Universidade de Aveiro Departamento de Química
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica do Professor Doutor João Manuel Costa Araújo Pereira Coutinho, Professor Catedrático do Departamento de Química da Universidade de Aveiro, e co-orientação da Doutora Carmen Sofia da Rocha Freire Barros, Investigadora Principal do CICECO- Instituto de Materiais da Universidade de Aveiro.

"Whether you think you can, or you think you can't... You're right."

Henry Ford

o júri

presidente

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Professora Catedrático da Universidade de Aveiro (orientador).

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palavras-chave Extração, compostos bioativos, gengibre, 6-gingerol, solventes eutécticos profundos.

resumo O gengibre é uma planta que tem atraído um interesse crescente tanto no sector alimentar como farmacêutico devido às suas propriedades medicinais. Como planta medicinal, é uma das mais antigas e populares do mundo. As suas propriedades terapêuticas advêm dos compostos bioativos presentes na sua composição, entre os mais importantes destacam-se os compostos fenólicos, nomeadamente o 6-shogaol e o 6-gingerol. Estes compostos despertam um grande interesse na comunidade científica devido ao seu poder antioxidante e anti-tumoral. Os processos convencionais de extração destes compostos requerem o uso de solventes orgânicos, no entanto, nos últimos anos tem-se intensificado a procura por solventes alternativos com maior capacidade de solvatação, eficientes e economicamente viáveis. Os solventes eutécticos profundos (DES) são uma classe de solventes novos, de baixo custo e de origem natural, baseados em percursores renováveis que apresentam baixa toxicidade, podendo ser catalogados como solventes amigos do ambiente. Neste contexto, o objetivo principal deste trabalho foca-se na obtenção de um extrato rico em compostos bioativos (nomeadamente 6-gingerol) a partir do gengibre utilizando DES como solventes de extração com possibilidade de recuperação do extrato e reciclagem dos DES, criando assim um processo sustentável. Para atingir este objetivo, foram estudadas as solubilidades de dois compostos fenólicos modelo, nomeadamente os ácidos siríngico e ferúlico em DES (puros e em solução aquosa), por forma a avaliar os DES mais promissores na solubilização dos compostos bioativos, assim como, as condições de extração. Entre as várias combinações entre doadores (ácidos orgânicos, polióis, açúcares e ureia) e aceitadores (ureia, cloreto de colina, betaína e prolina) de pontes de hidrogénio, o DES ácido propiónico:ureia foi o que apresentou melhores resultados. Com base nestes resultados e recorrendo a metodologias de superfície de resposta, foram avaliados e otimizados diversos parâmetros de extração do 6-gingerol usando soluções aquosas de DES. Simultaneamente, efetuaram-se extrações convencionais como controlo, seguido da identificação e quantificação do 6-gingerol presente nos extratos usando a cromatografia líquida de alta resolução (HPLC). A metodologia de extração do 6-gingerol a partir do gengibre usando os DES como solventes alternativos demonstrou ser mais seletiva, capaz de extrair mais 60 % de 6-gingerol quando comparado com os métodos de extração convencionais. Além disso, foi possível recuperar um extrato sólido rico a partir da solução de DES utilizando uma extração em fase sólida (SPE). Por fim, a caracterização dos extratos foi complementada com a análise da atividade antioxidante, usando o radical 2,2-difenil-1-picrilhidrazilo (DPPH). Os resultados indicam que os extratos obtidos a partir da metodologia proposta neste trabalho apresenta maior atividade antioxidante que os extratos obtidos com métodos de extração convencionais.

keywords Bioactive compounds, extraction, ginger, 6-gingerol, deep-eutectic solvents.

abstract Ginger is a plant that has attracted the attention from both the food and pharmaceutical sectors due to its medicinal properties. As a medicinal plant, it is one of the oldest and most popular in the world. Its therapeutic properties arise from the bioactive compounds it contains, among which stand the phenolic compounds, and in particular 6-shogaol and 6-gingerol. These compounds aroused great interest in the scientific community due to its antioxidant and antitumoral properties. The conventional extraction methods of these compounds require the use of organic solvents, however, in recent years, there has been an intensified search for alternative solvents with higher solvation capacity, efficient and cheap. Deep-eutectic solvents (DES) are a new class of solvents, of low cost and natural origin, based on renewable precursors that have a low toxicity and can be categorized as environmentally friendly solvents. The goal of this work focuses on obtaining an extract rich in bioactive compounds (namely 6-gingerol) from ginger using DES as extraction solvents, and to evaluate the possibility of recycling of DES, thus creating a sustainable process. For that purpose the solubility of two phenolic model compounds, namely syringic and ferulic acids, were studied in DES (neat and in aqueous solution) in order to identify the most promising DES for the bioactive compound solubilisation, as well as the best extraction conditions. Between the several combinations amongst hydrogen bond donors (organic acids, polyols, sugars and urea) and hydrogen bond acceptors (urea, choline chloride, betaine and proline), propionic acid:urea revealed to have the best results. Based on these results and using the surface response methodology, several extraction parameters of ginger extraction using aqueous solutions of DES were optimized. Simultaneously, conventional extraction assays were carried for comparison, followed by the identification and quantification of 6-gingerol present in extracts by high pressure liquid chromatography (HPLC). Once optimized the different parameters of extraction it was possible to achieve an efficient methodology capable to enhance the 6-gingerol extraction in 60% when compared with the conventional extraction methodology. Besides, an extract was successfully recovered from the DES extract solution using solid-phase extraction (SPE). The extract characterization was complemented with the analysis of the antioxidant activity using 2,2-diphenyl-1-picrylhydrazil (DPPH). Results show that the extracts obtained with the methodology proposed in this work present a higher antioxidant activity than those obtained with conventional extraction techniques.

