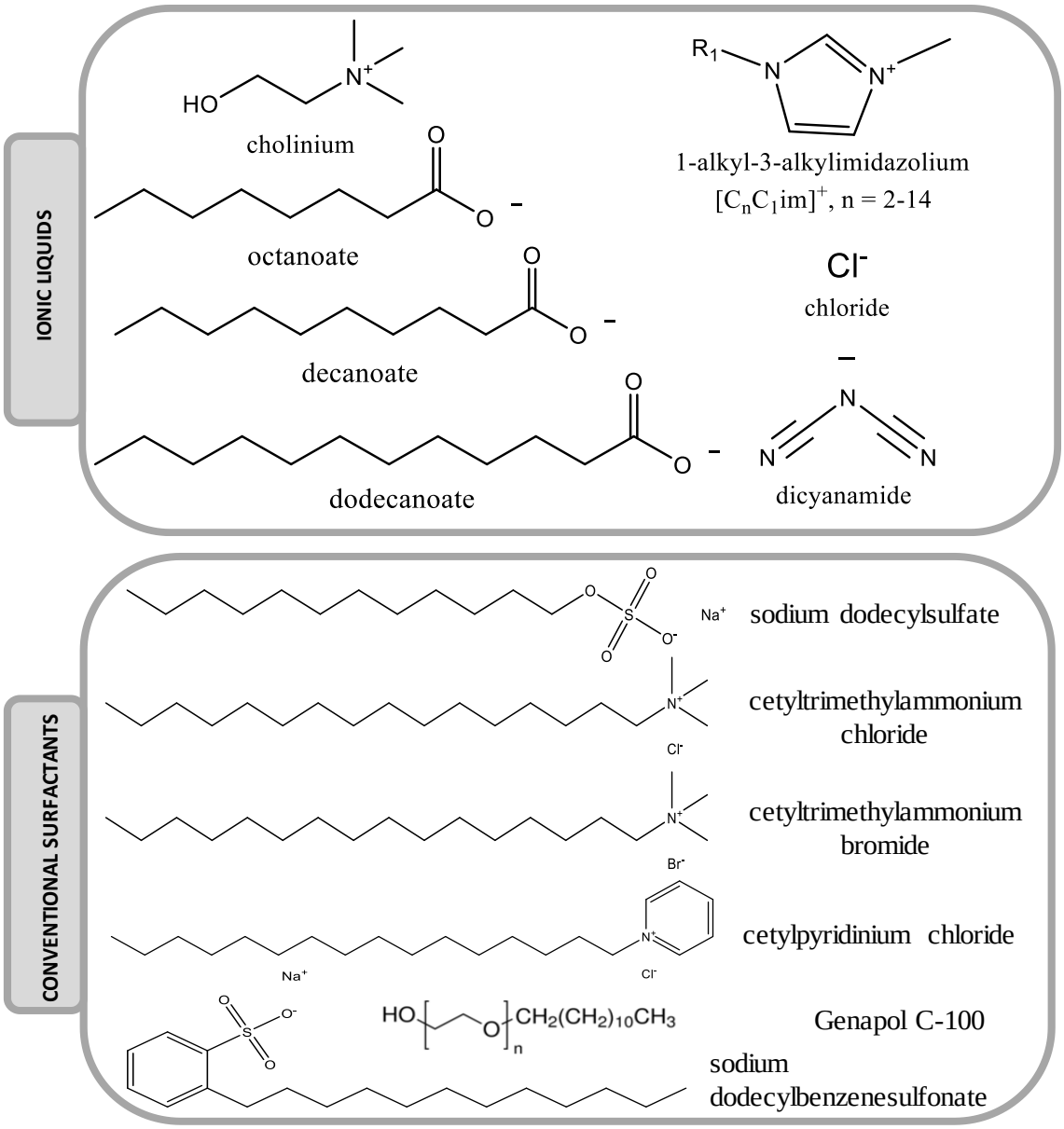


Fig. 4.2. Chemical structures of ionic liquids and conventional surfactants tested in the extraction of cynaropicrin.

Determination of critical micellar concentration (CMC)

Aiming at better understanding the role played by surface-active ILs in the extraction process, the critical micellar concentration (CMC) of the studied ILs, when unavailable in the literature,[37,47-51] was determined by electric conductivity.[52] The conductivity of several aqueous solutions of different concentrations of IL was determined using a Russel RL105 Conductivity Meter at 25°C, by continuous dilution of an IL concentrated solution in water. Each conductivity value was recorded when its fluctuation was less than 1% within 2 min.

Quantification, extraction and recovery of cynaropicrin

Solid-liquid mixtures were prepared with *C. cardunculus L.* leaves and aqueous solutions of ILs at different concentrations (100, 250, 500, 750, and 1000 mM) in closed glass vials. The mixtures were kept under controlled stirring and temperature (± 0.5°C). To optimize the extraction yield of cynaropicrin, different temperatures (25, 35 and 45°C), extraction time (30, 40, 50, 60, 120, 180, 300 and 1440 min) and solid-liquid (S/L) ratio (1:10, 1:20, 1:30 and 1:40, weight of dried biomass *per* weight of solvent, considering a fixed biomass weight of 0.25 mg) were investigated, while keeping constant the stirring speed (1000 rpm). A similar procedure was applied in the extraction using conventional organic solvents and conventional surfactants, in which the IL aqueous solutions were replaced by these solvents. A sample of *C. cardunculus* L. leaves (5 g, dried weight) was also Soxhlet extracted with dichloromethane (150 mL) for 7 h for comparison purposes.

After the extraction step, the mixtures were centrifuged, and the supernatant filtered using a 0.20 µm syringe filter. Before HPLC injection, a 200 µL aliquot was taken from the filtered supernatant, diluted with 800 µL of methanol, and filtered again. The identification and quantification of the extracted cynaropicrin was performed by HPLC-DAD (Shimadzu, model PROMINENCE) using an analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 µm C18 100 A from Phenomenex. The mobile phase consisted of 75% (v:v) of water and 25% (v:v) of acetonitrile. The separation was conducted in isocratic mode, at a flow rate of 0.5 $mL.min^{-1}$, using an injection volume of 10 µL. DAD was set at 198 nm. The column oven and the autosampler were operated at a constant temperature (30°C). The quantification of cynaropicrin was based on a calibration curve previously established under the same conditions, with a cynaropicrin concentration ranging between 4.98 $x10^{-3}$ and 4.98 mg/mL, R^2 = 0.9993. The cynaropicrin extraction yield is expressed as the percentage ratio between the total weight of extracted cynaropicrin and the total weight of the dried biomass. The quantitative results presented along the manuscript correspond to the average of at least three independent experiments.

To infer the maximum amount of cynaropicrin in the *C. cardunculus* L. leaves and on solvents saturation effects, six successive extraction cycles with the same biomass and new aqueous solutions of IL, and four successive cycles of extraction using the same solvent and fresh biomass, were carried out. After the four extraction cycles with the reuse of the same aqueous solution of IL, leading to the solvent saturation, the recovery of cynaropicrin by induced precipitation using water was evaluated as an anti-solvent. To this end, different amounts of water were added in order to decrease its solubility/saturation. 0.25 mL of the IL-water solution used in the

extraction experiments were diluted in 2.5 and 5 mL of distilled water. After the addition of water, the solution was centrifuged at 6000 rpm for 30 min, and vacuum filtered with a 0.45 µm microporous membrane. The solid residue was dried in the oven at 50°C for 2 days. A sample of the precipitated cynaropicrin was dissolved in methanol for identification and quantification by HPLC-DAD. The quantification of the residual cynaropicrin present in the IL aqueous solution after the dilution step was additionally determined.

Results and discussion

Effect of the ILs structural features and operational conditions

The remarkable potential of ILs as- solvents in biomass processing is mainly due to their tunable properties, enabling them to dissolve a wide variety of compounds of different hydrophobicity, as well as to swell and dissolve raw biomass which may lead to an improved access to the valuable ingredients embedded in biopolymer matrices.[19,21] In addition to the use of pure ILs, the use of aqueous solutions of ILs can avoid the cellulosic matrix dissolution while still allowing the extraction of target compounds,[23,53] e.g. cynaropicrin. Moreover, a higher selectivity is usually obtained with aqueous solutions of ILs, as well as improvements in the mass transfer phenomenon and energetic consumption due to a decrease on the overall solvent viscosity.[19]

Knowing that cynaropicrin has a hydrophobic character ($\log K_{ow}$ = 1.08) and is thus poorly water-soluble (774.6 mg/L at 25°C)[54] it is expected to achieve a good extraction performance using aqueous solutions of surface-active ILs. To confirm this hypothesis, we investigated the cynaropicrin extraction with aqueous solutions of both cationic, with [$C_n$mim]Cl ILs (*n* = 8-14), and anionic surface-active ILs, with [Ch][C_nCO_2] (*n* = 8-12). In the same line, i.e. to prove that surface-active ILs are really amongst the most relevant options and that aqueous solutions of ILs that act as hydrotropes[31] are not the most adequate solvents for cynaropicrin extraction, aqueous solutions of [C_2mim]Cl, [C_4mim]Cl and [C_4mim][$N(CN)_2$] were also investigated.

An initial screening with [C_4mim]Cl and [C_{14}mim]Cl at different concentrations (100, 500 and 1000 mM) was carried out in order to address the impact of using surface-active *versus* hydrotropic ILs and the most relevant concentrations which lead to high extraction yields of cynaropicrin – results shown in **Figure 4.3**. An increase in the concentration of [C_4mim]Cl, from 100 to 1000 mM, leads to an increase in the cynaropicrin extraction yield, from 0.83 to 1.19 wt%. On the other hand, with the surface-active [C_{14}mim]Cl, the extraction yield goes through a maximum (3.18 wt%), occurring at 500 mM of IL. However, a decrease in the yield above 500 mM of IL was observed, which might be due to an increased viscosity of the solvent at higher IL

concentrations, which is particularly more relevant for ILs with longer alkyl side chains, hindering thus mass transfer phenomena.

The described results allowed us to select the 500 mM concentration to screen the potential of different ILs in the extraction of cynaropicrin. The operational conditions used in the ILs screening were: S/L ratio of 1:10, extraction time of 60 min, and temperature of 25°C. The results obtained are shown in **Figure 4.3.** The detailed results are given in the Appendix (Table S4.1). As shown in **Figure 4.3**, higher extraction yields are obtained with aqueous solutions of surface-active ILs (mainly [C$_n$mim]Cl ILs, with *n* = 8-14) over those obtained with aqueous solutions of hydrotropic ILs ([C$_2$mim]Cl, [C$_4$mim]Cl and [C$_4$mim][N(CN)$_2$]). Although aqueous solutions of hydrotropic ILs have high potential to extract less hydrophobic compounds, such as alkaloids and phenolic acids,[31,55] when dealing with more hydrophobic compounds, such as cynaropicrin, aqueous solutions of surface-active ILs are known to exhibit a better extraction performance.[35]

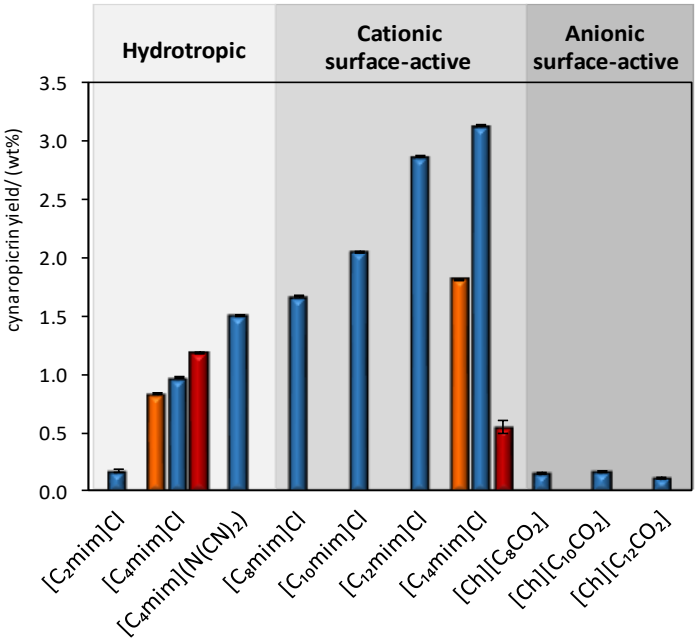


Fig. 4.3. Weight fraction percentage of cynaropicrin extracted from the leaves of *C. cardunculus L.* with several ILs at different concentration: 100 mM (orange), 500 mM (blue), 1000 mM (red) (S/L ratio = 1:10, T = 25°C and t = 60 min).

With surface-active cationic ILs ([C$_n$mim]Cl ILs, *n* = 8-14), there is an increase in the cynaropicrin extraction yield with the decrease of the ILs CMC.[30] Cationic ILs with longer alkyl side chains and lower CMC values are more effective solvents for the extraction of the target compound. On the other hand, the results obtained also show that anionic surface-active ILs ([Ch][C$_n$CO$_2$]), even with CMC values in the same order of magnitude as those of [C$_n$mim]Cl ILs, ranging from 4 to

383 mM, display the lowest cynaropicrin extraction performance (yield < 0.12 wt%). The CMC values of the surface-active ILs investigated were taken from the literature[37,47-51] and determined in this work when not available – *cf*. the Appendix with detailed data (Table S4.2).

In summary, these results clearly demonstrate the higher capability of cationic surface-active ILs, and in particular of [C_{14}mim]Cl at 500 mM, to extract cynaropicrin, in which a maximum extraction yield of 3.18 wt% was obtained. Based on these results, we then studied the influence of different concentrations of [C_{14}mim]Cl in aqueous solution, ranging from 0 to 1000 mM, on the cynaropicrin extraction yield. The results obtained are shown in **Figure 4.4a**, whereas the detailed results are provided in the Appendix (Table S4.3).

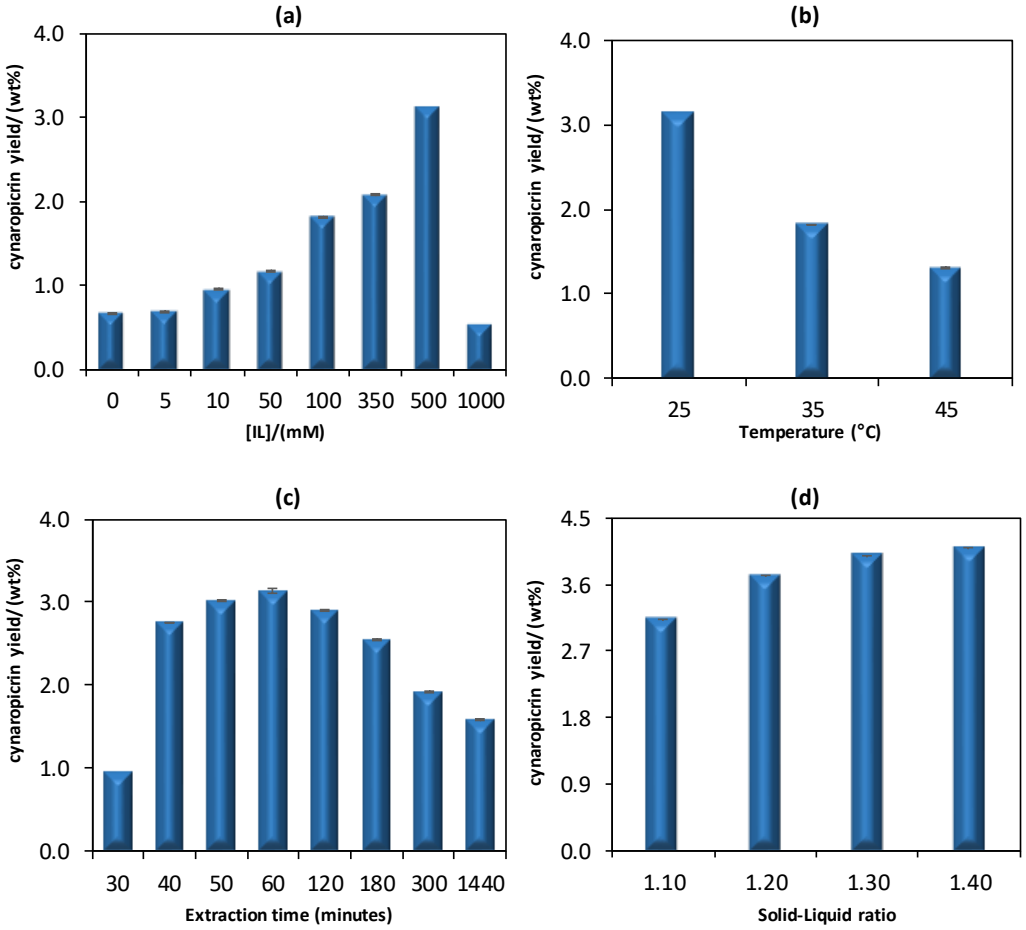


Fig. 4.4. Optimization of the operational conditions on the yields of cynaropicrin extracted from *C. cardunculus* leaves using aqueous solutions of [C_{14}mim]Cl at several conditions, namely: (a) concentration of IL in aqueous solution, [IL]; (b) Temperature (T); (c) extraction time (t), and (d) solid-liquid ratio (S/L ratio).

At low concentrations (5 mM) of [C_{14}mim]Cl and close to the CMC (4 mM)[50] of this IL, the cynaropicrin extraction yield is 0.69 wt%, which is similar to value obtained with pure water (0.67 wt%) under the same conditions. However, as the CMC of [C_{14}mim]Cl is overwhelmed, a significant increase in the cynaropicrin extraction yield is observed. The efficiency continuously increases with the IL concentration, but seems to reach a maximum around 500 mM, above which the extraction yield of cynaropicrin starts to decrease. This maximum in the extraction yield along the IL concentration occurs most probably due to viscosity problems, as mentioned above and as reported in the literature.[19]

After optimizing the concentration of [C_{14}mim]Cl aqueous solution, we proceeded to the optimization of the extraction temperature **(Figure 4.4b)**, performing extractions at 25, 35 and 45°C, keeping constant the remaining conditions, namely a S/L ratio of 1:10, a concentration of 500 mM, and an extraction time of 60 min. The detailed results are given in the Appendix (Table S4.4). As shown in **Figure 4.4b**, an increase in temperature is not favorable for the cynaropicrin extraction. Although an increase in the cynaropicrin extraction with temperature would obviously be expected, the observed reduction in the recovery of this compound is most probably due to the dissolution of polysaccharides at higher temperatures, as previously reported with this type of biomass yet using other solvents,[56] and given the extremely viscous solutions obtained after the extraction process at higher temperatures. However, this aspect can be seen as an advantage since good extraction yields of cynaropicrin are obtained at temperatures close to room temperature, without requiring additional energetic inputs.

The extraction time was also varied between 30 and 1440 min, keeping constant the remaining variables (S/L ratio of 1:10, a concentration of IL of 500 mM, and a temperature of 25°C). The influence of this variable towards the extraction yield of cynaropicrin is illustrated in **Figure 4.4c** (detailed data are provided in the Appendix – Table S4.5). An increase in the extraction time shows a positive effect on the cynaropicrin extraction efficiency, up to 60 min with a maximum yield of cynaropicrin of 3.13 wt%. However, and although a small decrease in the extraction yield is observed between 60 and 120 min, there is a dramatic decrease in the yield from 120 min onwards. This may again be associated to the dissolution of biomass polysaccharides in the IL aqueous solution, as discussed before with the temperature increase effect, becoming more relevant for longer extraction periods. In fact, aqueous solutions of high viscosity have been obtained for extraction times longer than 120 min. Similar effects with an increase in the extraction time were observed in the extraction of several value-added compounds from biomass using different solvents.[57,58]

If the saturation of the solvent is reached, the extraction yield usually increases with the decrease of the S/L ratio. However, an increase in the volume of the solvent used further results

in more expensive and less sustainable processes, in which higher amounts of solvents need to be recycled or disposed.[59] To address this issue, the S/L ratio was finally optimized - the data obtained are shown in **Figure 4.4d**, whereas the detailed results are provided in the Appendix (Table S4.6). S/L ratios of 1:10, 1:20 and 1:30, keeping constant the remaining conditions, namely an IL concentration of 500 mM, a temperature of 25°C, and an extraction time of 60 min, were tested. The results obtained **(Figure 4.4d)** show a positive effect on the cynaropicrin when increasing the amount of the solvent (liquid) used, reaching a maximum extraction yield of 4.09 wt%. However, this increment is more relevant between 1:10 and 1:20, while after that and up to a 1:40 S/L ratio the increase in the extraction yield is not significant enough to justify the use of larger volumes of solvent (taking into account the economic and environmental impact of the process). In this perspective, and considering the amounts of solvent used in the 1:20 and 1:40 ratios, and the slight differences in the extraction yields obtained (3.73 and 4.09 wt%, respectively), the whole process becomes more feasible with a S/L ratio of 1:20. Among the studied variables in the optimization of the extraction process, the S/L ratio was found to be the variable with the weakest influence on the extraction yield of cynaropicrin.

The results showed here clearly confirm the remarkable ability of aqueous solutions of surface-active ILs, namely [C_{14}mim]Cl, to extract cynaropicrin from the leaves of *C. cardunculus* L. To maximize the extraction yield of cynaropicrin under more sustainable conditions, a concentration of IL of 500 mM, a temperature of 25°C, an extraction time of 60 min, and a S/L ratio of 1:20, should be adopted. Still, and to demonstrate the high potential of ILs aqueous solutions as alternative solvents, and particularly the relevance of the surface-active [C_{14}mim]Cl IL, we further carried out the extraction of cynaropicrin from *C. cardunculus* L. leaves using several conventional surfactants, namely cationic (CPC, CTAC and CTAB), anionic (SDS and SDBS) and nonionic (Genapol C-100) ones, with or without aromatic rings, in concentrations ranging between 10 and 500 mM, under the previously optimized operational conditions. The results obtained are shown in **Figure 4.5** and in the Appendix (Table S4.7).