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List of Abbreviations

[Ch]Cl – Choline Chloride;

AcOH – Acetic acid;

Bet – Betaine;

DES – Deep eutectic solvent;

DNA – Deoxyribonucleic acid;

DPPH - 2,2-Diphenyl-1-picrylhydrazyl;

ETG – Ethylene glycol;

FA – Formic acid;

Fru – Fructose;

GA – Glycolic acid;

GC-FID – Gas chromatography coupled with flame ionization detection;

GC-MS – Gas chromatography coupled with mass spectrometry detection;

Glc – Glucose;

Gly – Glycerol;

HBA – Hydrogen bond acceptor;

HBD – Hydrogen bond donor;

HDL – High density lipoprotein;

HPLC – High performance liquid chromatography;

HPLC-UV – High pressure liquid chromatography coupled with UV detection;

HPLC-MS - High pressure liquid chromatography coupled with MS detection;

IL – Ionic liquid;

iNOS – Inducible nitric oxide synthase;

LA – Lactic acid;

LDL – Low density lipoprotein;

LPS – Lipopolysaccharide;

MA – Malic acid;

MS – Mass spectrometry;

NMR – Nuclear magnetic resonance;

PA – Propionic acid;

Pro – Proline;

RSM – Response surface methodology;

SPE – Solid phase extraction;

TLC – Thin layer chromatography;

TSA – ρ-Toluenesulfonic acid;

U – Urea;

UPLC – Ultra performance liquid chromatography.

Xyl – Xylose.

List of Symbols

Abs –Absorbance (dimensionless);

Cp - Replicate number of the central point;

IC_{50} - Half maximal inhibitory concentration (μg mL^{-1});

k – Factorial number;

N – Number of runs;

R^2 – Correlation coefficient (dimensionless);

S – Solubility of the tested compound (mg mL^{-1});

S/S_0 - Solubility enhancement (dimensionless);

S_0 – Solubility of the tested compound in water (mg mL^{-1});

w_{DES} – DES concentration in aqueous solution in weight percentage;

wt% – Weight fraction percentage.

1. Introduction

1.1. Scope and Objectives

The main objective of the present work is to develop an efficient and sustainable method to extract ginger bioactive compounds that have already demonstrated several beneficial properties for human health. Nowadays, industry gives an important relevance to more sustainable and environmentally friendly processes. In this context, deep eutectic solvents (DES) emerge as a novel type of solvents with interesting properties that can be an alternative to conventional organic solvents.

In a first part of this work, ethanol was applied to the extraction of dried ginger by the conventional methodologies. These results are used as control to evaluate the performance of the novel DESs solvents here studied. Then, solubility tests were performed in model phenolic compounds such as ferulic and syringic acids in order to identify the best DES to extract the target compound, 6-gingerol. The selected DES will then be used in a Response Surface Methodology (RSM) to optimize the extraction conditions and allow a comparison between the conventional and the novel solvents evaluated. In the optimized conditions, and in order to achieve a separation of the extracted compounds from the DES aqueous solution, a solid-phase extraction (SPE) procedure was applied. Finally, the antioxidant activity of the extracts is tested to evaluate their potential health effects.

1.2. Literature Review

1.2.1. Origin, chemical composition and properties of ginger

Ginger (*Zingiber officinale* Roscoe) is a perennial plant (Figure 1) that can grow up to one meter tall. Although the plant is indigenous to China, from where it spread to other parts of Asia and West Africa *(1)*, India is the largest world producer with 683,000 tonnes produced in 2013. In the same year, the total world ginger production accounted to 2 100,000 tonnes *(2)*.

Figure 1. Ginger plant (i.) and its rhizome, ginger root (ii.)*(3)*

Ginger root, with its spicy taste, is widely used in gastronomy and it may vary in taste, smell or pungency depending on the country of origin or the variety of the plant used *(4)*. Nowadays, ginger is commonly found in supermarkets but throughout history it was also highly valued as a traditional medicine. Indians and Chinese used it as a folk medicine and a tonic root for over 5000 years, the Roman Empire imported it from India, mainly due to its medicinal properties. Even after the fall of Rome, ginger continued to be an important article of trade in Europe, and in the thirteenth century the value of a pound of ginger was the same as the cost of a sheep *(5)*. Ginger extract has many uses in foods, condiments, candies, beverages, cosmetics and perfumes. Each use determines the time when ginger should be harvested. For the purpose of oil extraction, ginger should be harvested after 9 months as the concentration of essential oils increases with age *(5)*.

At least 115 constituents have been identified, in oil extracts from fresh and dried ginger root by Jolad and co-workers *(6)* using gas chromatography coupled with mass spectrometry (GC-MS), mostly being phenolic compounds and terpenes (Figure 2).

Zingirebene is the major component present in the essential oils of ginger, reaching up to 29.6% of the total oil content *(4)*. α-Curcumene, farnesene, sesquiphellandrene, camphene and citral are some of the other compounds found in substantial quantities (5-12%) in ginger oil *(4,7)*. Several other constituents, such as paradols, gingerdiols and gingerdiones, are present in concentrations below 5% *(4,6)*. In spite of gingerols and shogaols contributing only to 2-3% of the total ginger oil content, they are the responsible for the pungent taste of ginger *(4)*.

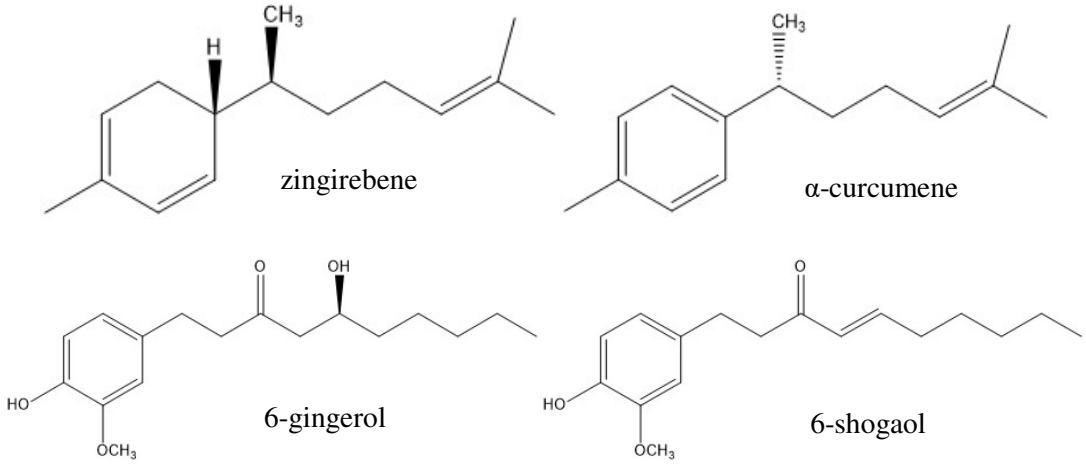


Figure 2. Examples of ginger root oleoresin compounds.