The best results were obtained with [C_{14}mim]Cl followed by CPC aqueous solutions. In general, it was found a relevant influence of the cationic/anionic nature of the surfactant and of the presence of aromatic rings towards the extraction of cynaropicrin from *C. cardunculus* L. leaves. This is in agreement with the results obtained with the two classes of ILs investigated and discussed above. However, the most relevant observation is that the surface-active [C_{14}mim]Cl leads to the highest cynaropicrin extraction yield, notably 36% higher than that obtained with the best conventional surfactant (CPC). These results thus demonstrate the remarkable potential of surface-active ILs on the cynaropicrin extraction from *C. cardunculus L.* leaves.

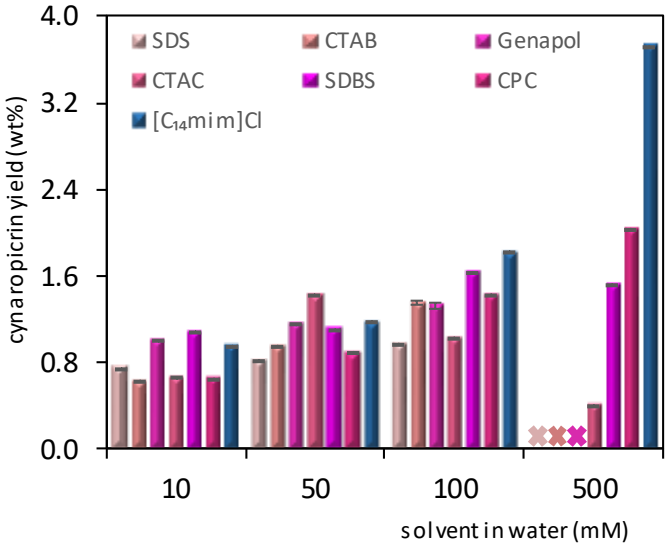


Fig. 4.5. Cynaropicrin extraction yields obtained with aqueous solutions of conventional surfactants and [C_{14}mim]Cl at fixed conditions: S/L ratio = 1:20, t = 60 min, T = 25°C. SDS, Genapol and CTAB were not applied at 500 mM due to their lower water solubility.

Finally, the extraction of cynaropicrin from *C. cardunculus* leaves using water, acetone, n-hexane and dichloromethane under the same conditions, as well as by Soxhlet extraction with dichloromethane, was performed for comparison purposes (detailed data are provided in the Appendix – Table S4.8). The maximum yield of cynaropicrin achieved was 8.65 wt% by Soxhlet extraction, in agreement with previously published values.[35] The higher yields obtained with this technique, when compared to solid-liquid extractions with the same solvent (4.53 wt%) can be explained by the specific conditions enabled by the Soxhlet technique (high temperature, time and number of extraction cycles with the use of fresh solvent) that allows to reach a maximum extraction capacity. Regarding the use of pure water, *n*-hexane and acetone at the same conditions used with the IL aqueous solutions, these lead to substantially lower extraction yields (0.68, 0.04 and 0.35 wt%, respectively, *versus* 3.73 wt% obtained with the IL aqueous solution). Although dichloromethane stands out as the best evaluated solvent for the extraction of cynaropicrin (4.53 wt%), the competitive results obtained with [C_{14}mim]Cl aqueous solutions (3.73 wt%) support their high potential as alternative solvents to extract this compound from biomass without requiring the employment of volatile organic solvents. Moreover, low concentrations of ILs and moderate temperatures and time are used, with no significant energetic requirements.

Recovery process for cynaropicrin from biomass

After the identification of the optimum extraction conditions, we turned our attention towards the development of scaled and more sustainable extraction/recovery processes. To this end, we addressed the use of the same biomass in consecutive extractions with "fresh" solvents samples (for six cycles) to appraise the maximum amount of cynaropicrin that can be extracted, and the use of the same [C_{14}mim]Cl aqueous solution using new biomass samples (for four cycles) to infer the solvent saturation. The detailed data are provided in the Appendix (Table S4.9 and Figure S4.2).

The first test allowed to determine the maximum amount of cynaropicrin that could be extracted from the same biomass; according to the gathered results, a total extraction yield of 6.47 wt% after six extraction cycles was obtained using fresh IL-water samples. The sum of the first three recycles of biomass lead already to a higher cynaropicrin extraction yield than that obtained with dichloromethane, namely 6.33 *versus* 4.53 wt%, under the same extraction conditions, yet lower than that obtained by Soxhlet extraction.

Tests with the same aqueous solution of [C_{14}mim]Cl for cynaropicrin extraction from new biomass samples were additionally performed. Detailed results are given in the Appendix (Table S4.10 and Figure S4.3). At the end of each extraction, the mixture was immediately filtrated in order to remove the biomass residue and to reuse the recovered IL-water solution. This process was repeated four times. The results obtained show that there is an increase in the extraction yield in each new cycle, from 3.73 to 4.16 wt%. However, this increment is of low significance meaning that the solvent almost reaches saturation in the first step of extraction under the optimized operational conditions.

Taking advantage of the solvent saturation and of effect of the IL concentration on the solubility/extraction of cynaropicrin, it is possible to envision a recovery process simply based on the addition of water, which will act as an anti-solvent. To confirm this hypothesis, at the end of the extraction process, the saturated solution was filtrated to remove solid biomass residues. The supernatant was then diluted with water in two different ratios (1:10 and 1:20 (v:v)), leading to the formation of a solid residue that was recovered by filtration. Detailed data are given in the Appendix. The HPLC analysis of the remaining aqueous solution, while taking into account the dilutions carried out, allowed to confirm the precipitation of 65% of the extracted cynaropicrin. Cynaropicrin can thus recovered by filtration and the surface-active IL can be again used after an evaporation step to remove the excess of water, falling within an integrated biorefinery approach.

In summary, cynaropicrin can be recovered from the IL aqueous solutions by the addition of water as an anti-solvent, inducing the precipitation/recovery of 65 wt% of the target compound, and further allowing the solvent recycling. An example of a DAD-HPLC chromatogram is shown in the Appendix. Although pure cynaropicrin was not obtained, a fact that only occurs if totally selective solvents could been identified, the obtained extracts may contain other valuable components, as reported in previous studies.[7,60] However, these cynaropicrin-rich extracts can be directly used in nutraceutical and cosmetic applications, as shown in the literature,[29] without the need of obtaining pure cynaropicrin.

Figure 4.6 depicts the developed process for the extraction and recovery of cynaropicrin from biomass. Although ILs have been described as greener replacements for harmful volatile organic solvents, numerous studies demonstrated that this group of solvents, yet depending on their chemical structure, can be less, equally or even more toxic than commonly used organic solvents.[61] However, ILs display negligible vapor pressures, and at least losses to atmosphere will not occur as it happens with conventional organic solvents. Moreover, aqueous solutions ILs are being proposed as remarkable solvents instead of pure ILs, being water the greenest solvent overall, thus contributing towards the sustainability of the proposed process and opening new perspectives for the development of biorefinery approaches based on the use of aqueous solutions of ILs as alternative and more efficient solvents.

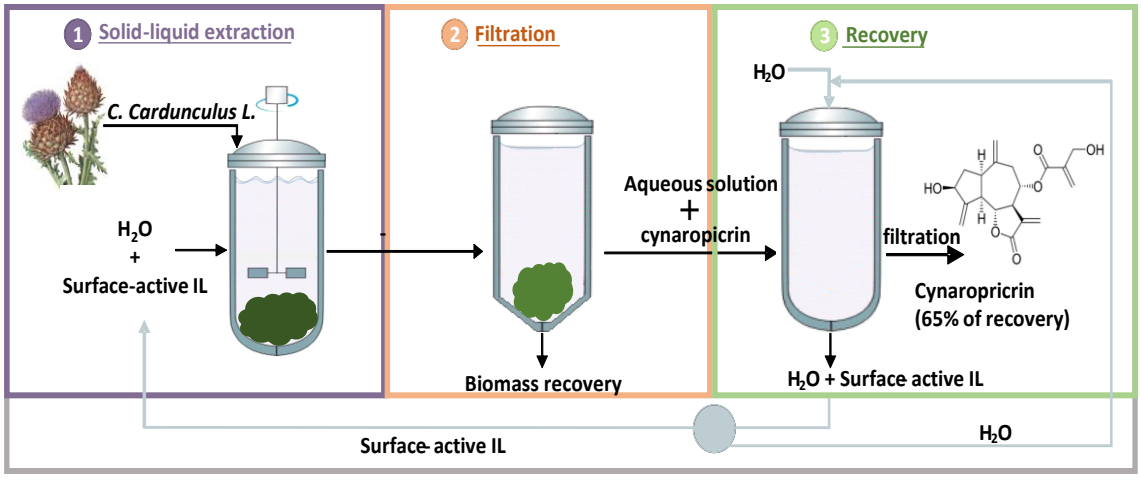


Fig. 4.6. Schematic representation of the extraction and recovery process for cynaropicrin from biomass using aqueous solutions of surface-active IL.

Conclusions

Aqueous solutions of surface-active ILs were shown to be efficient extraction media for cynaropicrin from *C. cardunculus L.* leaves and can effectively replace commonly used organic

solvents. Based on the cynaropicrin extraction profile, it was demonstrated that the extraction yield depends on the IL concentration and respective CMC, and that cationic surface-active ILs have a better performance as extraction solvents.

Several operational conditions were optimized to improve the extraction of cynaropicrin; the best conditions were obtained with a concentration of 500 mM of $[C_{14}mim]Cl$, a temperature of 25°C, an extraction time of 60 minutes and a S/L ratio of 1:20, allowing to obtain a cynaropicrin extraction yield of 3.73 wt%, a significantly higher value than that obtained with pure water, aqueous solutions of conventional surfactants or organic solvents under the same conditions. The recovery of the target compound by the addition of water as an anti-solvent was shown, as well as the reusability of the biomass and of the solvent, leading thus to the development of more sustainable extraction processes.

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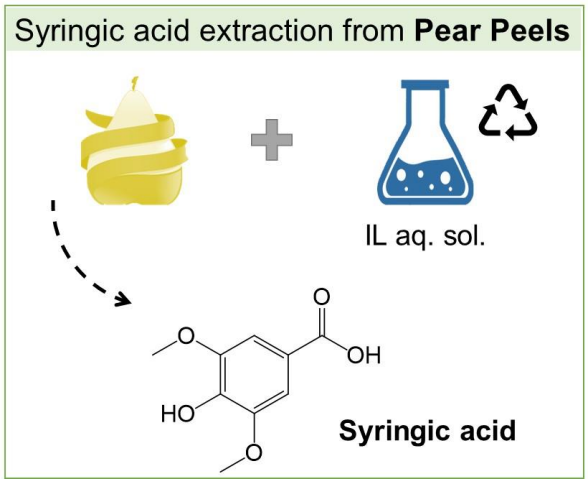
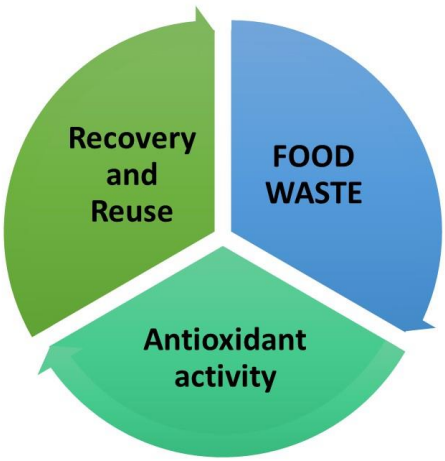
5. RECOVERY OF SYRINGIC ACID FROM INDUSTRIAL FOOD WASTE WITH AQUEOUS SOLUTION OF IONIC LIQUIDS

Chapter based on the submitted article:

E.L.P. Faria, A.M. Ferreira, A.F. Cláudio, J.A.P. Coutinho, A.J.D. Silvestre and M.G. Freire. Recovery of syringic acid from industrial food waste with aqueous solution of ionic liquids. *ACS Sustainable Chem. Eng.*, **2019**. Submitted.

Contributions: A.J.D.S. and M.G.F. conceived and directed this work. E.L.P.F. acquired the experimental data. E.L.P.F., A.M.F., A.F.C., J.A.P.C., A.J.D.S. and M.G.F. interpreted the experimental data. The manuscript was mainly written by E.L.P.F., A.M.F., A.J.D.S and M.G.F. with contributions from the remaining authors.





Abstract

Phenolic acids present in industrial food waste display a broad range of biological activities and related health benefits, among which the strong antioxidant and free radical scavenger activities are the most investigated. However, food waste or by-products are still scarcely considered as an alternative source for these compounds and volatile organic solvents for their extraction are still the preferred choice. In this work, aqueous solutions of ionic liquids (ILs) with hydrotropic or surfactant character were investigated to improve the solubility and effectively extract syringic acid from *Rocha* pear peels, a relevant waste of the food industry. The solubility of syringic acid in aqueous solutions of a wide variety of ILs at different concentrations at 30°C was first ascertained. The results obtained show that ILs that behave as cationic hydrotropes are the best option to enhance the solubility of syringic acid in aqueous media, with increases in solubility of up to 84-fold when compared with water. After identifying the most promising IL aqueous solutions, a response surface methodology was used to optimize operational extraction conditions (extraction time, solid-liquid ratio and temperature), leading to a maximum

extraction yield of syringic acid of 1.05 wt.% from pear peels. Both the solvent and biomass reuse were additionally investigated, demonstrating to overcome the biomass-solvent ratio constraints and mass transfer effects while leading to extraction yields of 2.04 and 2.22 wt.%. Taking advantage of the solubility of syringic acid dependency with the IL concentration, water was used as an anti-solvent, where 77% of the extracted phenolic acid with high purity can be obtained. A continuous countercurrent process conceptualized for large-scale applications, and that further allows the solvent recycling after the recovery of syringic acid, is finally proposed.

Introduction

Biomass is a source of high-value compounds with relevant biological activities such as phenolic compounds, one of the most abundant families of secondary metabolites in plants.[1] The interest in phenolic compounds results from their broad range of biological activities and related health benefits, among which the strong antioxidant and free radical scavenger activities are well-established.[2] These properties are responsible for their application in the food, nutraceutical, cosmetic and pharmaceutical industries.[3] Examples of phenolic compounds present in biomass are, among others, vanillic, gallic, protocatechuic, ellagic, and syringic acids, and quercetin, vanillin and resveratrol.[1]

Most of these compounds can be obtained from biomass byproducts, particularly those generated by agroforest and agrofood industries, thus contributing to the full valorization of feedstocks in an integrated biorefinery perspective, and, ultimately, contributing towards a circular economy.[4] Accordingly, the development of sustainable extraction, purification and recovery processes of high-value compounds from industry waste, suitable of industrial implementation, is nowadays a crucial need[5] and in line with the United Nations Sustainable Development Goals.[6]

Most of these compounds, and particularly those with recognized health benefits, are of high cost due to the complex, multistep methods required for their extraction, purification and recovery. The extraction of these compounds from biomass is commonly carried out using volatile organic solvents,[7] which recently have been combined with microwave- and ultrasound-assisted methods to improve the extraction efficiency.[8] Supercritical CO^2 extraction and alternative solvents, such as ionic liquids and deep eutectic solvents, have also been investigated.[8-10] In addition to the extraction step, further purification steps are required, typically carried out by solid-phase extraction, back-extraction with organic solvents, evaporation of the solvents or compounds (when applicable), and induced precipitation.[9]

Amongst the several solvents that can be used for the extraction of bioactive compounds from food industry waste, and taking into account their envisioned consumption by humans, water certainly corresponds to the most benign solvent and preferred choice. Nevertheless, many bioactive compounds display a limited solubility in water,[11] restricting its use for their effective extraction from biomass. To increase the solubility of bioactive compounds in aqueous solutions, additives such as surfactants or hydrotropes can be used.[12,13] Surfactants may be used in micelle-mediated extraction processes, which are particularly relevant to enhance the solubility and to extract highly hydrophobic compounds, such as triterpenic acids.[14,15] On the other hand, hydrotropes do not form micelles but can increase the solubility of given solutes in aqueous media by the formation of hydrotrope-solute micelles.[14] Hydrotropes are usually anionic or cationic aromatic ring functionalized compounds with a sulphate, sulfonate or carboxylate group.[16,17] Besides enhancing the solubility of target compounds in aqueous media, hydrotropes play an important role in the stabilization of aqueous solutions and on tailoring their viscosity.[18,19] Furthermore, since the solubility of a given solute in aqueous media depends on the hydrotrope concentration, it is possible to design strategies for the solute recovery using water as anti-solvent. In addition to the well-known ability of ionic liquids (ILs) to act as surfactants if properly designed, it was recently demonstrated that some ILs in aqueous media behave as hydrotropes.[13,14] This was shown by determining the solubility of vanillin and gallic acid in aqueous solutions of a wide range of ILs aqueous solutions, where an increase in the solubility of up to 40-fold was observed when using ILs with hydrotropic character.[13,14] Dynamic light scattering, nuclear magnetic resonance and molecular dynamics simulations studies were additionally employed, allowing to confirm the presence of IL-biomolecule micelles.[13,14]

Based on the need of developing cost-effective and sustainable processes to extract and recover bioactive compounds from industry waste, in this work we investigated a series of ILs to improve the solubility of syringic acid in aqueous media and for its extraction from *Rocha* pear peels. The interest in syringic acid is related with its bioactive properties, namely antioxidant, antiproliferative, antiendotoxic, antimicrobial, anti-inflammatory, and anticancer activities.[20] The *Rocha* pear was chosen because of its economic importance for the agrofood industry in Portugal, with an average annual production of 173,000 tons.[21] This pear is used to produce juices, jams and other food products, generating vast amounts of peel residues. Moreover, pears in general are rich in phenolic compounds, such as syringic, chlorogenic, ferulic and coumaric acids.[22-25] Syringic acid is one of the phenolic compounds present at higher concentrations in pears (9.5–21.26 mg/100 g fresh pears).[22-25]

We first measured the solubility of syringic acid in aqueous solutions of a wide range of ILs and concentrations to identify the most promising ILs, and then applied the best IL aqueous

solutions to extract the target phenolic acid from pear peels. In order to optimize the extraction operational conditions, namely temperature, solid-liquid (biomass-solvent) ratio and time of extraction, a factorial planning was applied. Both the solvent and biomass reuse have been investigated, allowing to propose a continuous countercurrent process conceptualized for large-scale applications, which further allows the solvent recycling after the recovery of syringic acid by the addition of water that acts as anti-solvent.

Materials and methods

Materials

Syringic acid (> 99 % pure), whose chemical structure is shown in **Figure 5.1**, was purchased from Sigma-Aldrich and used as received. The water employed was double distilled, passed across a reverse osmosis system, and further treated with a Milli-Q plus 185 water purification apparatus. A large variety of ILs with hydrotropic or surfactant character were investigated in aqueous solutions aiming at improving the solubility and extraction of syringic acid from pear peels. The chemical structures of the investigated ILs are depicted in Figure 1. The ILs investigated were 1-butyl-3-methylimidazolium tosylate ($[C_4C_1im][TOS]$, 98% pure), 1-butyl-3-methylimidazolium thiocyanate ($[C_4C_1im][SCN]$, >98% pure), 1-butyl-3-methylimidazolium hydrogensulfate ($[C_4C1im][HSO_4]$, >98% pure), 1-butyl-3-methylimidazolium chloride ($[C_4C_1im]Cl$, 99% pure), 1-butyl-3-methylimidazolium dicyanamide ($[C_4C_1im][N(CN)_2]$, >98% pure), 1-butyl-3-methylimidazolium acetate ($[C_4C_1im][Ac]$, >98% pure), 1-methyl-3-octylimidazolium chloride ($[C_8C_1im]Cl$, 99% pure), 1-butyl-3-methylpyridinium chloride ($[C_4C_1py]Cl$, 99% pure), 1-butyl-1-methylpiperidinium chloride ($[C_4C_1pip]Cl$, 99% pure), 1-butyl-1-methylpyrrolidinium chloride ($[C_4C_1pyrr]Cl$, 99% pure), tetrabutylammonium chloride ($[N_{4444}]Cl$, ≥97% pure), tetrabutylphosphonium chloride ($[P_{4444}]Cl$, 96% pure), triisobutyl(methyl)phosphonium tosylate ($[P_i(_{444})_1][TOS]$, 98% pure), cholinium chloride [Ch]Cl, ≥99% pure), cholinium acetate ([Ch][Ac], 98% pure), cholinium butanoate (Ch][But], >97 wt.% pure), cholinium hexanoate ([Ch][Hex], >97 wt.% pure), cholinium octanoate ([Ch][Oct], >97 wt.% pure), and cholinium decanoate ([Ch][Dec], >97 wt.% pure). The imidazolium-, pyridinium-, piperidinium- and pyrrolidinium-based were purchased from Iolitec. The tetrabutylphosphonium chloride and triisobutyl(methyl)phosphonium tosylate were kindly supplied by Cytec Industries Inc. Tetrabutylammonium chloride and cholinium chloride were acquired from Sigma-Aldrich. With the exception of cholinium acetate that was purchased from Iolitec, the remaining cholinium carboxylate ILs were synthesized by us, using a methodology

previously reported.[26] Before use, all ILs were dried under vacuum (10^{-2} Pa) at 30°C for a minimum of 48 h.