Besides gingerols and shogaols, ginger beneficial properties are also due to other important bioactive components, such as α-curcumene and gingerdiols *(8)*. Gingerols and shogaols content in ginger is highly dependable on its freshness, dryness or level of post-processing, along with the region where it was cultivated *(4)*, being 6-gingerol the main constituent in both fresh and dry ginger (Figure 3). However, in dry ginger its content is reduced due to dehydration, leading to the formation of several different molecules including shogaols (the major gingerol dehydration products) *(6)*.

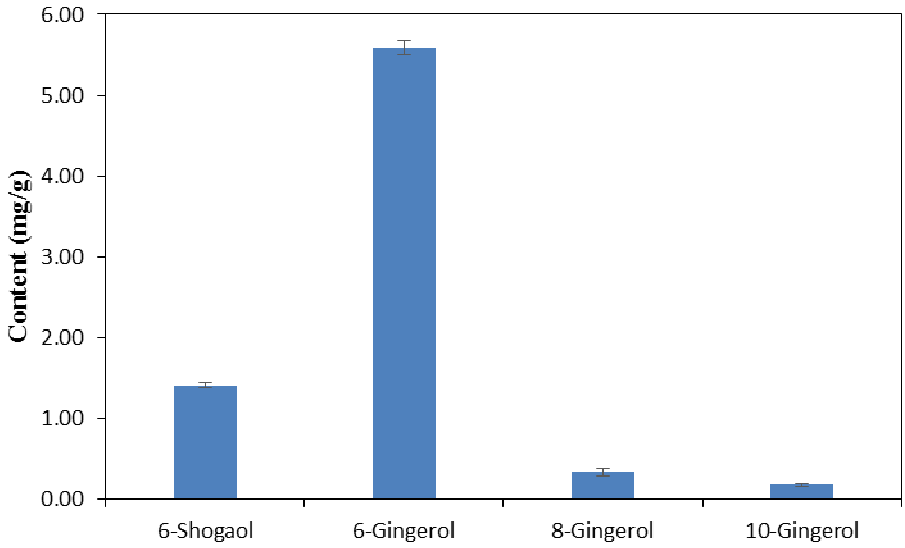


Figure 3. Contents of gingerols and shogaols for a particular variety of dry ginger *(9)*.

The stability of both 6-gingerol and 6-shogaol in aqueous solutions were studied by Bhattarai and co-workers *(10)*. The authors *(10)* demonstrated that 6-gingerol, the most abundant constituent in the gingerol series, was quite stable in a pH from 1 to 7 at 310.15 K, while exhibiting the highest stability at pH 4. At temperatures of 333.15 K or greater and in solutions with pH of 1 or 7, the compound starts to show some degradation in order to form 6-shogaol. This degradation has shown to be more favorable at higher temperatures and acidic conditions. The authors *(10)* proposed that in an acidic environment, the ß-hydroxy group of 6-gingerol undergoes catalytic dehydration to form 6-shogaol, while 6-shogaol suffers rehydration to form 6-gingerol. The research group *(10)* confirmed this by a test carried out at 353.15 K and pH of 1, which resulted in an almost even distribution of 6-shogaol (46%) and 6-gingerol (40%) at equilibrium.

1.2.2. Health benefits of ginger and its main constituents

Ginger has been cultivated for medicinal purposes for a long time. As ginger oil extract is mainly composed of phenolic compounds and terpenes, it shares many of their beneficial properties *(6)*. In recent years, several clinical trials have shown that ginger can be successfully used in the treatment of various diseases*(11–14)*.

A simple explanation of the actions and health benefits of ginger is associated with its antioxidant properties, considering that oxidative stress is commonly related to

numerous diseases *(13)*. Ginger root contains a very high concentration of antioxidants (3.85 mmol/100g), being surpassed only by few other fruits as pomegranate and some berries *(14)*. Orange, for example, although it has a high concentration of ascorbic acid shows a lower concentration of total antioxidants (1.14 mmol/100g) than ginger. Topic and co-workers *(12)* reported in 2002 that ginger can decrease age-related oxidative stress markers, such as the amount of oxidized proteins and lipid peroxidation. In ginger treated animals, these two indicators were significantly reduced. Ginger was also proposed to protect against ethanol-induced hepatotoxicity by suppressing its oxidative consequences in rats treated with ethanol *(11)*. 6-Gingerol was reported to inhibit nitric oxide production by reducing inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-stimulated mouse macrophages *(15)*. Reactive nitrogen species, such as nitric oxide, influence signal transduction and cause deoxyribonucleic acid (DNA) damage, which contributes to the disease processes. 6-Shogaol and 1-dehydro-10-gingerdione were both reported to effectively reduce iNOS expression and to decrease LPS-induced NO production *(16)*. In order to evaluate the relative antioxidant activity of some of the primary ginger constituents, amongst them 6-shogaol and 6-,8-, and 10-gingerol, Dugasani and co-workers *(17)* investigated stable free-radicals (DPPH), superoxide (O_2^- •) and hydroxyl radical (OH•) scavenging actions where 6-shogaol exhibited the most potent antioxidant activity with an IC_{50} value of 8.05±1.02 µM, while 6-gingerol exhibited the weakest antioxidant activity with an IC_{50} value of 26.3±1.42 µM. This potent antioxidant activity of 6-shogaol can be attributed to the presence of a α,ß-unsaturated ketone moiety *(17)*. Overall, several works support the hypothesis that some of ginger components are effective antioxidants *(18–21)*. Nevertheless, whether or how the physiological activity occurs in humans is still not clear and should be further investigated.