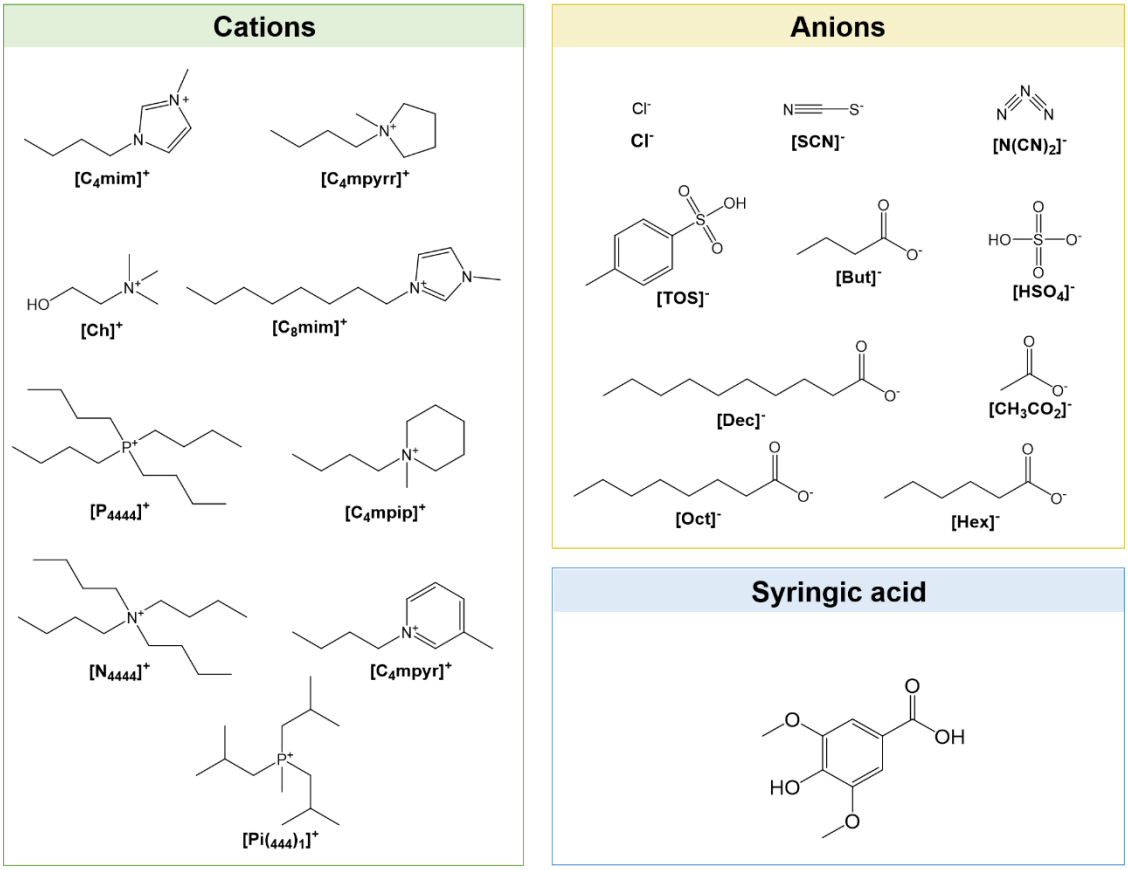


Fig. 5.1. Chemical structures of syringic acid and cations/anions of the ionic liquids investigated.

Solubility of syringic acid in ILs aqueous solutions

IL aqueous solutions were prepared with concentrations ranging from 0.1 to 4.0 mol·L^{-1}. Syringic acid was added in excess to each IL aqueous solution and equilibrated under constant stirring and fixed temperature using an Eppendorf Thermomixer Comfort equipment. Solubility data were determined at 30°C, using optimized equilibration conditions, namely a stirring velocity of 750 rpm and an equilibration time of at least 72 h. Samples were then centrifuged in a Hettich Mikro 120 centrifuge, during 20 min at 4500 rpm, to separate the macroscopic solid (syringic acid) and liquid (IL aqueous solution saturated with syringic acid) phases. After centrifugation, samples were placed in an air bath equipped with a Pt 100 probe and PID controller at the temperature used in equilibrium assays for more 2 h. Samples of the liquid phase were collected and diluted in ultra-pure water, and the amount of syringic acid was quantified through UV-spectroscopy, using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, at a wavelength of 271 nm, using an established calibration curve. Controls containing each IL at the same

concentration were used in all experiments as control samples. At least three individual samples were prepared and quantified. The same procedure was applied to determine the solubility of syringic acid in pure water.

Extraction of syringic acid from pear peels using IL aqueous solutions

Peels from fresh Portuguese *Rocha* pears, purchased in a local supermarket, were manually removed, dried at 25°C for 2 days, and ground with a commercial coffee grinder. Weighted amounts ($\pm 10^{-4}$ g) of ground pear peels were added to $[C_4C_1im]Cl$ aqueous solutions. This IL was used on this set of experiments due to its significant hydrotropic effect and ability to improve the solubility of syringic acid in aqueous media. Operational conditions, namely temperature, solid-liquid (biomass-solvent) ratio, and time of extraction were optimized by a 2^3 factorial planning to simultaneously analyze various operational conditions and to identify the most significant parameters that enhance the syringic acid extraction yield.[27] Further details and the 2^3 factorial planning used in this work are provided in the Appendix (Table S5.1). Student's t-test was used to evaluate the statistical significance of the adjusted data. The suitability of the model was determined by evaluating the lack of fit, the regression coefficient (R^2) and the F-value obtained from the analysis of variance (ANOVA). In the factorial planning the central point was experimentally considered at least in triplicate. Additional 12-20 experiments *per* factorial planning were carried out, for which several operational conditions were repeated to guarantee the accuracy of the data. The StatSoft Statistica 10.0© software was used for all statistical analyses.

After the extraction step, the IL aqueous solutions were separated from the biomass by centrifugation (at 5000 rpm for 10 min using an Eppendorf centrifuge 5804), and the supernatant was filtered using a 0.20 µm syringe filter. A 200 µL aliquot was taken, mixed with 800 µL of mobile phase (ultra-pure H_2O + 0.2% acetic acid) used in the HPLC-DAD analysis, and filtered over a 0.2 µm syringe filter. The quantification of syringic acid in each solution was carried out using a HPLC-DAD (Shimadzu, model PROMINENCE). HPLC analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), Kinetex 5 µm C18 100 Å, from Phenomenex. The mobile phase consisted of 77.5% of ultra-pure H_2O + 0.2% acetic acid and 22.5% of acetonitrile. The separation was conducted in isocratic mode, at a flow rate of 1.0 mL $\cdot min^{-1}$ and using an injection volume of 10 µL. DAD was set at 271 nm. Each sample was analyzed at least in duplicate. The column oven and the autosampler operated at 30°C. Calibration curves were prepared using pure and commercial syringic acid aqueous solutions.

For comparison purposes, methanol and dichloromethane were also used as solvents to carry out the extraction of syringic acid from pear at the optimized conditions.

The reported syringic acid extraction yield corresponds to the percentage ratio between the weight of pure syringic acid extracted and the total weight of dried biomass.

Reusability of biomass and solvent, and syringic acid recovery

In order to develop a sustainable extraction and recovery process, as well as to infer the solvent saturation effects and maximum amount of the target compound present in the biomass, two strategies were investigated: (i) reuse of the biomass employing 5 new aqueous solutions of ILs to the same biomass sample; and (ii) reuse of the solvent by applying 5 successive cycles of extraction using the same IL aqueous solution. Both approaches were applied at the optimum operational conditions. These assays also allowed us to propose the use of IL aqueous solutions in a continuous countercurrent mode extraction process.

After 5 extraction cycles with the reuse of the IL aqueous solution, which allowed the solvent saturation, water was finally added as an anti-solvent, inducing the precipitation and recovery of syringic acid. Different amounts of added water were investigated; 0.5 mL of the IL aqueous solutions containing syringic acid were diluted in 1, 5, 10, 15 and 25 mL of distilled water. After the addition of water, the solution was centrifuged at 5000 rpm for 15 min, and vacuum filtered with a 0.45 µm microporous membrane. HPLC-DAD analysis of the aqueous solutions was carried out to determine the recovery yield of syringic acid by precipitation. The recovery yield corresponds to the percentage ratio between the total weight of precipitated syringic acid and the weight of syringic acid initially present in the IL aqueous solution after the extraction step. The obtained precipitate/solid residue was dried in an air oven at room temperature for 2 days, and further analyzed by HPLC-DAD to guarantee its stability and purity.

Results and discussion

Solubility of syringic acid ILs aqueous solutions

In a first set of experiments we determined the solubility of syringic acid in aqueous solutions of ILs at concentrations ranging from 0.1 to 4.0 mol·L^{-1}, at 30°C, aiming at better understanding the role of ILs to improve the solubility of natural phenolic acids in aqueous media and to identify the most promising IL aqueous solutions to proceed with the syringic acid extraction from food industry waste, namely pear peels. The solubility results are given in the Appendix (Table S5.2). The solubility of syringic acid at 30°C in pure water determined by us is 1.43 ± 0.08 g·L^{-1} (7.22

mol·L^{-1}). The effect of the IL chemical structure and IL concentration on the solubility of syringic acid at 30°C is depicted in **Figure 5.2**. These results are shown as solubility enhancement, namely S/S_0, where S and S_0 represent the solubility of the syringic acid in each IL aqueous solution and in pure water, respectively.

The results obtained reveal an overall synergetic effect of the two solvents (water and IL) on the solubility of syringic acid, where a maximum in solubility occurs along the IL concentration. The IL concentration where this maximum occurs depends however on the IL chemical structure and hydrotrope or surfactant behavior, with surfactants usually performing better at lower concentrations. A similar behavior was reported by Cláudio et al.[14] on the solubility of vanillin and gallic acid in aqueous solutions of ILs. Amongst all the ILs studied, [C_4C_1im]Cl is the best IL identified with an increase in the solubility of syringic acid in aqueous media up to 84-fold (120.3 ± 0.3 g·L^{-1} in the IL aqueous solution versus 1.43 ± 0.08 g·L^{-1} in pure water).

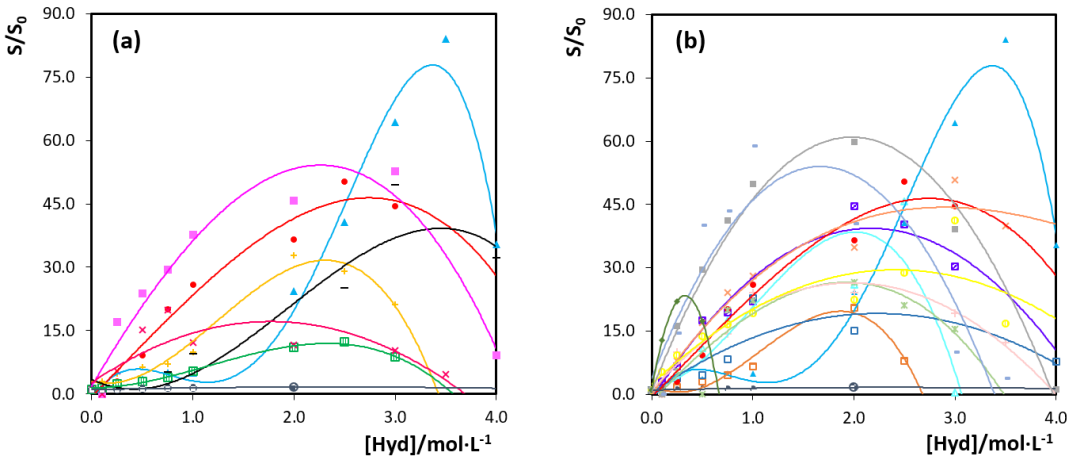


Fig. 5.2. Solubility enhancement of syringic acid in aqueous solutions of ILs (a): (▲) [C_4C_1im]Cl, (×) [C_8C_1im]Cl, (●) [P_{4444}]Cl, (+) [N_{4444}]Cl, (▥) [C_4C_1pyrr]Cl, (–) [C_4C_1pip]Cl, (■) [C_4C_1py]Cl, (◓) [Ch]Cl); (b) (▲) [C_4C_1im]Cl, (△) [C_4C_1im][TOS], (▣) [C_4C_1im][HSO_4], (◪) [C_4C_1im][$N(CN)_2$], (□) [C_4C_1im][SCN], (×) [C_4C_1im][Ac], (✳) [$Pi_{(444)1}$][TOS], (●) [P_{4444}]Cl, (◓) [Ch][Cl], (+) [Ch][Ac], (◍) [Ch][But], (■) [Ch][Hex], (–) [Ch][Oct] and (◆) [Ch][Dec]). Lines have no physical meaning and are only guides for the eye.

Figure 5.2a shows results for the following ILs: [C_4C_1im]Cl, [C_8C_1im]Cl, [P_{4444}]Cl, [N_{4444}]Cl, [C_4C_1pyrr]Cl, [C_4C_1pip]Cl and [C_4C_1py]Cl, which share a common anion (chloride), thus allowing to evaluate the IL cation effect on enhancing the syringic acid solubility. Among these only [C_8C_1im]Cl presents a surfactant behavior, with a previously reported critical micellar concentration (CMC) of 238 mM,[28] whereas the remaining ILs of this series are expected to act

as hydrotropes. The overall capacity of these ILs to improve the solubility of syringic acid (appraised at the maximum solubility of syringic acid) follows the order: $[C_4C_1im]Cl$ > $[C_4C_1py]Cl$ > $[P_{4444}]Cl$ > $[C_4C_1pip]Cl$ > $[N_{4444}]Cl$ > $[C_8C_1im]Cl$ > $[C_4C_1pyrr]Cl$ > [Ch]Cl. The obtained trend reflects the ability of the IL cation to act as hydrotrope in the solubility of syringic acid. With the exception of cholinium- and pyrrolidinium-based ILs that perform worst than the surfactant $[C_8C_1im]Cl$, all remaining ILs display a significant ability to increase the solubility of syringic acid while behaving as hydrotropes. In particular, with [Ch]Cl the solubility of syringic acid only slightly increases, reaching a maximum of solubility enhancement of 1.64, suggesting that neither $[Ch]^+$ nor Cl^- have a significant effect on improving the solubility of the target phenolic compound. When comparing the solubility of syringic acid in aqueous solutions containing $[C_4C_1im]Cl$ and $[C_8C_1im]Cl$, it is clear that ILs with shorter alkyl chains and with hydrotrope characteristics perform better at enhancing the solubility. When using ILs with surfactant characteristics, the solubility enhancement occurs due to IL self-aggregation and possibility of incorporating hydrophobic solutes in the micelle core. Therefore, with $[C_8C_1im]Cl$, hydrotropy is replaced by a micellar solubilization mechanism. However, for this type of ILs, the enhancement of solubility is more pronounced at low concentrations of IL, close to the critical micellar concentration, but seems to quickly saturate at around 0.75 $mol \cdot L^{-1}$, above which a much weaker effect on the solubility of the syringic acid is observed - **Figure 5.2a**. The same behavior was observed by Cláudio et al.[14] while evaluating the variation of the solubility of vanillin in aqueous solutions of $[C_nC_1im]Cl$ ILs, with $2 \leq n \leq 14$, concluding that ILs with shorter alkyl chain lengths ($[C_nC_1mim]Cl$, n = 2–6) behave as hydrotropes, whereas ILs with longer alkyl side chains ($[C_nC_1im]Cl$, n = 8–14)[28,29] behave as surfactants.

Although the formation of solute–hydrotrope complexes based on π…π interactions (between aromatic moieties of the substrate and aromatic hydrotropes) has been used to explain the hydrotropy concept,[30,31] the results obtained with non-aromatic ILs, such as $[N_{4444}]Cl$, $[P_{4444}]Cl$, and $[C_4C_1pip]Cl$, reveal that the hydrotropic effect cannot be explained as resulting from these interactions. This observation is in agreement with the findings of Cláudio et al.,[14] as well as with other works in which conventional hydrotropes have been studied,[32-35] demonstrating that π⋯π interactions are not the dominant effect of hydrotropy. These sets of results reveal that although most common hydrotropes present aromatic anions in their constitution, the hydrotropic effect can be induced by both aromatic and non-aromatic cations.

Figure 5.2b shows the results in solubility enhancement for the two following series of ILs: (i) $[C_4C_1im]Cl$, $[C_4C_1im][TOS]$, $[C_4C_1im][HSO_4]$, $[C_4C_1im][N(CN)_2]$, $[C_4C_1im][SCN]$, and $[C_4C_1im][Ac]$, and (ii) $[Pi_{(444)1}][TOS]$ and $[P_{4444}]Cl$. These two sets of ILs share a common cation, allowing to address the IL anion effect on the syringic acid solubility. In the same figure additional results for the ILs

[Ch][Ac], [Ch][But], [Ch][Hex], [Ch][Oct] and [Ch][Dec] are given to address the effect of carboxylate-based anions combined with the cholinium cation to improve the syringic acid solubility in water. Among these, [Ch][Oct] and [Ch][Dec] display surface-active properties.[36,37]

All ILs investigated with the $[C_4C_1im]^+$ cation display an important hydrotropic effect, thus improving the solubility of syringic acid in aqueous media according to the following anion trend (taken at the maximum solubility enhancement): $Cl^- > [Ac]^- > [TOS]^- > [N(CN)_2]^- > [SCN]^- > [HSO_4]^-$. With the $[P_{4444}]$-based ILs, the same trend was observed for the ILs comprising the Cl^- and $[TOS]^-$ anions. If compared with previous trends obtained for ILs used to improve the solubility of vanillin and gallic acid,[14] these results again confirm that the hydrotropy dissolution is solute dependent. According to the literature, most of the industrially used hydrotropes are anionic, and often contain a phenyl group, such as tosylate.[38] However, according to the results show in **Figure 5.2b**, $[C_4C_1im]Cl$ and $[C_4C_1im][Ac]$ perform better than $[C_4C_1im][TOS]$, and $[P_{4444}]Cl$ performs better than $[P_{i(444)1}][TOS]$, demonstrating that tosylate does not act as an hydrotrope able to improve the solubility of syringic acid in aqueous solutions. As observed with the cationic hydrotropes previously discussed, this result further supports the idea that π···π interactions do not contribute to the hydrotropic effect.[32-34,39] Although chloride is not an hydrotrope, and as a marginal effect upon the solubility of syringic acid as shown by the results of [Ch]Cl, aqueous solutions of $[C_4C_1im]Cl$ are the best solvent identified. All these results therefore indicate that for syringic acid there is not a synergetic effect of both IL ions to improve its solubility, as it would be expected by using ILs where both ions have hydrotrope characteristics, *e.g*. $[C_4C_1im][TOS]$ and $[C_4C_1im][N(CN)_2]$.

It has been previously reported that the solubility enhancement by hydrotropes is more effective for more hydrophobic solutes,[14] as ascertained when comparing the solubility of vanillin and gallic acid (with values of the logarithm of the octanol-water partition coefficient, K_{ow}, as follows: 1.23 and 0.72, respectively[40]). Syringic acid has an intermediate K_{ow} value of 1.04,[40] but more significant improvements in solubility have been observed if compared to vanillin and gallic acid. Furthermore, in the current work, the IL cation plays a major role on the design of effective hydrotropes. These results suggest that the strength of interactions between ILs and phenolic compounds are different, being solute-specific, which could be however useful to design selective solvents and effective recovery strategies.

In addition to $[C_4C_1im]$-based ILs, cholinium-based ILs have also been investigated. Both short alkyl side chain cholinium carboxylates (acetate, butanoate and hexanoate) that could act as hydrotropes, and long alkyl side chain cholinium carboxylate (octanoate and decanoate), able to form micelles in aqueous solutions and with reported CMCs,[36,37] were studied. Contrarily to the $[C_8C_1im]Cl$ previously discussed that is a cationic surfactant, [Ch][Oct] and [Ch][Dec] are

anionic surfactants. The results obtained show that the maximum in the syringic acid solubility occurs at lower IL concentrations as the alkyl side chain at the carboxylate anion is increased. Furthermore, the maximum in solubility enhancement occurs with [Ch][Hex] and [Ch][Oct], but strongly decreases with the better surfactant– [Ch][Dec]. The surfactant-based cholinium carboxylate ILs perform better than $[C_8C_1im]Cl$, meaning that anionic surfactants are more appropriate to increase the solubility of syringic acid in aqueous media. However, favorable and specific interactions occurring between the carboxylate anions and syringic acid cannot be discarded since $[C_4C_1im][Ac]$ is one of the best ILs (after $[C_4C_1im]Cl$) identified in the imidazolium-based series of ILs. Still, $[C_4C_1im][Ac]$ is a better hydrotrope to enhance the solubility of syringic acid than [Ch][Ac], reinforcing the relevance of cationic IL hydrotropes. These results disclose that the IL cation and anion may have different roles, being the hydrotropy effect towards syringic acid dominated by the IL cation.

Overall, according to the results discussed above and considering that aqueous solutions of $[C_4C_1im]Cl$ at 3.5 $mol \cdot L^{-1}$ were identified as the best solvent, able to increase 84-fold the solubility of syringic acid when compared to pure water, aqueous solutions of this IL were used to optimize the operational conditions of extraction of syringic acid from *Rocha* pear peels by a response surface methodology, as described below.