Alizadeh-Navaei and co-workers *(22)* demonstrated that ginger powder consumption significantly lowered the lipid levels in volunteer patients, in a controlled clinical trial study, supporting a protective role of ginger constituents in cardiovascular functions. Triglycerides and cholesterol were substantially decreased, as well as the low density lipoprotein (LDL) levels, when compared to a placebo group. Notably, the high density lipoprotein (HDL) level of the ginger consuming group was higher than that of the placebo group *(22)*. A test involving a cholesterol enriched diet and the effect of ginger consumption hints that ginger intake might boost lipid metabolism *(23)*. Recently, ginger has gained popularity for its potential to treat various aspects of cardiovascular

diseases, with its several properties such as anti-inflammatory, antioxidant, antiplatelet, hypotensive, and hypolipidemic effects *(24)*.

The most common and well-established use of ginger throughout history is its utilization in alleviating symptoms of nausea and vomiting. The effectiveness of ginger as an antiemetic has been attributed to its carminative effect, which helps to break up and expel intestinal gas. Furthermore, ginger root is commonly recommended to prevent seasickness *(25)*. At the same time, ginger continues to be recommended for alleviating nausea and vomiting during pregnancy and chemotherapy *(26,27)*.

One of the other health claims credited to ginger is its capability to decrease inflammation, swelling, and pain and the ability to fight osteoarthritis and rheumatism *(28)*. 6-Gingerol *(29)*, dried ginger extract, and gingerol-enriched extract *(30)* were reported to exhibit analgesic and potent anti-inflammatory effects. Most scientific evidence suggests that ginger and its various components have anti-inflammatory effects. However, the data supporting ginger as an effective anti-inflammatory agent in humans *in vivo* are still contradictory and incomplete *(31,32)*. For example, in humans, one study showed no difference between placebo and ginger treatment in patients with osteoarthritis of the hip or knee *(31)*; and in another study, consumption of 2 g of ginger before 30 minutes of cycling, at moderate pace, had no effect on quadriceps muscle pain, rating of perceived exertion, work rate, heart rate or oxygen uptake *(32)*.

A great deal of interest is now being focused on the cancer-preventive and potential cancer therapeutic applications of ginger and its various components *(33)*. Studies focused on the anticancer activities of various forms of ginger, from a crude or partially purified extract, to gingerols (especially 6-gingerol), shogaols, (especially 6-shogaol) and zerumbone, a sesquiterpene compound derived from ginger, and a number of minor components and metabolites *(34–37)*. The effectiveness of ginger in preventing or suppressing cancer cell growth has been examined in a variety of cancer types, including lymphoma, hepatoma, colorectal, breast, skin, liver, and bladder cancers *(33,38–42)*. Kim and co-workers *(43)* demonstrated that 6-shogaol exhibited the greatest cytotoxicity against human tumor cells when compared to 4-, 6-, 8-, and 10-gingerols. This compound also inhibited proliferation of several transgenic mouse ovarian cancer cell lines *(43)*. This characteristic was attributed to the fact that 6-shogaol presented the most potent antioxidant activity from all the ginger bioactive constituents *(17)*.

Ginger has also been proposed to be helpful to a number of other disease conditions, such as diabetes *(44)*. In a streptozotocin-induced diabetic rat model, rats that were fed ginger, displayed better glucose tolerance and higher serum insulin levels than untreated rats, suggesting that it can help control blood sugar levels *(44)*. Dried ginger may have also beneficial effects in treating dementia, including Alzheimer's disease *(45)*. Components of ginger root were reported to contain potent compounds with the ability to suppress allergic reactions and might be suitable for the treatment and prevention of allergic diseases *(46)*. Asthma is a chronic disease characterized by inflammation and hypersensitivity of airway muscle cells to diverse substances that induce spasms. Ginger has been used for centuries in treating respiratory illnesses *(46)*. In a mouse model of Th2-mediated pulmonary inflammation, an injection of ginger extract rich in gingerols decreased the recruitment of eosinophils to the lungs in ovalbumin-sensitized mice and also suppressed the Th2 cell-driven response to allergen *(47)*.

Taking into account ginger's medicinal properties, investigation of the available extraction methodologies for its bioactive compounds is important. Therefore, in the next section, conventional extraction methodologies of these compounds are presented, as well as alternative methodologies that make these processes more sustainable from an economical and environmental point of view.

1.2.3. Extraction of bioactive compounds from Ginger

Conventional extraction methods. Several methods of extraction of bioactive compounds from ginger have been reported in literature during the last 20 years as detailed in Table 1. Soxhlet extraction is the main technique used for the extraction of these compounds. In this extraction process *(48)*, the solvent is heated to reflux in the distillation flask, the vapors are then condensed in the condenser and drop into the chamber containing the material to be extracted. The chamber slowly fills with the heated solvent and some of the compounds are dissolved in this solvent. When the chamber is almost full it is automatically emptied by a siphon side arm, with the solvent running back to the distillation flask. This cycle can be repeated several times in order to further enrich the solvent with extracted material *(48)*. However, this technique presents several drawbacks such as long extraction times, high extraction temperatures, the use of large volumes and volatile, sometimes toxic, organic solvents *(49)*. Furthermore, the possibility of some

vestiges of these solvents to remain in the final extract is a key issue when the product is intended to be used in the food, cosmetics and medical industries *(49)*. Although Soxhlet extraction is the most widely used technique in literature, it should be taken into account that the high temperatures may cause degradation of the target compound, 6-gingerol. Due to this effect plus the fact that maceration is the technique most commonly applied when it comes to phenolic compounds, makes maceration a more appropriate technique for the extraction of 6-gingerol from ginger *(10,50–52)*.

Alternative extraction methods. Several alternative techniques have been studied in order to find a more efficient methodology for the extraction of bioactive compounds from ginger or to improve the selective extraction process. The accelerated water extraction, heat reflux extraction using ethanol, pressurized liquid extraction, steam distillation, enzyme-assisted extraction, microwave-assisted process and supercritical extraction, were some of the alternative techniques reported in literature (Table 1). Some of them even double the extraction yields when compared with conventional extraction techniques *(53)*. Even though these techniques offer an alternative approach using low organic solvents volumes, high energy powers are required for some extreme operation conditions (temperatures and pressures) used *(54,55)*. Moreover, these conditions may cause the target compounds degradation, due to the thermal instability of bioactive compounds presents in ginger extract, as previously discussed *(10)*. This should be taken into account when selecting an extraction methodology to extract a specific target compound as for example, in ginger, high temperatures cause 6-gingerol degradation favoring 6-shogaol formation, as previously discussed.