Extraction of syringic acid from *Rocha* pear peel using IL aqueous solution

Although pure ILs have been described as solvents for the extraction of value-added compounds from biomass, e.g. ellagic acid,[41] artemisinin,[42] limonene,[43] betulin,[44] among others, aqueous solutions of ILs display a high potential and additional advantages due to the use of lower amounts of IL, with intrinsic environmental and economic benefits. Aqueous solutions of ILs are solvents of lower toxicity and cost when compared to pure ILs, while decreasing the overall viscosity of the solvent and enhancing mass transfer. Furthemore, aqueous solutions of ILs are more selective, not allowing the dissolution of all biomass. Accordingly, whenever possible, IL aqueous solutions should be used instead of pure ILs.

After evaluating the best IL aqueous solutions as solvents for syringic acid, a factorial planning of 2^3 (3 factors and 2 levels) was used to optimize the operational conditions for the syringic acid extraction from *Rocha* pear peels. The results obtained were analyzed statistically with a confidence level of 95% (data shown in the Appendix – Figure S5.1). This methodology allows the study of the relationship between the response (extraction yield of syringic acid) and the independent variables/operational conditions that influence the extraction yield, namely extraction time (t, min), biomass-solvent weight ratio (S/L ratio, weight of dried biomass per

weight of solvent) and temperature (T, °C). The extraction yield corresponds to the percentage ratio between the weight of extracted syringic acid and weigth of dried biomass (pear peels). Based on the solubility results shown in **Figure 5.2** and maximum solubility enhancement of syringic acid of 84-fold, aqueous solutions of $[C_4C_1im]Cl$ at 3.5 $mol \cdot L^{-1}$ were used. Variance analysis (ANOVA) was used to estimate the statistical significance of the variables and their interactions. The experimental points used in the second factorial planning, the model equation, the extraction yield of syringic acid obtained experimentally and the respective calculated values using the correlation coefficients obtained in the statistical treatment, as well as all the statistical analysis, are shown in the Appendix. Based on the statistical model and results (Tables S5.3 and S5.4 in the Appendix), the average relative deviation between the experimental and the predicted values is 0.45%, supporting the good description of the experimental results by the statistical model.

The influence of the three variables on the extraction yield of syringic acid is illustrated in **Figure 5.3**, with the respective detailed data given in the Appendix (Table S5.4). It is evident that extraction time is a significant parameter leading to a region of maximum yield of extraction at 60 min. The solid-liquid ratio also leads to different yields of syringic acid. In general, the amount of extracted syringic acid increases with the solvent volume. Additionally, higher temperatures are more efficient for the extraction of syringic acid from pear peels, although this is the variable with the weakest influence on the extraction yield of syringic acid (between 45 and 60°C). Overall, the parameters that have a higher impact on the extraction yield of syringic acid (as can be seen in the pareto chart, Figure S5.1 in the Appendix), are the extraction time and the solid-liquid ratio. The optimized operational conditions found for the extraction of syringic acid occur at a temperature of 50°C, an extraction time of 60 min, and a solid-liquid ratio of 0.10, providing a syringic acid extraction yield of 1.05 wt.%.

Some organic solvents were also evaluated at the optimized conditions, with dichloromethane and methanol leading to extraction yields of 1.51 wt.% and 1.68 wt.%, respectively. Although a lower extraction yield is obtained with IL aqueous solutions, these are still competitive solvents since the use of volatile organic solvents is avoided. Furthermore, by reusing the IL aqueous solutions similar yields are obtained, as shown below, supporting their high potential as alternative solvents to extract syringic acid from biomass.

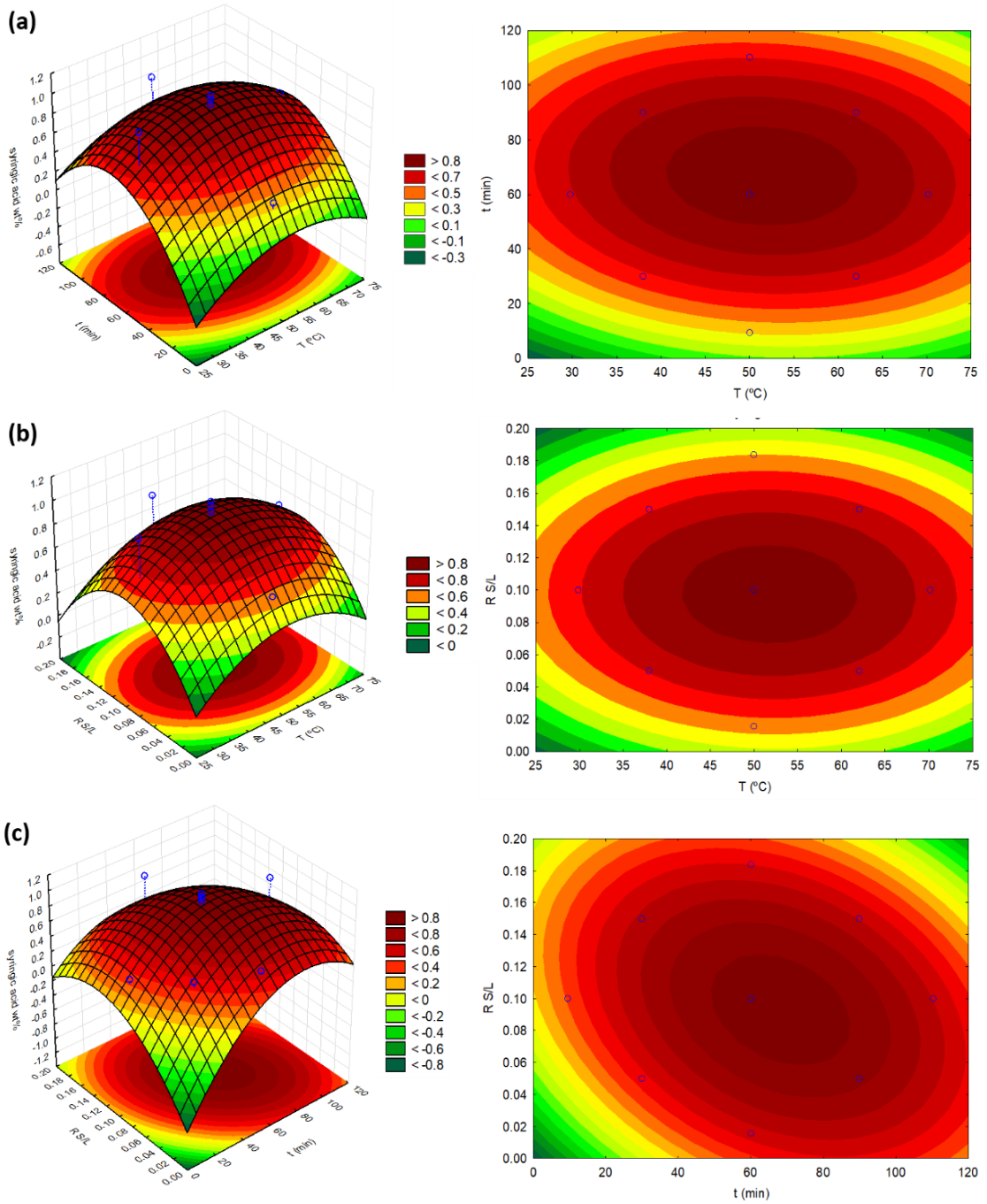


Fig. 5.3. Response surface plots (left) and contour plots (right) on the extraction yield of syringic acid with the combined effects of (a) T (°C) and t (min), (b) T and S/L ratio and (c) S/L ratio and t, using aqueous solutions of $[C_4C_1im]Cl$ at 3.5 M.

The two last sets of results on solubility and extraction from biomass suggest that the high performance demonstrated by ILs aqueous solutions in the extraction of biocompounds from biomass may be a major outcome of the enhanced solubility afforded by ILs with hydrotrope characteristics and not only from the biomass disruption as usually reported.

Reusability of biomass and solvent, and syringic acid recovery

Aiming to infer the maximum amount of syringic acid present in biomass, the same sample of *Rocha* pear peels was sequentially extracted with "fresh" IL aqueous solutions using the optimum conditions previously identified in five successive extraction cycles. The results obtained are shown in **Figure 5.4a** and given in detail in the Appendix (Table S5.5). It is shown that the syringic acid present in the biomass sample is not fully extracted in the first cycle (1.05 wt.%) of extraction and it is possible to achieve a maximum yield of 2.22 wt.% after 5 extraction cycles with "fresh" IL aqueous solutions. Thus, in a single extraction approach half of syringic acid still remains in biomass; however, if several extraction cycles are implemented the total extraction yield can surpass the ones obtained with conventional organic solvents.

The reusability of the extraction solvent using new *Rocha* pear peels was also investigated for 5 cycles to maximize the cost-efficiency and sustainability character of the developed process (**Figure 5.4b**; detailed results given in the Appendix - Table S5.6). After each extraction, the solid-liquid mixture was filtered, and the IL aqueous solution was reused with new pear peel samples. As summarized in **Figure 5.4b**, the extraction yield obtained in the first extraction cycle (1.07 wt.%) could be improved by reusing the solvent and applying it to new biomass samples, achieving a yield of 2.06 wt.% after the fifth cycle of reuse of the IL aqueous solution.

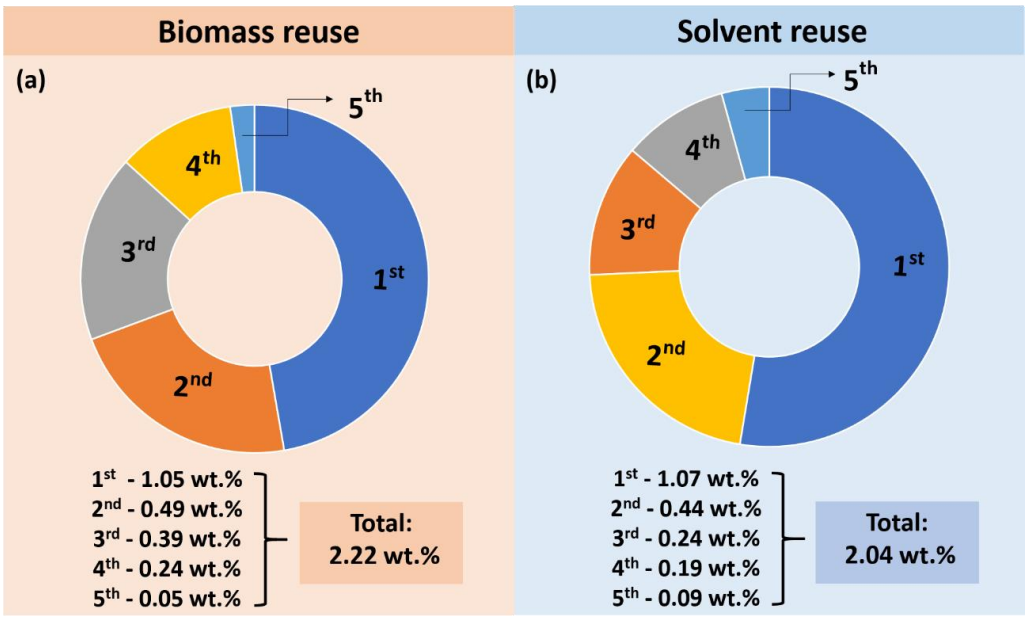


Fig. 5.4. Syringic acid extraction yields from *Rocha* pear peels with the biomass (a) and solvent (b) reuse at the optimized operational conditions.

These improvements in the extraction yield were not identified before in the factorial planning due to constraints of the solid-liquid ratio and mass transfer phenomenon, but if both the solvent and biomass are reused a significant increment on the extraction yields are obtained. Accordingly, an extraction continuous process operating in countercurrent, in which the solvent and biomass are reused in a continuous mode seems to be the most adequate option to be applied in large-scale applications. A schematic representation of the envisioned process is given in **Figure 5.5**.

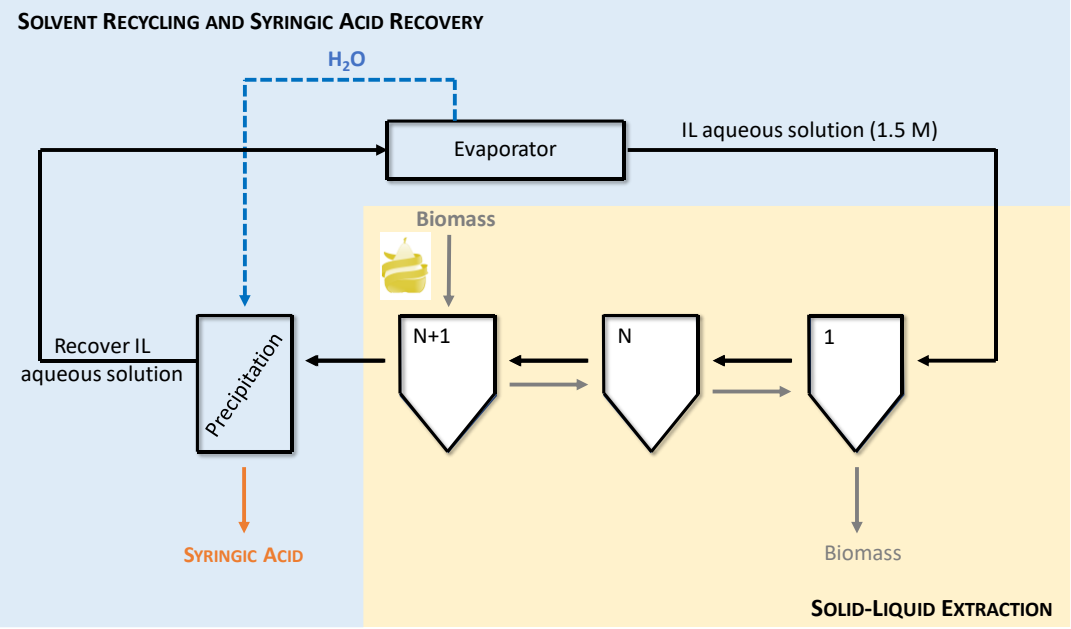


Fig. 5.5. Scheme of the continuous extraction process operating in countercurrent.

Although the use of aqueous solutions of aprotic ILs has shown to be an alternative over pure ILs to extract value-added compounds from biomass, the non-volatile nature of aprotic ILs represents a major drawback when envisaging the target compound recovery since a simple evaporation step cannot be applied. Based on this limitation and knowing that the solubility of the target compound largely depends on the IL (hydrotrope) concentration as shown in **Figure 5.2**, the recovery of syringic acid from the IL aqueous solution was carried out by dilution with water, which acts as anti-solvent. Therefore, at the end of the fifth extraction cycle of the previous experiments (reuse/saturation of the solvent), the saturated IL aqueous solution (0.5 mL) was diluted with increased volumes of water (1, 5, 10, 15 and 25 mL) (cf. in the Appendix - Table S5.7 and Figure S5.2). By applying this process, 77% of the extracted syringic acid can be recovered by precipitation by the addition of the largest volume of water, and with high purity as shown in the chromatogram given in the Appendix (Figure S5.3). Finally, the recovery of

syringic acid by this process allows the elimination of the IL from syringic acid, thus overcoming the toxicity concerns that could be associated to ILs, and the IL recycling after an evaporation step to decrease the water content, as shown in the process given in **Figure 5.5**.

The obtained results demonstrate that aqueous solutions of ILs with hydrotrope characteristics are excellent solvents to dissolve and extract phenolic compounds from biomass, allowing the target compounds recovery by precipitation by the addition of water as anti-solvent the IL recycling.

Conclusions

It was here shown that aqueous solutions ILs that behave as cationic hydrotropes are remarkable solvents for syringic acid, leading to enhancements up to 84-fold in solubility when compared to pure water. Due to these solubility enhancements, the same ILs aqueous solutions lead to good extraction yields of syringic acid from pear peels. The operational extraction conditions were optimized by factorial planning resulting in a maximum extraction yield of 1.05 wt.%. With the goal of developing sustainable extraction strategies, both the biomass and the IL aqueous solutions were reused, allowing to reach extraction yields of syringic acid of 2.06 wt.% and 2.22 wt.%, respectively. These values are significantly higher than those obtained with dichloromethane and methanol at the same operational conditions (1.51 wt.% and 1.68 wt.%, respectively). Based on these results, and possibility of reusing both the solvent and biomass, a continuous process was conceptualized and proposed for large-scale applications, acting in continuous and countercurrent mode, in which the solvent is recycled after the recovery of syringic acid by precipitation with water as anti-solvent. This strategy allows to recover 77% of the extracted phenolic acid with high purity. The results reported here have a significant impact on the understanding of the role of ILs aqueous solutions in the extraction of value-added compounds from biomass as well as in the design of sustainable processes for their recovery.

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6. DEEP EUTECTIC SOLVENTS AS EFFICIENT MEDIA FOR THE EXTRACTION AND RECOVERY OF CYNAROPICRIN FROM *CYNARA CARDUNCULUS L.* LEAVES

Chapter based on the published article:

E.L.P. Faria, R.S. do Carmo, A.F. Cláudio, C.S.R. Freire, M.G. Freire and A.J.D. Silvestre. Deep eutectic as efficient media for the extraction and recovery of cynaropicrin from *Cynara cardunculus L.* leaves. *Int. J. Mol. Sci.*, **2017**, 18, pp. 2276.

Contributions: M.G.F. and A.J.D.S conceived and directed this work. E.L.P.F. and R.S.C. acquired the experimental data. E.L.P.F., A.F.C. C.S.R.F., A.J.D.S. and M.G.F. interpreted the experimental data. The manuscript was mainly written by E.L.P.F., M.G.F. and A.J.D.S with contributions from the remaining authors.

Abstract

In the past few years a high demand for natural ingredients with nutraceutical properties has been witnessed, for which the development of more environmentally-friendly and cost-efficient extraction solvents and methods play a primary role. In this perspective, in this work it was studied the application of deep eutectic solvents (DES), composed of quaternary ammonium salts and organic acids, as alternative solvents for the extraction of cynaropicrin from *Cynara cardunculus* L. leaves. After selecting the most promising DES, their aqueous solutions were investigated, allowing to obtain a maximum cynaropicrin extraction yield of 6.20 wt.%, using 70 wt.% of water. The sustainability of the extraction process was further optimized by carrying out several extraction cycles, reusing either the biomass or the aqueous solutions of DES. A maximum cynaropicrin extraction yield of 7.76 wt.% by reusing the solvent, and of 8.96 wt.% by reusing the biomass, have been obtained. Taking advantage of the cynaropicrin solubility limit in aqueous solutions according to the DES concentration, water was added as an anti-solvent, allowing to recover 73.6 wt.% of the extracted cynaropicrin. This work demonstrates the potential of aqueous solutions of DES for the extraction of value-added compounds from biomass and the possible recovery of both the target compounds and solvents.

Introduction

A growing awareness of human activities on the environment, as well as the need to obtain non-contaminated products with hazardous solvents, stimulated the development of "greener extraction" processes.[1] Green technologies seek for new solvents to replace common organic ones that present inherent toxicity and volatility problems.[2,3] Several types of alternative solvents, including deep eutectic solvents (DES), have been suggested as candidates within the development of greener processes.[4] First reported by Abbot et al.,[5] DES are composed of at least a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) species, which upon mixing establish strong hydrogen bond interactions leading to the formation of eutectic mixtures, often becoming liquid at conditions close to room temperature. The most common DES are based on choline chloride ([Ch]Cl) as the HBA, and HBDs comprising urea, polyols and carboxylic acids.[2] With respect to environmental and economic benefits, DES offer many advantages, including low volatility, simple preparation, low cost, and low toxicity profile.[1] As far as applications are concerned, it has been demonstrated that DES can be successfully used for the extraction of different types of natural bioactive compounds from biomass, including flavonoids,[6] ginsenosides,[7] anthocyanins,[8] catechins,[9] among others. Furthermore, it was recently demonstrated that they can also be used in the dissolution of more complex biomacromolecules, such as lignin.[10] Also worth noting, and similarly to ionic liquids,[11-13] it was demonstrated that aqueous solutions of DES can perform better than the pure solvents,[14] with inherent economic and process benefits. Finally, if benign or natural-based DES (the so called NADES) are used, these may be kept in the final formulations, in some cases also contributing to the improvement of the extracts biological properties,[15,16] again with a close similarity to ILs.[12]

Cardoon or wild artichoke (*C. cardunculus* L., Compositae) is a perennial plant, sharing a recent common ancestor with the modern cultivated ''globe artichoke", *C. scolymus L.*.[17] Both plants have their origin in edible *Cynara* cultivars, used by early farmers in the Mediterranean basin and Macaronesia (Madeira and Canary Islands).[18] Several bioactive compounds were already identified in *C. cardunculus* leaves and seeds,[17-19] namely saponins, flavones, sterols, coumarins and lignans, as well as sesquiterpene lactones, such as cynaropicrin.[20,21] Cynaropicrin **(Figure 6.1)**, is the main responsible for the bitter taste of *C. cardunculus* leaves, displaying a wide range of biological activities. These include anti-inflammatory,[22,23] antispasmodic,[24] antitrypanosomal,[25] and proapoptotic[26] properties, being therefore currently used as part of crude extracts in several nutraceutical formulations.

The extraction of cynaropicrin is most often performed using conventional and in some cases toxic organic solvents, such as chloroform,[27] and dichloromethane.[23] Other cynaropicrin

extraction methods/solvents include, for example, water at elevated temperatures,[28] or supercritical CO_2.[29] These methods require however the use of sophisticated equipment and are often highly energy consuming, in addition to the problems associated to the organic solvents commonly employed, which may lead to several human risks, safety issues and environmental impact.[30] To overcome some of these concerns, either regarding their environmental footprint or when used for the extraction of target compounds envisioned for human use, DES have emerged as promising alternative solvents.[2]

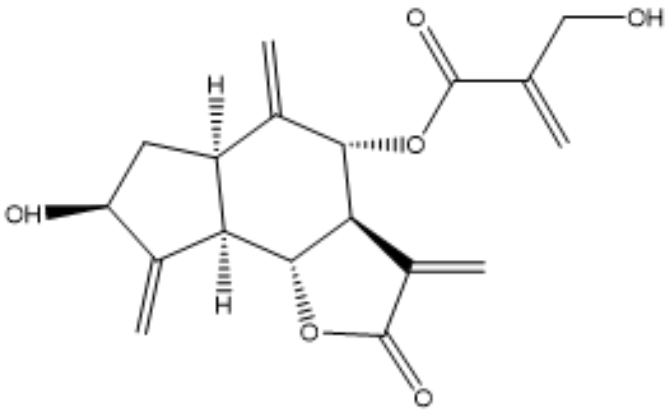

Fig. 6.1. Chemical structures of cynaropicrin.

Taking into account the biological potential of cynaropicrin, together with its abundance in *C. cardunculus* leaves (accounting for up to 87 g/kg),[23] its high commercial value[31] and nutraceutical applications, we propose here the use of DES as alternative solvents for the extraction of cynaropicrin from *C. cardunculus* leaves. An initial screening on DES (chemical structure and HBD: HBA molar ratio) was performed by the mixture of several carboxylic acids as HBD and quaternary ammonium salts as HBA. Some well-studied organic solvents were also investigated for comparison purposes. After identifying the most promising DES, the process variables (namely, solid–liquid ratio (S/L ratio), temperature (T), time of extraction (t) and percentage of water added to the DES) were optimized to maximize the cynaropicrin extraction yield. Finally, both the solvent and biomass reuse were addressed, and water was added as an anti-solvent allowing to recover the extracted cynaropicrin and to recycle the DES aqueous solution.

Materials and methods

Materials

C. cardunculus L. leaves were supplied in the dry form with a granulometry between 40-60 mesh, by the Experimental Centre of Agriculture School of the Polytechnic Institute of Beja, southern Portugal. A cynaropicrin standard was commercially purchased from Extrasynthesis, with a purity ≥ 97.5 wt.%. The compounds used in the formation of the DES, composed of quaternary ammonium salts as HBA and carboxylic acids as HBD, are the following: decanoic acid (purity of 99% purity) and cholinium chloride ([Ch]Cl, purity of 99%), both from Acros Organics, hexanoic acid (purity of 99%) and myristic acid (purity of 98%), both from Fluka, 12-hydroxystearic acid (purity of 95%) supplied by Alfa Aesar, and tetraethylammonium chloride ($[N_{2222}]Cl$), tetrabutylammonium chloride ($[N_{4444}]Cl$), tetrapropylammonium chloride ($[N_{3333}]Cl$), tetrapropylammonium bromide ($[N_{3333}]Br$), and tetrabutylammonium bromide ($[N_{4444}]Br$), all with a purity of 98% and purchased from Sigma-Aldrich. Common organic solvents were also tested as extraction media, namely acetone and dichloromethane with a purity of ≥ 99.99%, and n-hexane with a purity of 95%, all acquired from Sigma.

DES preparation

The preparation of DES was carried out by adding a known mass of HBD and HBA to a closed glass vial, where the solid mixture was homogenized by heating under constant stirring. The sample was heated gradually until the mixture formed a clear liquid inside the vial and was left for 1 h at this temperature under constant stirring. DES samples were then placed under constant stirring under vacuum at a temperature of 25°C for a minimum period of 48 h to remove possible volatile impurities. The list containing all DES prepared and used is provided in the Appendix Data. Aqueous solutions of DES were prepared gravimetrically, ± 10^{-4} g.

Cynaropicrin Extraction

Solid-liquid extractions of cynaropicrin from *C. cardunculus* leaves were carried out using a home-made aluminum dry oven, with controlled stirring and able to keep the temperature within ± 0.5°C. In all experiments stirring was kept at 1000 rpm. The extraction conditions ascertained and optimized to increase the cynaropicrin extraction yield were: temperature (25, 35 and 45°C); extraction time (30, 40, 50, 60, 120, 300 and 1440 min); solid-liquid (S/L) ratio (weight of biomass per weight of solvent, 1:10, 1:20, 1:30, 1:40 and 1:50); and percentage weight fraction of water added to the DES (0, 5, 10, 20, 30, 40, 50, 60, 70 and 100 wt.%). For

comparison purposes, the optimized conditions were applied using the conventional organic solvents and water. A sample of *C. cardunculus* leaves (5 g dry weight) was also Soxhlet extracted with dichloromethane (150 mL) for 7 h for comparison. After the extraction step, samples were centrifuged (Centrifuge 5804, Eppendorf), and the supernatant containing cynaropicrin filtered using a 0.20 µm syringe filter acquired at GE healthcare, Whatman. A 200 µL aliquot was taken from the supernatant of each assay, diluted with 800 µL of methanol, and filtered again. The quantification of cynaropicrin in each solution was carried out by HPLC-DAD (Shimadzu, model PROMINENCE). HPLC analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 µm C18 100 A, from Phenomenex. The mobile phase consisted of 75% (v:v) of water and 25% (v:v) of acetonitrile. The separation was conducted in isocratic mode, at a flow rate of 0.5 $mL.min^{-1}$, and using an injection volume of 10 µL. DAD was set at 198 nm. The column oven and the autosampler were operated at a controlled temperature of 30°C. The quantification of cynaropicrin was performed based on a calibration curve prepared using the pure cynaropicrin sample (sample) dissolved in methanol, R^2 = 0.9993. The cynaropicrin extraction yield is expressed as the percentage ratio between the weight of cynaropicrin and the total weight of the dried biomass. The results presented along the manuscript correspond to the average of three independent experiments with an associated error of < 5% in the extraction yield.

Reusability of the solvent and biomass

In order to develop a more sustainable extraction-recovery process, both 4 successive cycles of extraction using the same solvent and 6 new aqueous solutions of DES were applied to the same biomass. The main objective of this study was to appraise the solvent saturation and to infer the maximum amount of the target compound present in the biomass.

Recovery of the extracted cynaropicrin

After the 4 extraction cycles with the reuse of the same aqueous solution of DES, which allowed the solvent saturation, water was added as an anti-solvent, inducing the precipitation of cynaropicrin. Different amounts of added water were investigated; 0.5 mL of the DES aqueous solutions containing cynaropicrin were diluted in 5, 10, 15, 25 and 50 mL of distilled water. After the addition of water, the solution was centrifuged at 6000 rpm for 30 min, and vacuum filtered with a 0.45 µm microporous membrane. The remaining cynaropicrin in the solvent was quantified by HPLC-DAD as described before. Further, the solid residue was dried in an air oven

at 50°C for 2 days. A sample of the recovered precipitate was redissolved in 9 mL of methanol and cynaropicrin identified and quantified by HPLC-DAD.

Results and discussion

Screening of DES as solvents for the extraction of cynaropicrin

Several DES were investigated to identify the best solvents for cynaropicrin extraction from *C. cardunculus* leaves. The studied DES, described in detail in the Appendix Data, were prepared combining HBDs (carboxylic acids) with HBAs (quaternary ammonium salts) at different molar ratios. Those identified as liquid at 25°C were then used in the extraction assays. The respective pure acids that are liquid at the same temperature were also investigated for comparison purposes. Fixed operational conditions were used in this screening, namely a S/L ratio of 1:10, an extraction time of 120 min and a temperature of 25°C. The results obtained are depicted in **Figure 6.2** (detailed data is provided in the Appendix Data).

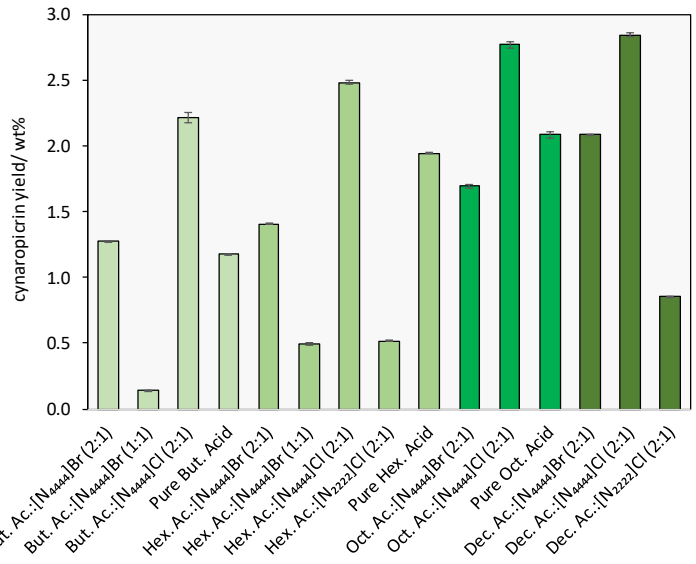

Fig. 6.2. Cynaropicrin extraction yield from *C. cardunculus* leaves using several DES at different molar ratios (HBD:HBA) and the corresponding pure acids; T = 25°C, t = 120 min and S/L ratio = 1.10.

Cynaropicrin extraction yields obtained with DES ranged from 0.14 to 2.84 wt.%. In general, there is an increase in the extraction efficiency of cynaropicrin with the increase of the carboxylic acid alkyl chain. If the HBA is kept constant, e.g. $[N_{4444}]Cl$ or $[N_{4444}]Br$, the DES comprising butanoic and decanoic acids are the ones which exhibit lower and higher extraction yields, respectively **(Figure. 6.2)**. This finding is in agreement with previous observations showing that

the extraction of volatile aliphatic components (namely aliphatic acids) increases with the alkyl chain length of both the HBA and HBD.[32] Furthermore, chloride-based salts lead to higher extraction yields of cynaropicrin when compared to the bromide counterparts.

Figure 6.2 also shows an increase in the cynaropicrin extraction efficiency by decreasing the amount of the HBA species, with DES based on a 2:1 molar ratio of HBD:HBA leading to a higher extraction yield, which may be due to a decrease in the viscosity of the solvent, as reported in previous studies.[33,34]

The potential of DES was further evaluated by comparison with the corresponding pure HBDs components. To this end, the cynaropicrin extraction yields using the liquid carboxylic acids at 25°C, namely butanoic, hexanoic and octanoic acids were investigated (it was not possible to test pure decanoic acid because it is solid at 25°C). The results obtained show that the cynaropicrin extraction yields are higher when using DES instead of the corresponding pure HBDs. In summary, the overall results demonstrate that among the tested DES, the most efficient for the extraction of cynaropicrin from *C. cardunculus* leaves is decanoic acid:[N_{4444}]Cl, in a 2:1 molar ratio.

Optimization of the cynaropicrin extraction conditions

Extraction conditions were optimized to improve cynaropicrin extraction yield using the decanoic acid:[N_{4444}]Cl 2:1 DES. The parameters studied were the extraction time (t, min), solid-liquid ratio (S/L ratio), temperature (T, °C) and water content (wt.%). The influence of each variable in the cynaropicrin extraction yield is illustrated in **Figure 6.3**. The respective detailed data are provided in Appendix Data.

The extraction temperature was optimized, performing extractions at 25, 35 and 45°C, while keeping constant the remaining conditions (S/L ratio of 1:10, 120 min) – **Figure 6.3A**. The maximum extraction yield, 2.84 wt.%, was achieved at 25°C. Higher temperatures lead to a decrease in the cynaropicrin extraction yield. Although this behavior was expected, since an increase in temperature should promote an increased solubility and thus extraction efficiency, the lower extraction yields at higher temperatures can be due to the simultaneous dissolution of polysaccharides, which at higher temperatures lead to extremely viscous solutions and hampers the filtration process and the recovery of cynaropicrin. However, these results can be considered as promising, since high extraction yields are obtained at low temperatures with minimum energy consumptions.

The influence of the extraction time (30, 40, 50, 60, 120, 300 and 1440 min) was further evaluated, keeping constant the remaining extraction conditions (S/L ratio of 1:10 and a temperature of 25°C (detailed data are provided in the Appendix Data). Considering the results

presented in **Figure 6.3B**, a remarkable increase in the extraction yield up to 60 min, with a maximum extraction yield of 3.13 wt.% was observed. From 60 min onwards, the extraction efficiency tends to gradually decrease. This tendency to pass through a maximum in the extraction yield has been reported before by several authors, either in the extraction of bioactive compounds from natural sources using deep eutectic solvents,[35,36] or even with conventional solvents such as ethanol.[37] In the present study, these results are again most probably due to the co-dissolution of polysaccharides from biomass, since an increase in the solvent viscosity is observed after 60 min, which again is expected to hinder the recovery of cynaropicrin.

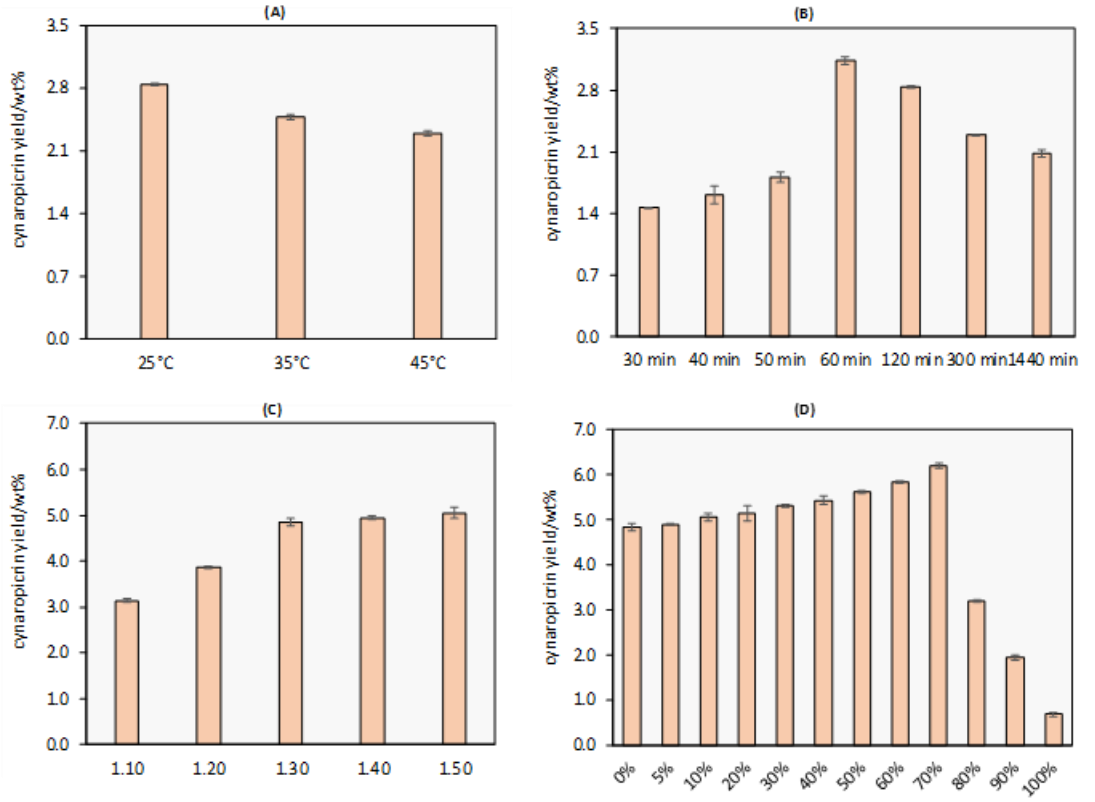

Fig. 6.3. Optimization of cynaropicrin the extraction yields from *C. cardunculus* leaves using decanoic acid:[N_{4444}]Cl at 2:1: (A) temperature (T); (B) extraction time (t); (C) S/L ratio; and (D) wt.% of water in DES.

The third tested operational condition in the extraction process was the S/L ratio, an essential parameter since a lower ratio is associated with higher costs of the process and eventually solvent wastes.[34] We tested the 1:10, 1:20, 1:30, 1:40 and 1:50 S/L ratios, keeping constant the remaining conditions, namely a temperature of 25°C and an extraction time of 60 min (detailed data are provided in the Appendix Data). According to the results shown in **Figure 6.3C**, the S/L ratio, shows to be a significant parameter. Decreasing the S/L ratio leads to an increase in the

cynaropicrin extraction yield, reaching a maximum of 5.06 wt.% using a ratio of 1:50, although between the S/L ratios of 1:30 and 1:50, the obtained yields are similar (4.84 and 5.05 wt.%, respectively). A similar behavior was observed by Wei et al.,[38] who studied the extraction of bioactive compounds from *Cajanus cajan* using DES and found that the extraction yields increase by decreasing the S/L ratio (from 1:10 to 1:40). Therefore, we propose the S/L ratio of 1:30 as the most adequate for cynaropicrin extraction since it implies a lower amount of solvent, and thus lower costs and generated wastes.

Finally, the effect of the water percentage added to the DES was investigated **(Figure 6.3D)**, keeping the temperature at 25°C, an extraction time of 60 min and a S/L ratio of 1:30 (detailed data are provided in the Appendix Data). An increase in the water content from 5 wt.% up to 70 wt.% promotes a gradual increase in the cynaropicrin extraction yield, up to a maximum of 6.20 wt.%. In fact, the addition of water to DESs has shown to be advantageous either to improve the solubility or extraction of target bioactive compounds.[10,34,38] The results obtained with aqueous solutions of DES are advantageous over pure DES, either from an environmental or economic point of view when envisaging the development of large scale processes. In summary, the overall results highlight the potential of aqueous solutions of DES to extract cynaropicrin from biomass, using mild conditions (25°C, 60 min, S/L ratio of 1:30 and 70 wt.% of water added to DES).

The best extraction conditions were then applied in a comparative extraction study using different organic solvents, namely n-hexane, acetone, and dichloromethane, commonly used in the extraction of cynaropicrin, as well as pure water. A soxhlet extraction with dichloromethane was also carried out. The results obtained are depicted in **Figure 6.4** whereas the detailed results are provided in the Appendix Data.

The maximum cynaropicrin extraction yield was achieved by Soxhlet extraction with dichloromethane (8.65 wt.%), in agreement with previously published results.[23] The higher yields obtained with this technique, when compared to solid-liquid extractions with the same solvent (4.53 wt.%) are due to the specific extraction conditions enabled by the Soxhlet extraction (high temperature and reuse of fresh solvent in multiple extraction cycles) that allows to reach the maximum extraction capacity. All the remaining solvents lead to lower cynaropicrin extraction yields when compared to those obtained with the above studied DES aqueous solution (6.20 wt.%), further supporting the high potential of this type of solvents to extract cynaropicrin from *C. cardunculus* leaves under mild conditions.

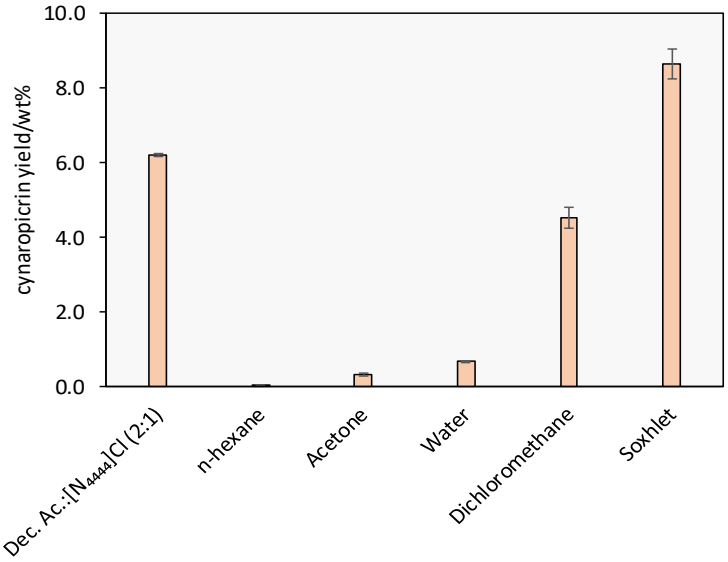

Fig. 6.4. Cynaropicrin extraction yields from *C. cardunculus* leaves with the solution composed of 70% of water in DES and several molecular solvents at fixed conditions: T = 25°C, t = 60 min, a S/L ratio = 1:30.

Reusability of the solvent and biomass

Aiming to maximise the cynaropicrin recovery, the same sample of *C. cardunculus* leaves was sequentially extracted with fresh DES aqueous solutions (70 wt.% of water in decanoic acid:[N_{4444}]Cl 2:1) in six successive cycles, keeping the remaining optimized extraction conditions reported above. The results obtained are shown in **Figure 6.5** and given in detail in the Appendix Data.