The type of substrate used has also a great influence on the efficiency extraction process, as in this case, dried root and powder ginger are the conventional substrates used in ginger extraction (Table 1) that generally produce better extraction yields. For example, Liu and co-workers *(56)* extracted 6-gingerol from fresh and dry ginger using 95% ethanol which resulted, respectively, in 2.08±0.54 wt% and 4.05±0.36 wt% content of 6-gingerol. This can be explained by the fact that in dried ginger the parenchyma cell wall is ruptured and exposed, facilitating the extraction of ginger bioactive compounds *(57)*. There is a wide range of target compounds that have been extracted from ginger. Although 6-shogaol and 6-gingerol were the most reported in literature, several others such as 8-gingerol, 10-gingerol, terpenes, polyphenols or even ginger oils in general have also been examined, as described before *(4)*.

Analytical techniques. After the extraction process, the characterization of the resulting extract is a key step since it allows an insight into the compounds extracted and the richness of the extract. Regarding the separation and analysis of target compounds from ginger extract two main analytical techniques were highlighted in the several works reported in literature: GC-MS and high pressure liquid chromatography coupled with UV detection (HPLC-UV) or high pressure liquid chromatography coupled with MS detection (HPLC-MS) as summarized in Table 1*(49,58,59)*. GC-MS technique has been often used to separate and quantify bioactive compounds from ginger extract but gingerol-related compounds may have long side chains (Figure 2) and may be difficult to detect by this method, due to their low volatility. Besides, due to the thermal instability of ginger constituents most of them might be products of thermal degradation as a consequence of high temperatures used in GC techniques *(60)*. The HPLC-UV technique is the most used approach for quantitative analysis of these extracts as it is based on a simple but effective process. The HPLC technique separates the sample into its constituent parts based on differences in the relative affinities of the different molecules for the mobile and stationary phases used in the separation *(61)*.

Table 1. Summary of some examples regarding the extraction of bioactive compounds from ginger, concerning to ginger substrate, target components, extraction methodology and analysis/separation technique.

Substrate	Target Component	Extraction method	Solvent	Quantification method	Reference
Conventional Methods					
Dried ginger root	6-Shogaol	Reflux extraction	Ethanol	HPLC-UV	*(62)*
Dried ginger root	6-,8-,10-Gingerol,6-Shogaol	Soxhlet	Ethanol	HPLC-UV	*(63)*
Dried ginger root	6-Gingerol, 6-Shogaol	Soxhlet	Ethanol	UPLC-UV	*(58)*
Dried ginger root	Ginger oils	Soxhlet	Ethanol/Hexane/Dichloromethane/Petroleum Ether	GC-MS	*(59)*
Dried ginger root/Fresh ginger	6-,8-,10-Gingerol,6-Shogaol	Blender/low pressure/high pressure-high temperature	95% Ethanol/Ethanol	HPLC-UV	*(56)*
Ginger powder	6-,8-,10-Gingerol	Soxhlet	Bioethanol	HPLC-MS	*(49)*
Ginger powder	Polyphenols	Soxhlet	Ethanol	Folin-Ciocalteu	*(64)*
Ginger powder	Oleoresin, Gingerol	Soxhlet	Acetone/Ethanol	HPLC-UV	*(53)*
Ginger powder	Oleoresin, 6-Gingerol, 6-Shogaol	Maceration	Ethanol	HPLC-UV	*(50)*

Alternative Methods					
Dried ginger root	6-,8-,10-Gingerol;6-Shogaol	Accelerated water extraction	Water	HPLC-UV	*(63)*
Dried ginger root	6-Gingerol, 6-Shogaol	Hot-compressed Water	Water	HPLC-UV	*(65)*
Dried ginger root	Ginger oils	Microwave-assisted process	Ethanol/hexane	GC-MS	*(59)*
Dried ginger root	Ginger oils	Supercritical Extraction	CO_2 (Ethanol as entrainer)	GC-Flame ionization detector (GC-FID)	*(66)*
Dried ginger root/Fresh ginger	6-,8-,10-Gingerol,6-Shogaol	Blender/low pressure/high pressure-high temperature	Water	HPLC-UV	*(56)*
Freeze-dried ginger root	Gingerols	Supercritical extraction with ultrasonic enhancement	CO_2	HPLC-MS	*(54)*
Fresh ginger	Ginger Oils	Steam distillation	Water	GC-MS	*(55)*
Ginger powder	Oleoresin, Gingerol	Enzyme-assisted extraction	Acetone/Ethanol	HPLC-UV	*(53)*
Ginger powder	6-,8-,10-Gingerol	Heat reflux extraction	Bioethanol	HPLC-MS	*(49)*
Ginger powder	6-,8-,10-Gingerol	Pressurized liquid extraction	Bioethanol	HPLC-MS	*(49)*
Peeled fresh ginger root	Terpenes (Terpinene-4-ol)	Pressurized liquid extraction	Ethanol/Methanol	GC-FID	*(67)*
Peeled fresh ginger root	Terpenes (Terpinene-4-ol)	Superheated water extraction	Water	GC-FID	*(67)*

1.2.4. Deep eutectic solvents: properties and applications

In 2003, Abbott and co-workers *(68)* presented a new type of solvents, the DESs, prepared by mixing urea and choline chloride ([Ch]Cl), two solid materials with high melting points that form a liquid at room temperature. Accordingly, DESs can be described as the result of a combination of solid starting materials that produce eutectic mixtures by formation of a hydrogen bond donor-acceptor complex, with a wide liquid range and unusual solvent properties. (Figure 4)

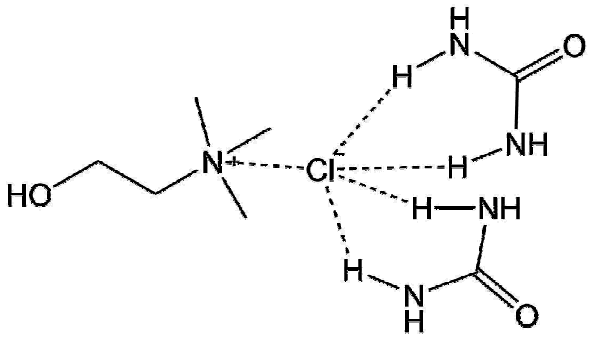


Figure 4. Example of a DES and its hydrogen bond donor-acceptor complex: [Ch]Cl and urea with a molar proportion of 1:2 *(69)*.