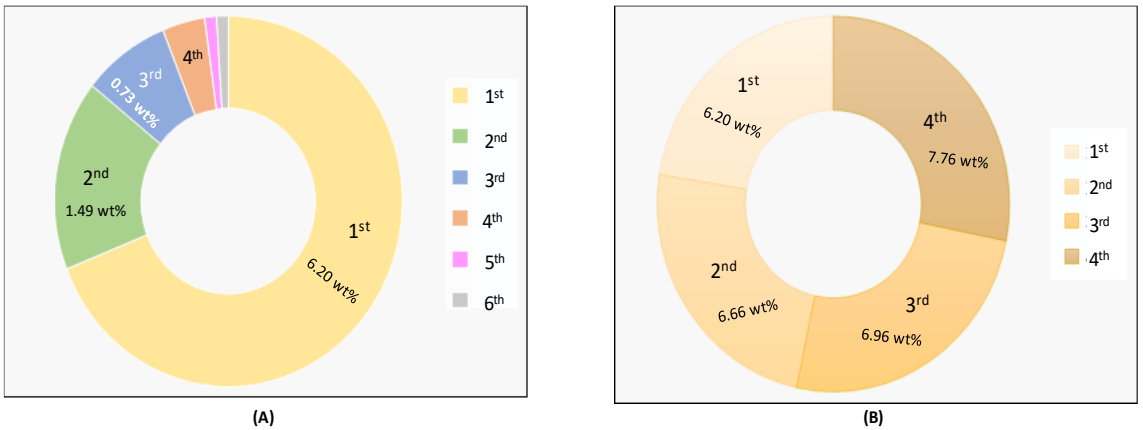

Fig. 6.5. Cynaropicrin extraction yields from *C. cardunculus* leaves with the biomass (A) and solvent (B) recycle at fixed conditions: 70 wt.% of water in decanoic acid:[N_{4444}]Cl (2:1) , T = 25°C, t = 60 min and S/L ratio = 1:30.

According to **Figure 6.5**, it is observed that the cynaropicrin present in the biomass sample is not fully extracted in the first cycle (6.20 wt.%) and it is possible to achieve a maximum yield of 8.96 wt.% after 6 extraction cycles with fresh solvent, leading to an extraction yield similar to the one obtained by Soxhlet extraction with dichloromethane (8.65 wt.%). However, it should be remarked that aqueous solutions of more environmentally friendly compounds are being used in the first case, together with significantly lower temperatures and times of extraction. Overall, after the third cycle, the amount of cynaropicrin extracted is not significant to justify additional extraction cycles (taking into account the economic impact).

In the same line, the use of the same DES aqueous solution in the cynaropicrin extraction using new *C. cardunculus* leaves samples was performed, for 4 cycles. The results obtained show that the extraction yield increases in each cycle; however, this increase is not significant, meaning that the solvent almost reached its saturation in the first cycle.

Recovery of cynaropicrin from the DES-based solvent

Taking into account the low solubility of cynaropicrin in water (1.75 g/L at 20°C),[39] the recovery of cynaropicrin from the DES aqueous solution was finally evaluated using water as anti-solvent. At the end of the 3rd extraction cycle of the previous experiments (reuse/saturation of the solvent), the saturated aqueous solution of DES (0.5 mL) was then diluted with increasing volumes of water (5, 10, 15, 25 and 50 mL). The gathered data are provided in the Supplementary Data. HPLC analysis of the remaining aqueous solution showed that up to 73.6% of the extracted cynaropicrin can be recovered by the addition of the largest volume of water. The recovery of cynaropicrin by this process further enables the recovery of the DES extraction solvent through the evaporation of the excess of water, allowing it further use.

Conclusions

The use of DES and their aqueous solutions have been investigated as alternative solvents for the extraction of the high-value cynaropicrin from *C. cardunculus* leaves. The use of long chain aliphatic organic acids as HBD, and chloride quaternary ammonium salts as HBA, allows to achieve improved cynaropicrin extraction yields, where the combination of decanoic acid:[N_{4444}]Cl 2:1 was found to be the most efficient. Several extraction conditions were then optimized, namely the extraction time, S/L ratio, temperature and content of water, leading to a maximum extraction yield of 6.20 wt.% of cynaropicrin using the decanoic acid:[N_{4444}]Cl (2:1) aqueous solution (70 wt.% of water), at 25°C, 60 min, and a S/L ratio of 1:30. Aiming the development of more sustainable extraction processes the reuse of the solvent and of the

biomass were also evaluated. Under the optimized conditions and with three cycles of fresh DES-water solvent, it was obtained an extraction yield of cynaropicrin of 8.96 wt.%, a similar value to that obtained with volatile organic solvents under soxhlet extractions yet applying milder conditions of temperature and time. Finally, 73.6% of the extracted cynaropicrin was recovered by the addition of water to the aqueous solutions of DES. In summary, DES and their aqueous solutions are remarkable alternatives to volatile organic solvents for the extraction of cynaropicrin from the *C. cardunculus* leaves and are therefore envisaged as promising candidates for the extraction of other compounds with nutraceutical interest.

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7. CONCLUSIONS & FUTURE WORK

Biomass and related by-products are key raw materials for the sustainable production of a wide variety of value-added chemicals. Accordingly, this thesis was focused on the study of alternative solvents, namely aqueous solutions of ILs and DES, for the extraction and separation of high-value compounds from vegetable biomass and related wastes, aiming at designing sustainable, cost-effective and integrated extraction/recovery strategies. In **Chapter 2** it was shown the successful extraction of triterpenic acids (TTAs, with antimicrobial, antitumor, hepatoprotective, anti-inflammatory, cytotoxic, anti-allergic and anti-HIV activities) from olive tree leaves (abundant residues of olive oil industries) using aqueous solutions of surface-active ionic liquids (ILs). Several operational conditions were optimized, such as the IL concentration, temperature and time of extraction. The results obtained confirm that aqueous solutions of surface-active ILs (in the range from 100 mM to 1000 mM) are outstanding solvents for the extraction of triterpenic acids from biomass residues and can provide improved extraction yields of up to 2.5 wt.% of oleanolic acid. In the same line, in **Chapter 3**, it was demonstrated that aqueous solutions of surfactant ILs are promising solvents for the extraction of TTAs over the commonly used volatile organic solvents, allowing the simultaneous extraction of betulinic, oleanolic and ursolic acids from apple peels. It was additionally demonstrated that surface-active ILs allow a significant increase in the solubility of ursolic acid in aqueous media – with an enhancement of 8 orders of magnitude when compared with its solubility in pure water. A total extraction yield of TTAs of 2.62 wt.% at moderate conditions was achieved with ILs aqueous solutions, overwhelming the extraction yields of 2.48 wt.% obtained with chloroform and 1.37 wt.% with acetone at similar conditions. With the objective of extracting cynaropicrin from the leaves of *C. cardunculus L.*, and with this to overcome common problems associated with volatile organic solvents, aqueous solutions of surface-active ILs were also investigated as alternative solvents (**Chapter 4**). The extraction yields of cynaropicrin depend on the CMC and chemical structure of the IL used. The concentration of IL, temperature, extraction time and solid-liquid ratio were tailored to increase the extraction yield of cynaropicrin. The best conditions were obtained with a concentration of 500 mM of $[C_{14}mim]Cl$, a temperature of 25°C, an extraction time of 60 minutes and a solid-liquid ratio of 1:20. Water was added as anti-solvent to induce the precipitation of the extracted compound from the IL-rich media.

In addition to the works in which it was demonstrated that aqueous solutions of surface-active ILs are promising solvents, in **Chapter 5** it is demonstrated that the extraction of syringic acid from pear peels is enhanced using aqueous solutions of ILs with hydrotrope characteristics. The results obtained reveal an enhancement in the solubility of syringic acid in aqueous solutions of ILs up to 84-fold when compared to pure water. The best ILs aqueous solutions were used to extract the target phenolic compound from *Rocha* pear peels, in which a response surface

methodology was applied to optimize the operational conditions. The sustainability of the extraction process was further optimized by carrying out several extraction cycles, reusing either the biomass or the IL aqueous solution. A maximum extraction yield of syringic acid of 2.06 wt.% by reusing the solvent and of 2.22 wt.% by reusing the biomass have been obtained. These values are higher than those obtained with dichloromethane and methanol at the same operational conditions (1.51 wt.% and 1.68 wt.%, respectively). After the syringic acid extraction and taking advantage of its solubility dependence with the IL concentration, water was added as an anti-solvent, allowing to recover 77 wt.% of the extracted target compound.

In **Chapter 6**, DES (composed of quaternary ammonium-based salts and organic acids) and their aqueous solutions were investigated for the extraction and recovery of cynaropicrin from the same biomass samples of Chapter 4. In this work it is shown that the size of the aliphatic chain of the organic acid used as HBD in the DES influences the extraction efficiency. Overall, the use of long-chain aliphatic organic acids as HBD allows the achievement of superior cynaropicrin extraction yields. It was additionally shown that the use of aqueous solutions of DES allows a higher extraction of cynaropicrin than pure DES and traditional organic solvents. It was also evaluated the reuse of the solvent and of the biomass aiming the development of more sustainable extraction and recovery processes, allowing a recovery yield of 73.6 wt.% of cynaropicrin. In summary, DES are here presented as an interesting alternative over organic solvents in the extraction of value-added compounds from biomass.

Although the general high performance of ILs and DES to extract value-added compounds from biomass, as demonstrated in this work, the best identified solvents are of different chemical structure, which further depend on the nature of the value-added compounds and on the biomass used. At this point, the crucial step to be developed is to go further into the biorefinery and circular economy concepts. The next step should consist on the study of more environmentally benign and biocompatible ILs (such as amino-acid-based, glycine-betaine derived, among others) instead of the imidazolium-based ones largely investigated up to date. After identifying more promising IL alternatives, the selective and sequential extraction of different types of compounds from the same biomass should be addressed. According to the literature and data gathered in this work, the selectivity of ILs for value-added compounds from biomass can be manipulated by the adequate selection of the cation/anion combination. Therefore, the selective and sequential extraction of phenolic compounds and triterpenic acids from the same biomass sample is possible, for instance by applying sequentially two types of ILs aqueous solutions, namely hydrotropic and surface-active ILs. Finally, with the aim of developing sustainable strategies for the extraction and recovery of natural compounds it is essential to

further optimize the products recovery and IL or DES recycling steps, and to consider the technologies scale-up.

LIST OF PUBLICATIONS

List of publications in the current thesis

1. Ana Filipa M. Cláudio, Alice Cognigni, **Emanuelle L. P. de Faria**, Armando J. D. Silvestre, Ronald Zirbs, Mara G. Freire, Katharina Bica, "Valorization of olive tree leaves: Extraction of oleanolic acid using aqueous solutions of surface-active ionic liquids," Sep. Purif. Technol., 2018, 204, pp. 30-37.
2. **Emanuelle L. P. de Faria**, Selesa V. Shabudin, Ana Filipa M. Cláudio, Mónica Válega, Fernando M.J. Domingues, Carmen S. R. Freire, Armando J. D. Silvestre, Mara G. Freire, "Aqueous solutions of surface-active ionic liquids: remarkable alternative solvents to improve the solubility of triterpenic acids and their extraction from biomass", ACS Sustain. Chem. Eng., 2017, 5, pp. 7344–7351.
3. **Emanuelle L. P. de Faria**, Melissa V. Gomes, Ana Filipa M. Cláudio, Carmen S. R. Freire, Armando J. D. Silvestre, Mara G. Freire, "Extraction and recovery processes for cynaropicrin from *Cynara cardunculus L.* using aqueous solutions of surface-active ionic liquids," Biophys. Rev., 2018, 10, pp. 915-925.
4. **Emanuelle L. P. de Faria**, Ana Filipa M. Cláudio, Carmen S. R. Freire, João A. P. Coutinho, Armando J. D. Silvestre, Mara G. Freire, "Recovery of syringic acid from industrial food waste with aqueous solution of ionic liquids", ACS Sustain. Chem. Eng., 2019. Submitted.
5. **Emanuelle L. P. de Faria**, Rafael S. Carmo, Ana Filipa M. Cláudio, Carmen S. R. Freire, Mara G. Freire, Armando J. D. Silvestre, "Deep eutectic solvents as efficient media for the extraction and recovery of cynaropicrin from *Cynara cardunculus L.* leaves," Int. J. Mol. Sci., 2017, 18, pp. 2276-2285.

Other publications

1. Ranyere L. de Souza, **Emanuelle L. P. de Faria**, Renan T. Figueiredo, Silvana Mettedi, Onélia A. A. dos Santos, Álvaro S. Lima, Cleide M. F. Soares, "Protic ionic liquid applied to enhance the immobilization of lipase in sol–gel matrices", J. Therm. Anal. Calorim., 2017, 128, pp. 833-840.
2. Ana Karine F. Carvalho, **Emanuelle L. P. de Faria**, Juan D. Rivaldi, Grazielle S. S. Andrade, Pedro Carlos Oliveira, Heizir F. de Castro, "Performance of whole-cells lipase derived from *Mucor circinelloides* as a catalyst in the ethanolysis of non-edible vegetable oils under batch and continuous run conditions", Ind. Crops Prod., 2015, 67, pp. 287-294.
3. Ranyere L. Souza, **Emanuelle L. P. de Faria**, Renan T. Figueiredo, Alini Fricks, Gisella M. Zanin, Onélia A. A. dos Santos, Álvaro S. Lima, Cleide M. F. Soares, "Use of polyethylene glycol in the process of sol-gel encapsulation of *Burkholderia cepacia* lipase", J. Therm. Anal. Calorim., 2014, 117, pp. 301-306.
4. Ranyere L. de Souza, **Emanuelle L. P. de Faria**, Rena T. Figueiredo, Lisiane S. Freitas, Miguel Iglesias, Silvana Mattedi, Onélia A. A. dos Santos, João A. P. Coutinho, Álvaro S. Lima, Cleide M. F. Soares, "Protic ionic liquid as additive on lipase immobilization using silica sol-gel", Enzyme Microb. Technol., 2013, 52, pp. 141-150.

Conference Papers

5. **Emanuelle L. P. de Faria**, Juan D. Rivaldi, Ana Karine F. de Carvalho, Heizir F. de Castro, "Síntese enzimática de monoésteres de etila catalisada por células íntegras de fungo filamentoso em reator de leito fixo", Conference - XX Congresso Brasileiro de Engenharia Química, 2015. DOI: 10.5151/chemeng-cobeq2014-1554-18689-149861.
6. **Emanuelle L. P. de Faria**, Daniela V. Cortez, Pedro C. Oliveira, Heizir F. de Castro, Influência do tempo espacial na produção de monoésteres de etila por células íntegras de *Mucor circinelloides* em reator de leito fixo a partir da etanólise de óleo de coco", Conference - Simpósio Nacional de Bioprocessos e Simpósio de Hidrólise Enzimática de Biomassa, 2015. DOI: 10.17648/sinaferm-2015-33476.

APPENDIX

Appendix A

Chapter 2. Valorization of olive tree leaves: extraction of oleanolic acid using aqueous solutions of surface-active ionic liquids.

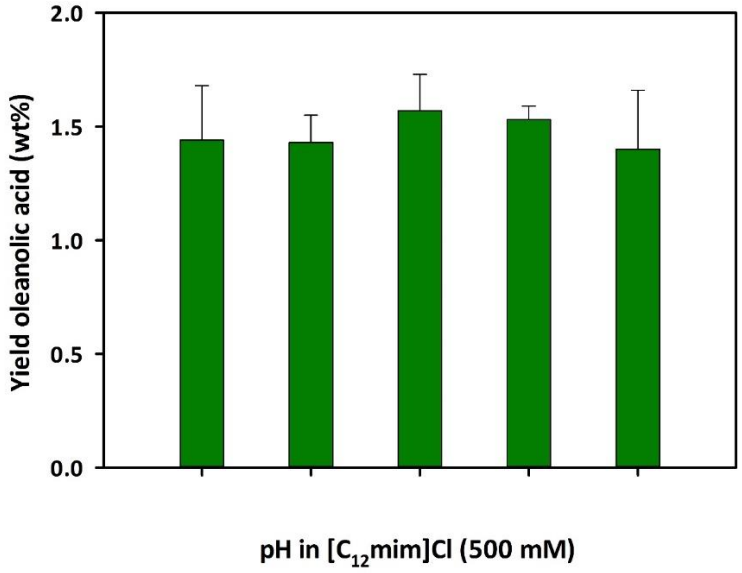

Fig. S2.1. Impact on pH variation on the extraction yield of oleanolic acid in aqueous solutions of the surface-active ionic liquid [C_{12}mim]Cl.

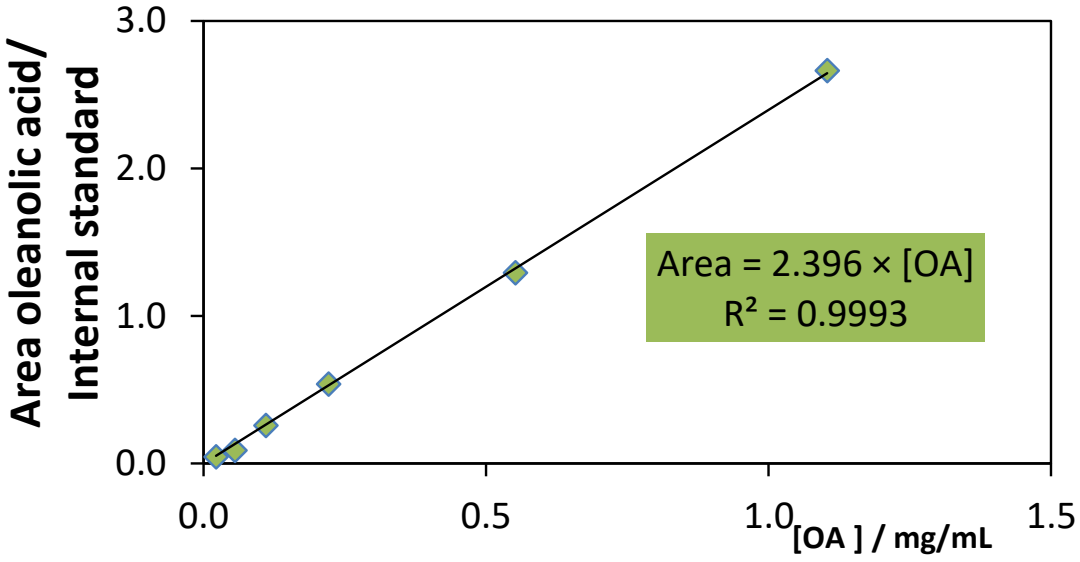

Fig. S2.2. Calibration curve for the quantification of oleanolic acid (OA) in the range of 0.01 – 1.2 mg/mL using 1-methylcyclohexene as internal standard.

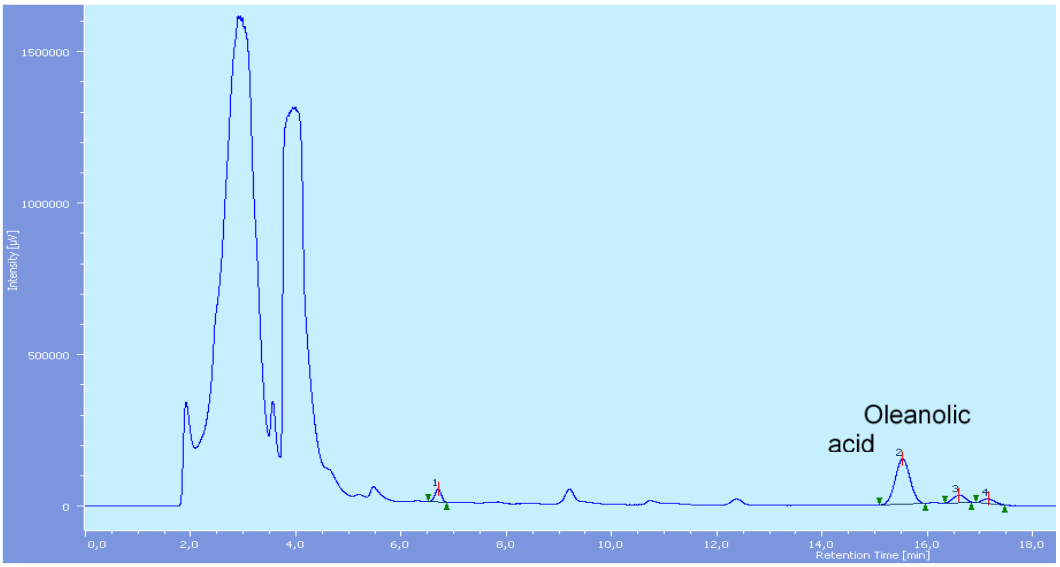

Fig. S2.3. Representative chromatogram of an olive tree leave sample extracted with aqueous ionic liquid solution. Oleanolic acid is eluted at a retention time of 15.5 min.

Appendix B

Chapter 3. Aqueous solutions of surface-active ionic liquids: remarkable alternative solvents to improve the solubility of triterpenic acids and their extraction from biomass.

Table S3.1. Solubility of ursolic acid in aqueous solutions of ILs at 30°C.

Ionic Liquid/Concentration	[Ursolic Acid] ± σ / (g.L^{-1})					
	50 mM	150 mM	250 mM	500 mM	750 mM	1000 mM
$[C_8C_1im]Cl$	-	0.004 ±0.001	0.028 ±0.001	0.070 ±0.003	0.280 ±0.016	0.435 ±0.007
$[C_{10}C_1im]Cl$	0.007 ±0.000	0.152 ±0.001	0.468 ±0.009	1.047 ±0.439	1.562 ±0.048	1.612 ±0.014
$[C_{12}C_1im]Cl$	0.059 ±0.001	0.523 ±0.009	0.719 ±0.045	1.071 ±0.068	1.435 ±0.028	1.714 ±0.052
$[C_{14}C_1im]Cl$	0.324 ±0.007	0.417 ±0.023	0.826 ±0.008	1.670 ±0.038	1.913 ±0.005	2.851 ±0.009
$[C_{16}C_1im]Cl$	0.227 ±0.005	0.895 ±0.010	1.403 ±0.036	2.489 ±0.057	0.826 ±0.110	ND
$[C_{18}C_1im]Cl$	0.418 ±0.019	1.202 ±0.018	ND	ND	ND	ND
$[C_4C_1im][C_8H_{17}SO_4]$	0.181 ±0.007	1.909 ±0.001	3.130 ±0.001	1.849 ±0.005	0.948 ±0.005	1.343 ±0.050
$[P_{444,14}]Cl$	0.235 ±0.003	0.298 ±0.003	0.348 ±0.003	0.280 ±0.004	0.389 ±0.006	0.813 ±0.029

*ND – Not determined.

Table S3.2. Solubility of ursolic acid in aqueous solutions of conventional surfactants at 30°C.

Conventional Surfactant/Concentration	[Ursolic Acid] ± σ / (g.L^{-1})					
	50 mM	150 mM	250 mM	500 mM	750 mM	1000 mM
CTAB	0.488 ±0.001	1.350 ±0.044	ND	ND	ND	ND
SDS	0.079 ±0.001	0.259 ±0.002	0.980 ±0.036	2.181 ±0.009	ND	ND
SDBS	1.240 ±0.077	2.088 ±0.066	2.480 ±0.045	ND	ND	ND

*ND – Not determined.

Table S3.3. CMC values of the studied surface-active ILs at 30°C.

[Ionic Liquids]	CMC (mM) ± σ (measured)	Literature
$[C_8C_1im]Cl$	233.0 ± 1.4	238.0[133]
$[C_{10}C_1im]Cl$	54.4 ± 0.8	57.2[175]; 55.0[176]
$[C_{12}C_1im]Cl$	14.4 ± 0.9	15.1[176]; 13.5[177]
$[C_{14}C_1im]Cl$	3.1 ± 0.1	3.8[239]; 3.6[177]
$[C_{16}C_1im]Cl$	1.0 ± 0.2	0.9[176,177]/ 1.1[176]
$[C_{18}C_1im]Cl$	0.43 ± 0.03	0.4[175]; 0.4[176]
$[C_4C_1im][C_8H_{17}SO_4]$	30.5 ± 0.7	31.0[168]

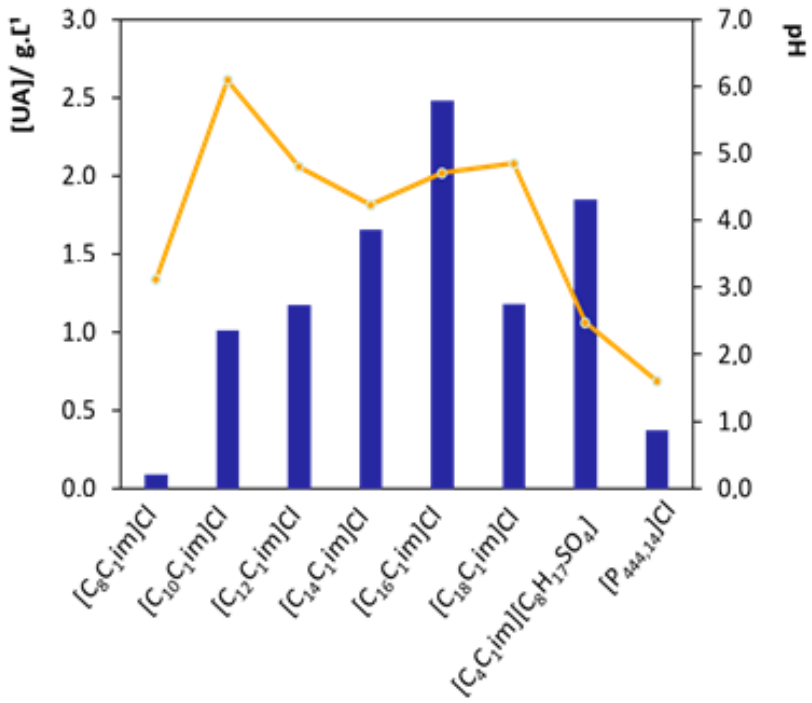

Fig. S3.1. pH (—) and UA solubility (—) in ILs aqueous solutions at 30°C.

Table S3.4. Extraction yields (EY) of TTAs (BA (betulinic), OA (oleanolic) and UA (ursolic) acids) from green apple peels with $[C_{14}C_1im]Cl$ at different temperatures and fixed conditions ([IL] = 500 mM, t = 60 min, $R_{S:L}$ = 1:10).

$[C_{14}C_1im]Cl$	Triterpenic Acids	T / °C	EY ± σ / wt.%
500 mM	UA	25	ND
		50	0.071 ± 0.001
		80	0.898 ± 0.001
		90	0.105 ± 0.002
500 mM	OA	25	ND
		50	0.437 ± 0.002
		80	1.638 ± 0.001
		90	1.561 ± 0.001
500 mM	UA	25	ND
		50	0.042 ± 0.001
		80	0.081 ± 0.002
		90	0.072 ± 0.002

*ND – Not determined.

Table S3.5. Extraction yields of TTAs (BA, OA and UA) extracted from green apple peels with volatile organic solvents at fixed conditions (T = 80°C under reflux, t = 60 min, $R_{S:L}$ = 1:10).

Solvent	Triterpenic Acids	EY ± σ / wt.%
Acetone	BA	0.824 ± 0.003
	OA	0.450 ± 0.006
	UA	0.090 ± 0.002
Chloroform	BA	1.393 ± 0.005
	OA	1.049 ± 0.001
	UA	0.032 ± 0.002

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Appendix C

Chapter 4. Extraction and recovery processes for cynaropicrin from *Cynara cardunculus L.* using aqueous solutions of surface-active ionic liquids.

Table S4.1. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with several ILs at different concentrations and other fixed conditions (S/L ratio = 1:10, T = 25°C and t = 60 min.

Ionic Liquid/Concentration	Cynaropicrin (wt%)		
	100 mM	500 mM	1000 mM
$[C_2mim]Cl$	ND	0.175 ± 0.009	ND
$[C_4mim]Cl$	0.833 ± 0.001	0.974 ± 0.001	1.186 ± 0.003
$[C_4mim](N(CN)_2)$	ND	1.512 ± 0.000	ND
$[C_8C_1im]Cl$	ND	1.669 ± 0.005	ND
$[C_{10}C_1im]Cl$	ND	2.050 ± 0.004	ND
$[C_{12}C_1im]Cl$	ND	2.867 ± 0.004	ND
$[C_{14}C_1im]Cl$	1.820 ± 0.002	3.182 ± 0.001	0.544 ± 0.005
$[Ch][C_8CO_2]$	ND	0.155 ± 0.001	ND
$[Ch][C_{10}CO_2]$	ND	0.174 ± 0.002	ND
$[Ch][C_{12}CO_2]$	ND	0.122 ± 0.002	ND

*ND – Not determined.

Table S4.2. CMC values of the studied surface-active ILs at 30ºC.

[Ionic Liquids]	CMC (mM) ± σ (measured in this work)	Literature
[C_8C_1im]Cl	233.0 ± 1.4	238.0[133]
[$C_{10}C_1$im]Cl	58.7 ± 0.8	57.2[175]; 55.0[176]
[$C_{12}C_1$im]Cl	15.2 ± 0.9	15.1[176]; 13.5[177]
[$C_{14}C_1$im]Cl	3.9 ± 0.1	3.8[239]; 3.6[177]
[Ch][C_8CO_2]	300.3 ± 0.9	383.0[179]
[Ch][$C_{10}CO_2$]	104.3 ± 0.7	103.3[179]
[Ch][$C_{12}CO_2$]	25.8 ± 0.3	ND

*ND – Not available in the literature.

Table S4.3. Optimization of the weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with aqueous solutions of [C_{14}mim]Cl at different concentrations and other fixed conditions (S/L ratio = 1:10, T = 25°C and t = 60 min).

Ionic Liquid/ Concentration	Cynaropicrin (wt%)							
	0 mM	**5 mM**	**10 mM**	**50 mM**	**100 mM**	**350 mM**	**500 mM**	**1000 mM**
[C_{14}mim]Cl	0.676 ±0.004	0.691 ±0.002	0.957 ±0.006	1.175 ±0.005	1.820 ±0.002	2.079 ±0.002	3.132 ±0.006	0.544 ±0.005

Table S4.4. Optimization of the weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with aqueous solutions of [C_{14}mim]Cl at different temperatures and other fixed conditions (S/L ratio = 1:10, [IL] = 500 mM and t = 60 min).

Ionic Liquid/Temperature	Cynaropicrin (wt%)		
	25ºC	**35ºC**	**45ºC**
[C_{14}mim]Cl	3.132 ±0.006	1.826 ±0.003	1.307 ±0.001

Table S4.5. Optimization of the weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with aqueous solutions of [C_{14}mim]Cl using different extraction times and other fixed conditions (S/L ratio = 1:10, [IL] = 500 mM and T = 25°C).

Ionic Liquid/ Extraction time	Cynaropicrin (wt%)							
	30 min	40 min	50 min	60 min	120 min	180 min	300 min	1440 min
[C_{14}mim]Cl	0.948 ±0.002	2.760 ±0.026	3.024 ±0.001	3.132 ±0.005	2.897 ±0.006	2.557 ±0.005	1.923 ±0.006	1.581 ±0.001

Table S4.6. Optimization of the weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with aqueous solutions of [C_{14}mim]Cl in different solid-liquid ratio and other fixed conditions ([IL] = 500 mM, T = 25°C and t = 60 min).

Ionic Liquid/Solid-liquid ratio	Cynaropicrin (wt%)			
	1:10	1:20	1:30	1:40
[C_{14}mim]Cl	3.132 ±0.006	3.727 ±0.006	4.001 ±0.001	4.093 ±0.003

Table S4.7. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with conventional surfactants and [C_{14}mim]Cl in different concentrations and other fixed conditions (S/L ratio = 1:20, t = 60 min, T = 25°C).

Ionic Liquid and Conventional Surfactant/ Concentration	Cynaropicrin (wt%)			
	10 mM	50 mM	100 mM	500 mM
[C_{14}mim]Cl	0.957 ±0.006	1.175 ±0.005	1.820 ±0.002	3.727 ±0.006
CTAB	0.627 ±0.009	0.946 ±0.007	1.346 ±0.018	ND
CTAC	0.658 ±0.006	1.420 ±0.000	1.019 ±0.005	0.403 ±0.012
CPC	0.650 ±0.009	0.890 ±0.000	1.417 ±0.001	2.033 ±0.000
SDS	0.745 ±0.001	0.818 ±0.001	0.962 ±0.012	ND
SDBS	1.082 ±0.001	1.106 ±0.006	1.629 ±0.008	1.518 ±0.001
Genapol	0.998 ±0.009	1.151 ±0.001	1.329 ±0.026	ND

*ND – Not determined.

Table S4.8. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with several solvents and [C_{14}mim]Cl at the following fixed conditions: S/L ratio = 1:20, t = 60 min, T = 25°C (IL at 500 mM).

Solvent	Cynaropicrin (wt%)
[C_{14}mim]Cl	3.727 ± 0.006
***n*-hexane**	0.037 ± 0.001
Acetone	0.346 ± 0.002
H_2O	0.676 ± 0.004
Dichloromethane	4.529 ± 0.027
Soxhlet	8.652 ± 0.041

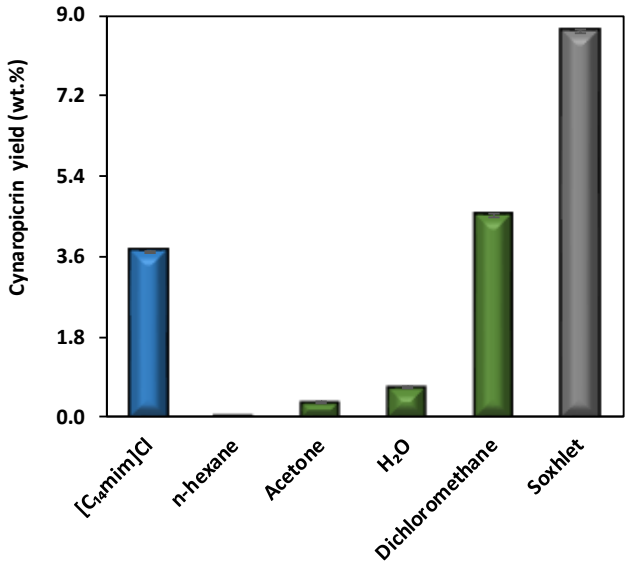

Figure S4.1. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with several solvents at the following fixed conditions: S/L ratio = 1:20, t = 60 min, T = 25°C ([C_{14}mim]Cl at 500 mM).

Table S4.9. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with several solvents and [C_{14}mim]Cl after 3 cycles of extraction using the same biomass. The fixed conditions are S/L ratio = 1:20, t = 60 min, T = 25°C and IL at 500 mM.

Solvent	Cynaropicrin (wt%)
[C_{14}mim]Cl (3cycles)	6.326 ± 0.006
Dichloromethane	4.529 ± 0.003
Soxhlet	8.652 ± 0.004

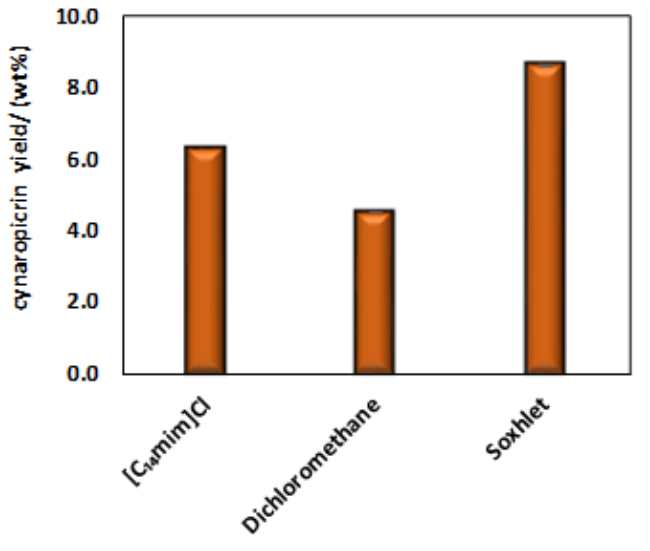

Figure S4.2. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L*. with several solvents and [C_{14}mim]Cl after 3 cycles of extraction using the same biomass. The fixed conditions are S/L ratio = 1:20, t = 60 min, T = 25°C and IL at 500 mM.

Table S4.10. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* using the surface-active IL solution and fresh biomass samples, at the following fixed conditions: S/L ratio = 1:20, t = 60 min, T = 25°C and [C_{14}mim]Cl at 500 mM.

Recycle	Cynaropicrin (wt%)
1 st	3.727 ± 0.006
2 nd	3.816 ± 0.004
3 rd	4.142 ± 0.008
4 th	4.160 ± 0.002

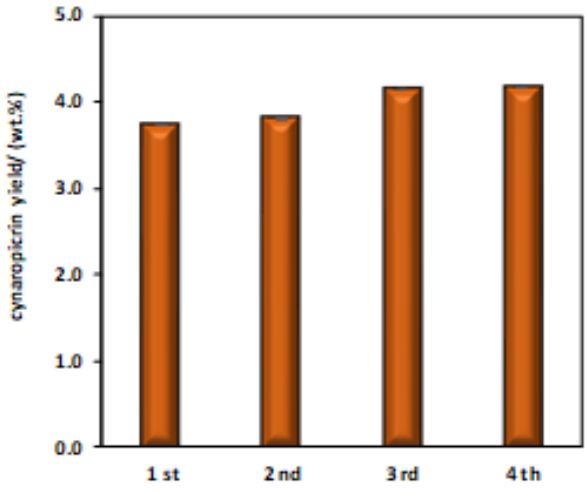

Figure S4.3. Weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* using the surface-active IL solution and fresh biomass samples, at the following fixed conditions: S/L ratio = 1:20, t = 60 min, T = 25°C and [C_{14}mim]Cl at 500 mM.

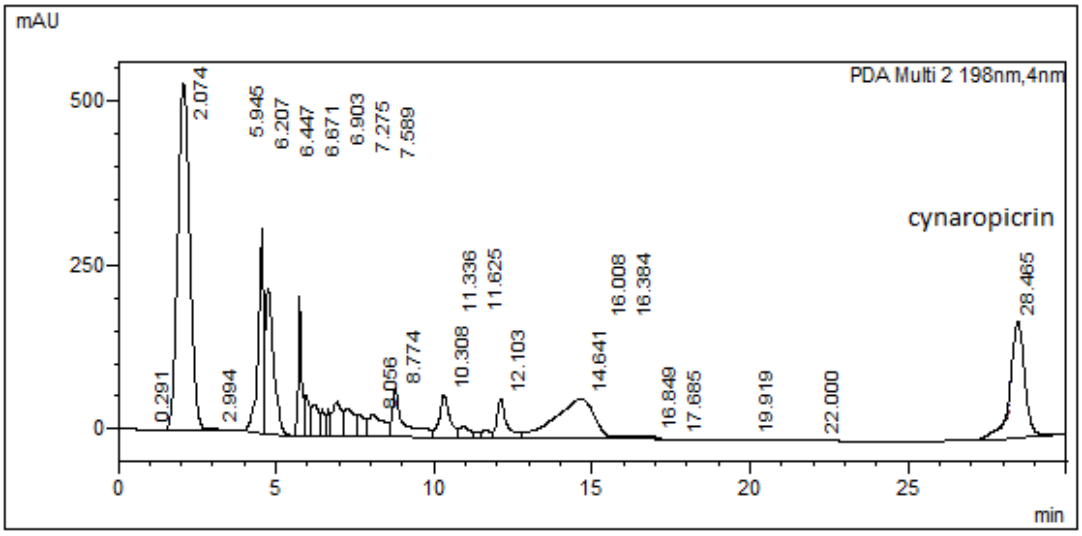

Figure S4.4. HPLC chromatogram of the cynaropicrin recovered from the leaves of *Cynara cardunculus* L. using the surface-active IL aqueous solution.

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Appendix D

Chapter 5. Recovery of syringic acid from industrial food waste with aqueous solution of ionic liquids.

Experimental section

Surface response methodology. In a 2^k factorial planning there are *k* factors that can contribute to a response described by a second order polynomial equation:

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \qquad \text{(S1)}$$

where y is the response variable (in this work, syringic acid extraction yield), and β_0, β_i, β_{ii} and β_{ij} are the adjusted coefficients for the intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables.

Figures/Tables

Table S5.1. 2^3 factorial planning.

	X_1	X_2	X_3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1.68	0	0
10	1.68	0	0
11	0	-1.68	0
12	0	1.68	0
13	0	0	-1.68
14	0	0	1.68
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

Table S5.2. Solubility of syringic acid in aqueous solutions of ILs.