The charge delocalization occurring through hydrogen bonding between a hydrogen bond-donor (HBD) and a hydrogen bond-acceptor (HBA) is responsible for the large decrease in the melting point of the mixture relative to its individual components *(70)* as shown in figure 5. The number of publications on DESs is still scarce when compared, for instance, with other neoteric solvents such as ionic liquids (IL). However, over the past few years it has been increasing exponentially mainly due to its interesting properties and potential applications.

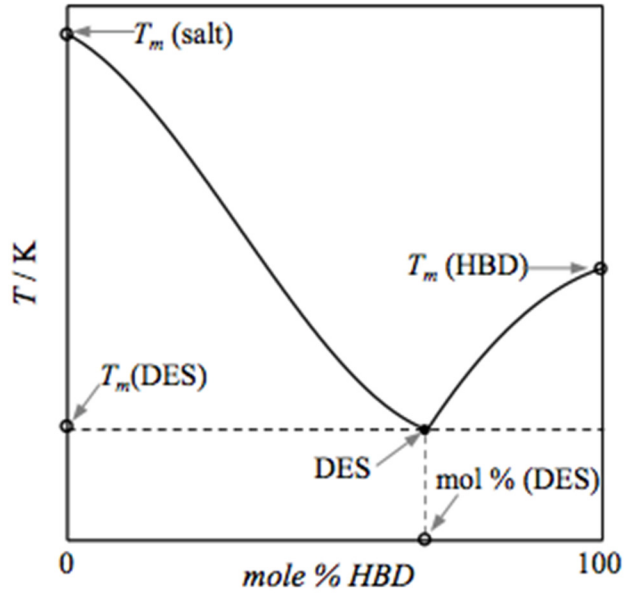


Figure 5. Schematic representation of a eutectic point on a two-component phase diagram *(71)*.

DESs can be described by the general formula $Cat^+X\text{-}zY$, where Cat^+ can be, in principle, any ammonium, phosphonium or sulfonium cation and X is a Lewis base, normally a halide anion, as the likes of Cl^-, F^- or Br^-. Complex anionic species are formed between X and either a Lewis or Brønsted acid, Y while *z* refers to the number of Y molecules that interact with X. Based on this general formula, three main types of DESs can be classified *(70)*:

- Type I: $Y=MCl_x$, where M=Zn, Sn, Fe, Al, Ga or In;
- Type II: $Y=MCl_x \cdot yH_2O$, where M=Cr, Co, Cu, Ni or Fe;
- Type III: Y=RZ, where Z= $CONH_2$, COOH or OH.

A fourth type of DESs can be formed mixing metal chlorides with different HBDs such as urea, ethylene glycol, acetamide or 1,6- hexanediol. It could be expected that these metal salts would not normally ionize in non-aqueous media but, $ZnCl_2$ has been shown to form eutectics with the different HBDs previously mentioned *(72)*. The number of non-hydrated metal halides which have a suitably low melting point to form type I DESs is restricted, nevertheless, the prospect of forming DESs can be increased by using hydrated metal halides (type II). Type III DESs have been the most studied class in literature. This class of DESs is quite simple and cheap to prepare and particularly versatile, with a large range of possible applications including bioactive compound extraction from plants,*(73,74)*.

DESs are now widely acknowledged as a novel class of solvents, sharing some properties with ILs such as a wide liquid temperature range, low vapor pressure and non-flammability. Furthermore, DESs overcome some of the ILs limitations. These new solvents are easy to prepare in a pure state through the mixing of the starting materials at moderate temperatures *(70)*. Moreover, the starting materials are usually cheaper and may often be obtained from renewable sources *(75)*. As in the case of ILs, innumerable combinations can be made (Figure 6) and DESs may also be classified as "designer solvents". Compared to ILs this characteristic is even more flexible due to the fact that DESs have no limitations in terms of stoichiometry. As no reaction takes place during their preparation, the intermolecular interactions lead to formation of a liquid within a range of relative molar compositions. This feature allows their physical properties and phase behavior to be tuned by varying the ratio of their components, and thus adding one more degree of freedom to the design of the solvent *(75,76)*. For all these reasons DESs are potential alternative solvents to extraction processes.

Even though DESs may offer a "greener" alternative to many traditional ILs and organic solvents, they are not by definition "green". While its individual components tend to be individually well toxicologically characterized, there is very little information about the toxicological properties of the eutectic mixture. This is reinforced by the fact that DESs have special properties that their individual components do not present. Hayyan and co-workers *(77,78)* studied the toxicity and cytotoxicity of choline chloride with several HBDs, such as glycerine, ethylene glycol and urea. This research group found that DESs cytotoxicity was much higher than their individual components and it varied depending on the structure of the components. Radosevic and co-workers *(79)* tested three [Ch]Cl-based DES, classifying them as "readily biodegradable" and as having a potential green profile. Morais and co-workers *(80)* also tested several [Ch]Cl-based DESs for their toxicity, classifying these DESs as "moderately toxic" and showing that the studied DESs were more toxic than the congener ILs. Juneidi and co-workers *(81)* also tested several DESs and concluded that Type III DES were the least toxic whereas type I were the most toxic. This could be expected as types I, II and IV all contain metal salts with their innate toxicity. Besides that, they proved that some DESs as [Ch]Cl-Ethylene Glycol or [Ch]Cl-Urea were practically harmless and that all the studied DESs in aqueous solution were 'readily

biodegradable'. However, it is clear that more investigation is needed in this field before DESs can be truly claimed as green, nontoxic and biodegradable solvents.

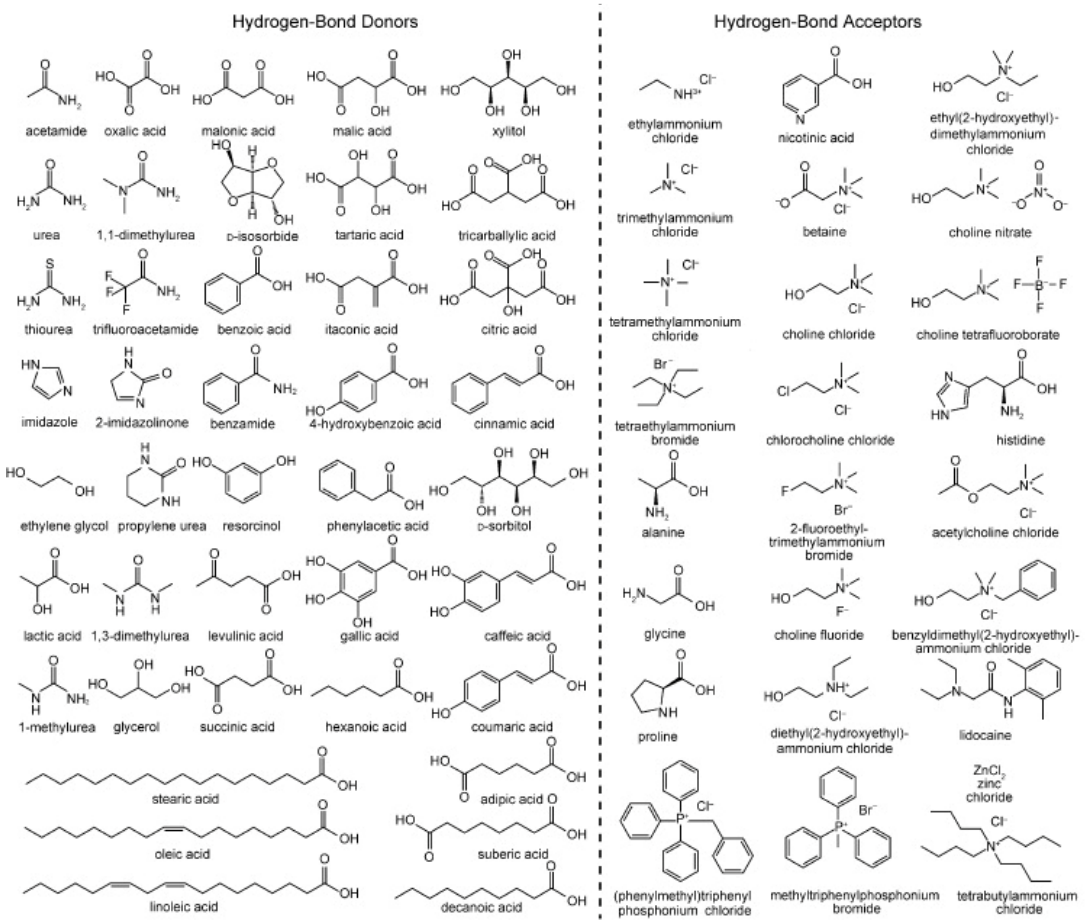


Figure 6. Molecular structures of HBDs and HBAs that can be combined to form a DES *(82)*.

Properties. Density and viscosity are two of the most important physical properties of a solvent. Most DESs exhibit higher densities than water, for example at 298.15 K, [Ch]Cl-urea with an 1:2 molar proportion has a density of 1.25 g.cm^{-3} *(83)* and [Ch]Cl-ethylene glycol with the same molar proportion has a density of 1.10 g.cm^{-3} *(84)*, at the same temperature water has a density of 0.997 g. cm^{-3}. This may be attributed to the composition of DESs, presenting some heavy atoms such halogenates. Most DESs exhibit high viscosities (>100 cP) at room temperature *(85)*. This characteristic is explained by the extensive hydrogen bond network between each counterpart of the DES, which results in a lower mobility of free species within the DES. Also the large ion size, very small void volume of DESs and the possibility of other forces such as electrostatic or Van der Waals interactions

may contribute to the high viscosity of DESs. The viscosities of eutectic mixtures are affected by the chemical nature of its components, temperature and water content *(85)*. Like ILs, DESs viscosity-temperature profile also follows an Arrhenius-like behavior. Owing to its potential applications as green media, and for the industrial applications, the development of DESs with low viscosity is of high importance *(85)*.

DESs present great solvation properties *(86)*. This is mainly due to their capacity of donating or accepting electrons or protons to form hydrogen bonds. For example, in a DES involving [Ch]Cl and urea in a 1:2 molar proportion at 323.15 K, a large number of compounds can be dissolved, including salts that are sparingly soluble in water (e.g. AgCl solubility of 0.66 mol dm^{-3}), aromatic acids (e.g.benzoic acid solubility of 0.82 mol dm^{-3}) and amino acids (e.g. D-alanine solubility of 0.38 mol dm^{-3}) *(68)*. Interestingly, DESs are capable of dissolving a wide range of molecules, including various metal oxides, becoming of great interest for surface cleaning, metallurgy or even in a "green" strategy for the separation and recycling of metals *(85–87)*.

Applications. DESs show the potential for several applications such as catalysis, where the choice of solvent is crucial *(85)*, in organic synthesis and in the preparation of inorganic materials, and can contribute to the design of eco-efficient processes. They offer many advantages such as the potential to selectively and conveniently extract reaction products and the possibility of the DES to be recycled *(85)*. Also in the field of [Ch]Cl-derived DESs there has been progress in the catalytic conversion of valuable renewable raw materials such as starch, lignin and cellulose *(75,88)*.