IL	IL concentration / (mol/L)	Solubility / (g/L)	SD[a]	S/S_0
	0.00	1.43	0.08	1.00
$[C_4C_1im][TOS]$	0.10	1.88	0.02	1.31
	0.25	2.38	0.02	1.66
	0.50	15.64	0.01	10.93
	0.75	25.12	0.15	17.56
	1.00	28.77	0.23	20.12
	2.00	37.13	0.01	25.97
	2.50	65.36	0.38	45.71
	3.00	0.65	0.19	0.46
$[C_4C_1im][N(CN)_2]$	0.10	4.41	0.01	3.09
	0.25	9.49	0.06	6.64
	0.50	25.12	0.03	17.56
	0.75	27.69	0.15	19.36
	1.00	31.60	0.01	22.10
	2.00	63.88	0.08	44.67
	2.50	57.51	0.06	40.22
	3.00	43.43	0.01	30.37
$[C_4C_1im]Cl$	0.10	2.05	0.01	1.43
	0.25	2.99	0.02	2.09
	0.50	5.06	0.01	3.54
	0.75	7.06	0.01	4.94
	1.00	7.07	0.06	4.94
	2.00	34.82	0.08	24.35
	2.50	58.13	0.03	40.65
	3.00	92.00	0.07	64.34
	3.50	120.28	0.27	84.11
	4.00	50.60	0.29	35.38
$[C_4C_1im][HSO_4]$	0.10	1.88	0.01	1.31
	0.25	2.60	0.01	1.82
	0.50	4.43	0.01	3.10
	0.75	6.61	0.01	4.62
	1.00	9.47	0.26	6.62
	2.00	29.42	0.01	20.58
	2.50	11.27	0.01	7.88
$[C_4C_1im][SCN]$	0.10	2.19	0.02	1.53
	0.25	5.39	0.05	3.77
	0.50	6.54	0.03	4.57

	0.75	11.85	0.08	8.29
	1.00	32. 52	0.29	22.74
	2.00	23.46	0.04	16.41
	2.50	21.58	0.05	15.09
	4.00	11.18	0.09	7.82
$[P_{4444}]Cl$	0.10	2.47	0.01	1.73
	0.25	4.32	0.01	3.02
	0.50	13.17	0.03	9.21
	0.75	28.78	0.13	20.13
	1.00	37.06	0.20	25.92
	2.00	52.21	0.05	36.51
	2.50	71.99	0.13	50.34
	3.00	63.68	0.47	44.53
$[N_{4444}]Cl$	0.10	4.35	0.01	3.05
	0.25	6.92	0.03	4.84
	0.50	9.13	0.01	6.38
	0.75	10.03	0.01	7.02
	1.00	14.09	0.08	9.85
	2.00	46.91	0.55	32.80
	2.50	41.48	0.01	29.00
	3.00	30.22	0.04	21.13
$[C_4C_1pip]Cl$	0.10	1.93	0.01	1.35
	0.25	2.23	0.01	1.56
	0.50	4.84	0.01	3.38
	0.75	7.34	0.05	5.13
	1.00	13.72	0.01	9.60
	2.00	15.77	0.06	11.02
	2.50	35.83	0.31	25.06
	3.00	70.68	0.15	49.43
	4.00	46.04	0.31	32.20
$[C_8C_1im]Cl$	0.25	7.80	0.03	5.46
	0.50	21.64	0.03	15.13
	0.75	28.32	0.13	19.80
	1.00	17.42	0.01	12.18
	2.00	16.54	0.05	11.56
	3.00	14.69	0.04	10.27
	3.50	6.59	0.05	4.61
$[C_4C_1im][CH_3CO_2]$	0.25	11.45	0.03	8.01
	0.50	24.21	0.19	16.93
	0.75	34.53	0.15	24.15
	1.00	40.17	0.31	28.09
	2.00	49.73	0.25	34.78

	3.00	72.75	0.21	50.87
	3.50	57.18	0.21	39.99
$[C_4C_1pyrr]Cl$	0.10	2.08	0.01	1.45
	0.25	3.46	0.01	2.42
	0.50	4.17	0.01	2.92
	0.75	5.60	0.01	3.92
	1.00	7.65	0.01	5.35
	2.00	15.91	0.08	11.12
	2.50	17.56	0.04	12.28
	3.00	12.45	0.03	8.71
$[C_4C_1py]Cl$	0.25	24.41	0.18	17.07
	0.50	33.92	0.15	23.72
	0.75	42.13	0.01	29.46
	1.00	53.79	0.09	37.61
	2.00	65.44	0.25	45.76
	3.00	75.28	0.05	52.64
	4.00	13.03	0.00	9.11
$[P_{i(444)_1}][TOS]$	0.25	7.74	0.03	5.41
	0.75	28.59	0.33	19.99
	1.00	31.85	0.20	22.27
	2.00	38.00	0.43	26.57
	2.50	30.18	0.17	21.10
	3.00	22.18	0.14	15.51
[Ch]Cl	0.10	1.51	0.01	1.05
	0.25	1.64	0.01	1.15
	0.50	1.86	0.01	1.30
	0.75	1.97	0.01	1.38
	1.00	1.98	0.01	1.38
	2.00	2.35	0.01	1.64
[Ch][Ac]	0.25	14.43	0.11	10.09
	0.50	19.02	0.08	13.30
	0.75	20.50	0.33	14.34
	1.00	34.42	0.01	24.07
	2.00	34.53	0.33	24.15
	3.00	27.36	0.17	19.13
	3.50	17.59	0.13	12.30
[Ch][But]	0.10	7.68	0.01	5.37
	0.25	13.20	0.02	9.23
	0.50	19.58	0.17	13.69
	0.75	23.60	0.08	16.50
	1.00	27.60	0.25	19.30
	2.00	32.04	0.08	22.41

	2.50	41.11	0.25	28.75
	3.00	59.07	0.50	41.31
	3.50	23.92	0.12	16.73
[Ch][Hex]	0.25	23.20	0.06	16.22
	0.50	42.25	0.12	29.54
	0.75	59.07	0.34	41.31
	1.00	71.31	0.21	49.87
	2.00	85.69	0.28	59.92
	3.00	55.93	0.04	39.11
	4.00	1.74	0.05	1.22
[Ch][Oct]	0.25	20.77	0.13	14.53
	0.50	57.37	0.33	40.12
	0.75	62.23	0.16	43.52
	1.00	84.27	0.38	58.93
	2.00	57.91	0.50	40.50
	3.00	14.40	0.06	10.07
	3.50	5.41	0.05	3.79
[Ch][Dec]	0.10	18.28	0.02	12.78
	0.25	31.42	0.06	21.97
	0.50	24.86	0.03	17.38

[a]standard deviation in solubility results (in g/L)

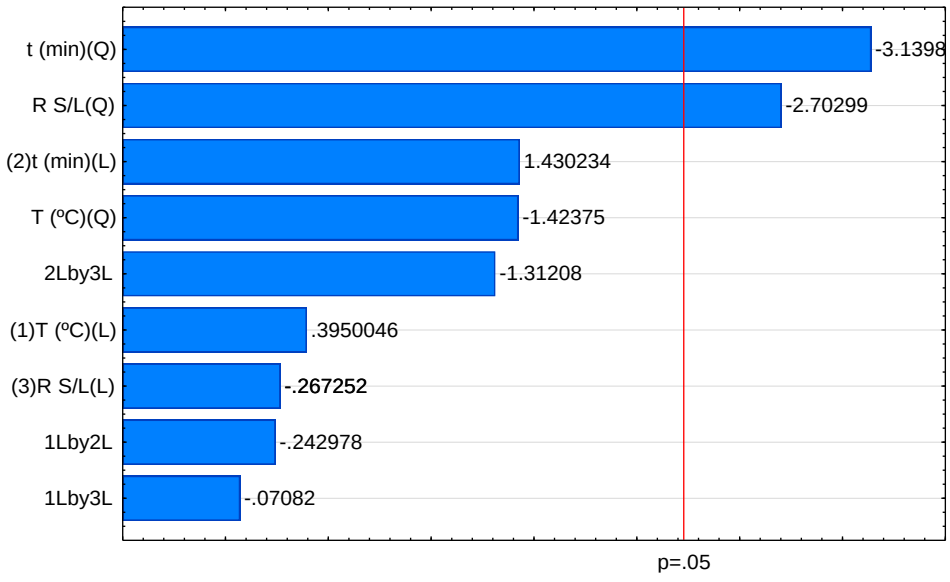

Fig. S5.1. Pareto chart for the standardized main effects in the factorial planning for the syringic acid extraction yield. The vertical line indicates the statistical significance of the several effects.

Table S5.3. Coded levels of independents variables used in the first and second factorial planning.

	Axial	**Factorial**	**Central**	**Factorial**	**Axial**
	-1.682	**-1**	**0**	**1**	**1.68**
Temperature (T)	29.8	38.0	50.0	62.0	70.16
Extraction time (t)	9.54	30.000	60.000	90.000	110.400
Solid-liquid ratio (R)	0.016	0.050	0.100	0.150	0.184

Table S5.4. Experimental data at several conditions evaluated.

Experiment	**T (^{o}C)**	**t (min)**	**Ratio S/L**	**Extraction yield of syringic acid (wt. %)**
1	38.00	30.0000	0.050	0.28
2	62.00	30.0000	0.050	0.46
3	38.00	90.0000	0.050	0.56
4	62.00	90.0000	0.050	0.73
5	38.00	30.0000	0.150	0.31
6	62.00	30.0000	0.150	0.53
7	38.00	90.0000	0.150	0.26
8	62.00	90.0000	0.150	0.34
9	29.82	60.0000	0.100	0.99
10	70.16	60.0000	0.100	0.78
11	50.00	9.5400	0.100	0.36
12	50.00	110.4000	0.100	0.86
13	50.00	60.0000	0.016	0.57
14	50.00	60.0000	0.184	0.80
15	50.00	60.0000	0.100	1.05
16	50.00	60.0000	0.100	0.87
17	50.00	60.0000	0.100	0.96
18	50.00	60.0000	0.100	0.91
19	50.00	60.0000	0.100	0.87
20	50.00	60.0000	0.100	1.01

Table S5.5. Syringic acid extraction yield according to the biomass reuse at best conditions (T = 50°C, t = 60 min and S/L ratio = 0.10, using aqueous solutions of $[C_4C_1im]Cl$ at 3.5 M).

Nº of extraction cycle	Syringic acid total yield (wt.%)
1st	1.05
2nd	0.49
3rd	0.39
4th	0.24
5th	0.05
Total	**2.22**

Table S5.6. Syringic acid extraction yield according to the reusability of the aqueous solution of $[C_4C_1im]Cl$ at 3.5 M.

Nº of extraction cycle	Syringic acid total yield (wt.%)
1st	1.07
2nd	0.44
3rd	0.24
4th	0.19
5th	0.09
Total	**2.04**

Table S5.7. Amount of water (mL) added and percentage of precipitation of syringic acid.

Amount of water (mL)	Syringic acid precipitation (%)
1	6.98
5	18.60
10	30.23
15	51.16
25	76.74

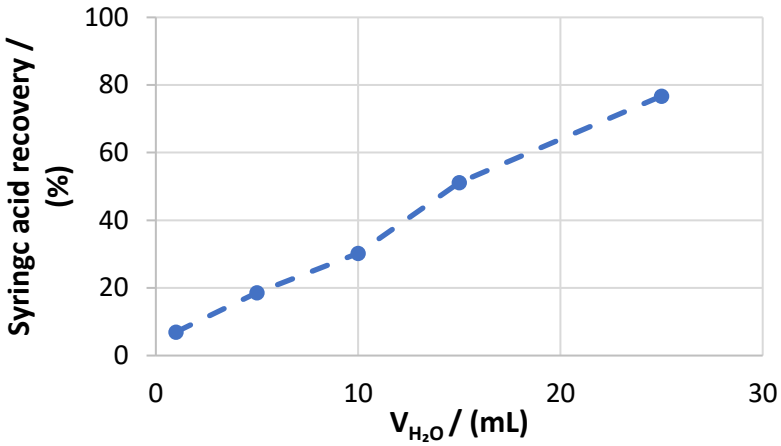

Fig. S5.2. Amount of water (mL) added and percentage of precipitation/recovery of syringic acid to 0.5 mL of the IL aqueous solution after the extraction.

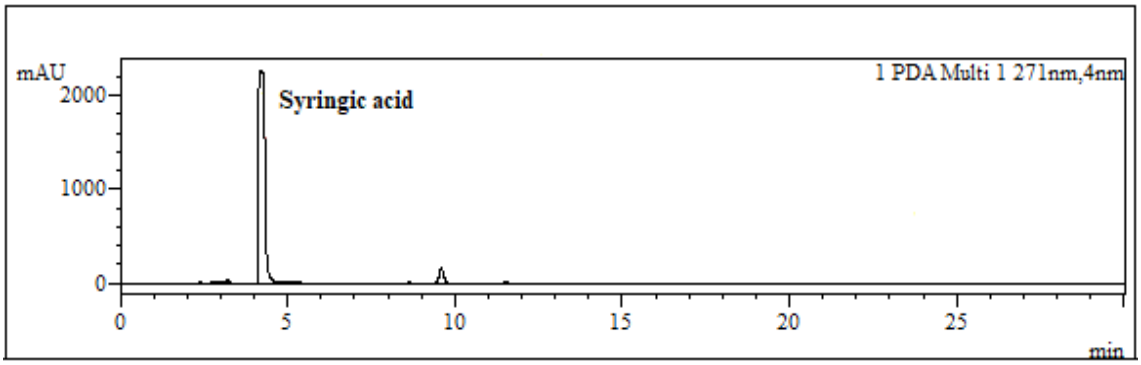

Fig. S5.3. HPLC-DAD chromatogram of the precipitated sample from the IL aqueous solution after addition of water.

Appendix E

Chapter 6. Deep eutectic solvents as efficient media for the extraction and recovery of cynaropicrin from *Cynara cardunculus L.* Leaves.

Table S6.1. Prepared DES, HBD:HBA ratio and physical state at room temperature (ca. 25^{o}C).

Hydrogen bond donor	Hydrogen bond Acceptor	HBD:HBA Ratio	Physical state
Decanoic acid	Choline chloride	1:1 / 2:1 / 1:2	Solid
Hexanoic acid	Choline chloride	2:1	Solid
Hexanoic acid	Choline chloride	4:1	Solid
Hexanoic acid	$[N_{2222}]Br$	2:1	Solid
Decanoic acid	$[N_{2222}]Br$	2:1	Solid
Hexanoic acid	$[N_{3333}]Br$	2:1	Solid
Decanoic acid	$[N_{3333}]Br$	2:1	Solid
Butanoic acid	$[N_{4444}]Br$	2:1	Liquid
Butanoic acid	$[N_{4444}]Br$	1:1	Liquid
Butanoic acid	$[N_{4444}]Br$	1:2	Solid
Caprylic acid/ hexanoic	$[N_{4444}]Br$	1:1	Liquid
Caprylic acid/ hexanoic	$[N_{4444}]Br$	2:1	Liquid
Caprylic acid/ hexanoic	$[N_{4444}]Br$	1:2	Solid
Octanoic acid	$[N_{4444}]Br$	2:1	Liquid
Octanoic acid	$[N_{4444}]Br$	1:1	Solid
Octanoic acid	$[N_{4444}]Br$	1:2	Solid
Decanoic acid	$[N_{4444}]Br$	2:1	Liquid

Decanoic acid	$[N_{4444}]Br$	1:1	Solid
Decanoic acid	$[N_{4444}]Br$	1:2	Solid
Lauric acid/ dodecanoic	$[N_{4444}]Br$	2:1	Liquid
Lauric acid/ dodecanoic	$[N_{4444}]Br$	1:1	Solid
Lauric acid/ dodecanoic	$[N_{4444}]Br$	1:2	Solid
Miristic acid/ tetradecanoic	$[N_{4444}]Br$	2:1	Solid
Hexanoic acid	$[N_{2222}]Cl$	2:1	Liquid
Decanoic acid	$[N_{2222}]Cl$	2:1	Liquid
Caprilic acid / hexanoic	$[N_{3333}]Cl$	2:1	Liquid
Caprilic acid/ Hexanoic	$[N_{3333}]Cl$	1:1	Solid
Caprilic acid/ Hexanoic	$[N_{3333}]Cl$	1:2	Solid
Decanoic acid	$[N_{3333}]Cl$	2:1	Liquid
Lauric acid/ dodecanoic	$[N_{3333}]Cl$	2:1	Liquid
Lauric acid/ dodecanoic	$[N_{3333}]Cl$	1:1	Solid
Lauric acid/ dodecanoic	$[N_{3333}]Cl$	1:2	Solid
Butanoic acid	$[N_{4444}]Cl$	2:1	Liquid
Butanoic acid	$[N_{4444}]Cl$	1:1	Solid
Butanoic acid	$[N_{4444}]Cl$	1:2	Solid
Hexanoic acid	$[N_{4444}]Cl$	2:1	Liquid
Hexanoic acid	$[N_{4444}]Cl$	1:1	Solid
Hexanoic acid	$[N_{4444}]Cl$	1:2	Solid

Octanoic acid	$[N_{4444}]Cl$	2:1	Liquid
Octanoic acid	$[N_{4444}]Cl$	1:1	Solid
Octanoic acid	$[N_{4444}]Cl$	1:2	Solid
Decanoic acid	$[N_{4444}]Cl$	2:1	Liquid
Decanoic acid	$[N_{4444}]Cl$	1:1	Liquid
Decanoic acid	$[N_{4444}]Cl$	1:2	Solid
12-hydroxystearic acid	$[N_{4444}]Br$	1:1 / 2:1 / 1:2	Solid
12-hydroxystearic acid	$[N_{3333}]Cl$	1:1 / 2:1 / 1:2	Solid
12-hydroxystearic acid	Choline chloride	1:1 / 2:1 / 1:2	Solid

Table S6.2. Weight fraction percentage (extraction yield, wt.%) of cynaropicrin from the leaves of *Cynara cardunculus L.* with several DES at different molar ratio and fixed conditions, S/L ratio= 1:10, T = 25°C and t = 120 min.

DES	Cynaropicrin (wt.%)
But. Acid:$[N_{4444}]Br$ (2:1)	1.273 ± 0.003
But. Acid:$[N_{4444}]Br$ (1:1)	0.138 ± 0.003
But. Acid:$[N_{4444}]Cl$ (2:1)	2.216 ± 0.036
But. Acid:$[N_{2222}]Cl$ (2:1)	ND
Pure But. Acid	1.175 ± 0.000
Hex. Acid:$[N_{4444}]Br$ (2:1)	1.405 ± 0.004
Hex. Acid:$[N_{4444}]Br$ (1:1)	0.498 ± 0.010
Hex. Acid:$[N_{4444}]Cl$ (2:1)	2.483 ± 0.017
Hex. Acid:$[N_{2222}]Cl$ (2:1)	0.518 ± 0.007

Pure Hex. Acid	1.944 ± 0.002
Oct. Acid:[N_{4444}]Br (2:1)	1.694 ± 0.016
Oct. Acid:[N_{4444}]Br (1:1)	ND
Oct. Acid:[N_{4444}]Cl (2:1)	2.768 ± 0.023
Oct. Acid:[N_{2222}]Cl (2:1)	ND
Pure Oct. Acid	2.083 ± 0.028
Dec. Acid:[N_{4444}]Br (1:1)	ND
Dec. Acid:[N_{4444}]Br (2:1)	2.085 ± 0.001
Dec. Acid:[N_{4444}]Cl (2:1)	2.842 ± 0.014
Dec. Acid:[N_{2222}]Cl (2:1)	0.851 ± 0.003
Pure Dec. Acid	ND

*ND – Not determined.

Table S6.3. Weight fraction percentage (extraction yield, wt.%) of cynaropicrin from the leaves of *Cynara cardunculus L.* with decanoic acid:[N_{4444}]Cl (2:1) at different temperatures and other fixed conditions (S/L ratio = 1:10 and t = 120 min).

DES/Temperature	**Cynaropicrin (wt.%)**		
	25 °C	**35 °C**	**45 °C**
Decanoic acid:[N_{4444}]Cl	2.842 ± 0.014	2.473 ± 0.030	2.292 ± 0.022

Table S6.4. Weight fraction percentage (extraction yield, wt.%) of cynaropicrin from the leaves of *Cynara cardunculus L.* with decanoic acid:[N_{4444}]Cl (2:1) in different extraction time and fixed conditions, S/L ratio = 1:10 and T = 25°C.

DES/ Extraction time	Cynaropicrin (wt.%)						
	30 min	**40 min**	**50 min**	**60 min**	**120 min**	**300 min**	**1440 min**
Decanoic acid:[N_{4444}]Cl	1.464 ±0.005	1.614 ±0.099	1.812 ±0.052	3.130 ±0.044	2.842 ±0.014	2.295 ±0.000	2.080 ±0.042

Table S6.5. Optimization of weight fraction percentage of cynaropicrin extracted from the leaves of *Cynara cardunculus L.* with decanoic acid:[N_{4444}]Cl (2:1) at solid-liquid ratio and other fixed conditions (T = 25°C and t = 60 min).

DES/Solid-liquid ratio	Cynaropicrin (wt.%)				
	1:10	**1:20**	**1:30**	**1:40**	**1:50**
Decanoic acid:[N_{4444}]Cl	3.130 ±0.044	3.853 ±0.026	4.842 ±0.086	4.942 ±0.045	5.055 ±0.117

Table S6.6. Weight fraction percentage (extraction yield, wt.%) of cynaropicrin from the leaves of *Cynara cardunculus L.* using aqueous solutions of decanoic acid:[N_{4444}]Cl (2:1), and other fixed conditions (T = 25°C, S/L ratio = 1:30 and t = 60 min).

DES/ Added water (wt.%)	Cynaropicrin (wt.%)											
	0	**5**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**Pure water**
Decanoic acid: [N_{4444}]Cl	4.842 ± 0.086	4.896 ± 0.012	5.065 ± 0.080	5.134 ± 0.164	5.309 ± 0.021	5.431 ± 0.089	5.622 ± 0.030	5.832 ± 0.030	6.202 ± 0.048	3.188 ± 0.025	1.928 ± 0.056	0.676 ± 0.036

Table S6.7. Weight fraction percentage (extraction yield, wt.%) of cynaropicrin from the leaves of *Cynara cardunculus L.* with several solvents and decanoic acid:[N_{4444}]Cl (2:1) (70 wt.% of water) at the following fixed conditions: S/L ratio = 1:30, t = 60 min and T = 25°C.

Solvent	Cynaropicrin (wt.%)
Decanoic acid:[N_{4444}]Cl	6.202 ± 0.048
n-hexane	0.037 ± 0.010
Acetone	0.346 ± 0.045
H_2O	0.676 ± 0.017
Dichloromethane	4.529 ± 0.266
Soxhlet	8.652 ± 0.407

Table S6.8. Weight fraction percentage (extraction yield, wt.%) of cynaropicrin from the leaves of *Cynara cardunculus L.* with the biomass recycle at fixed conditions (S/L ratio= 1:30, t = 60 min, T = 25°C).

Cycle	Cynaropicrin (wt.%)
1st	6.202 ± 0.048
2nd	1.491 ± 0.003
3rd	0.732 ± 0.018
4th	0.351 ± 0.003
5th	0.103 ± 0.009
6th	0.097 ± 0.012

Table S6.9. Weight fraction percentage (extraction yield, wt.%) of cynaropicrin from the leaves of *Cynara cardunculus L.* with the aqueous solution of DES recycle at fixed conditions (S/L ratio = 1:30, t = 60 min and T = 25°C).

Cycle	Cynaropicrin (wt.%)
1st	6.202 ± 0.048
2nd	6.664 ± 0.009
3rd	6.958 ± 0.109
4th	7.760 ± 0.093

Table S6.10. Amount of water (mL) added and percentage of precipitation/recovery of cynaropicrin (0.5 mL of the DES-water solution used).

Amount of water (mL)	Cynaropicrin recovery (%)
5	38.26
10	48.28
15	52.51
25	65.70
50	73.61