Applications as solvent in the extraction of bioactive compounds. More importantly for this work, there has been a growing interest in the application of DESs to the extraction of bioactive compounds from natural sources, such as plants or other forms of biomass. Table 2 lists several studies using DESs. Flavonoids, terpenoids and phenolic compounds are three of the main families of compounds extracted from natural sources using DESs *(74,89–93)*. Type III DESs are the principal DESs used in these extractions. The resulting extracts are generally analyzed through HPLC, as DESs have very low vapor pressure, creating difficulties to analyze by GC technique *(94)*. Extraction methods vary from simple stirring and heating to more complex methods using microwave-assisted extraction or ultrasonic-

assisted extraction *(89,95)*. Several of these works proved that extraction using DESs was at least, as efficient as conventional organic solvents *(96)*. For example, Zhang and co-workers *(96)* extracted catechin, epicatechin gallate and epigallocatechin gallate from green tea using aqueous solutions of DESs and several organic solvents such as methanol, ethanol, hexane and acetonitrile. Only methanol proved to extract more catechin (4.106±0.001 mg g^{-1}) than the aqueous solution of DES (2.302±0.002 mg g^{-1}). Still, the aqueous solution of DES obtained more epicatechin gallate (81.470±0.004 mg g^{-1}) and epigallocatechin gallate (25.150±0.003 mg g^{-1}) than methanol (66.81±0.001 mg g^{-1}) and 20.49±0.001 mg g^{-1}, respectively) or any other organic solvent used. Nam and co-workers *(73)* extracted flavonoids from Flos Sophorae, the dry flower from *Sophora japonica,* using DESs. Their study shows again the potential of aqueous solutions of DESs for the extraction of bioactive compounds, as the ultrasound assisted extraction with DESs allowed a higher extraction efficiency than ultrasound assisted and heat reflux extraction with conventional solvent (methanol). Another study involving the extraction of flavonoids from *Chamaecyparis obtuse* using DESs as solvent, proved that the extraction process was at least as efficient as using conventional solvents *(89)*. An investigation using aqueous solutions of natural DESs to extract phenolic metabolites in *Carthamus tinctorius,* led to a "simple, low-cost, green, and efficient method that can be applied to the extraction and isolation of natural products" from biomass *(74)*. Rajan and co-workers *(97)* investigated the extraction of 6-gingerol and 6-shogaol from ginger using DESs. The quantification of ginger bioactive components was missed in Rajan and co-workers *(97)* study where only the antioxidant activity was measured. Thus, this work was performed on a qualitative basis, having no quantitative evaluation whereby it is not possible to truly assess the efficiency and selectivity of this process or to compare to conventional methodology. The works presented on Table 2 shows the potential that DESs may have in the field of extraction of bioactive compounds.

Table 2. Extraction of bioactive compounds using DESs with details on substrate, target component, extraction methodology and characterization technique.

Substrate	Target Component	Extraction method	Solvent	Quantification method	Reference
Chamaecyparis obtusa	Flavonoids	Extraction with DES using stirring, heating and ultrasonic irradiation	Alcohol-based DESs	HPLC-UV	*(89)*
Carthamus tinctorius	Phenolic metabolites	Extraction with DES using stirring and heating	Sugar-based DESs	HPLC-UV	*(74)*
Model Oil	Phenolic compounds	Ultrasonic wave-assisted liquid phase microextraction	[Ch]Cl-Ethylene Glycol	HPLC-UV	*(98)*
Flos Sophorae	Flavonoids	Extraction with DES using stirring, heating and ultrasonic irradiation	Sugar-based DESs	LC-UV	*(73)*
Herba Artemisiae Scopariae	Phenolic acids	Extraction with DES using stirring, heating and ultrasonic irradiation	Tetramethyl ammonium chloride-Urea; Alcohol-based DESs	HPLC-UV	*(99)*
Agave sisalana; Ziziphus joazeiro	Saponins;polyphenols	Extraction with DES using stirring and heating	Organic acid-based and Alcohol-based DESs; [Ch]Cl-Urea	Vanillin-sulfuric acid method and Folin Denis method using spectrophotometry	*(100)*
Cajanus cajan	Phenolic compounds	Microwave-assisted extraction	Organic acid-based, Alcohol-based DESs and sugar-based DESs	UPLC-UV	*(95)*
Pyrola incarnata	Phenolic compounds	Microwave assisted extraction; Ultrasonic assisted extraction and Heat-stirring extraction	Polyol-based DESs	HPLC-UV	*(101)*
Shrimp byproducts	Astaxanthin (carotenoid)	Ultrasound-Assisted Method	Alcohol-based DESs	HPLC-UV	*(102)*

Green tea	Catechin compounds	Extraction with DES using stirring, heating and ultrasonic irradiation	Alcohol-based DESs	HPLC-UV	*(96)*
Chamaecyparis obtusa	Terpenoids	Headspace-solvent microextraction	[Ch]Cl-Ethylene Glycol	GC-FID	*(90)*
Ginger root	Gingerol and shogaol (phenolic compounds)	Extraction using stirring; Heat reflux and ultra-sonication method	Sucrose-citric acid; L-proline-oxalic acid; L-proline-lactic acid and trehalose-citric acid	DPPH test	*(97)*
Virgin olive oil	Phenolic compounds	Extraction with DES using stirring and heating	[Ch]Cl-based DESs	HPLC-UV	*(91)*
Mangosteen pericarp	α-Mangostin	Extraction with DES using agitation	[Ch]Cl-based DESs	HPLC-UV	*(103)*
Chinese herbal medicines	Alkaloids, flavonoids, saponins, anthraquinones and phenolic acids	Ultrasound-Assisted Method	[Ch]Cl-, betaine- and L-proline-based DESs	HPLC-UV	*(104)*
Grape skin	Phenolic compounds	Microwave assisted extraction; Ultrasonic assisted extraction and Heat-stirring extraction	[Ch]Cl-based DESs	HPLC-UV	*(92)*
Korean ginseng	Ginsenosides	Microwave assisted extraction; Ultrasonic assisted extraction and Heat-stirring extraction	[Ch]Cl-, citric acid and glycerol based DESs	HPLC-UV	*(93)*

Recovery of target compounds and recyclability of DES. The low volatility that makes DESs "greener" creates also a challenge for product separation and recovery. There is still scarce evidence to what can be done in order to achieve this in the case of DESs, but some techniques already applied to ILs, that are analogue to DESs, may be used. There is already some work published in extractions with ILs for the recovery and separation of the product. Supercritical extraction with CO_2, the use of anti-solvents, recrystallization, back extraction and chromatographic techniques have been successfully used for this purpose with ILs *(105–108)*. Despite this fact, few studies have been working on the recoverability of the products from DESs solutions using chromatographic techniques such as solid-phase extraction (SPE) and the use of anti-solvents *(73,74,93)*. Nam and co-workers*(73)*, after using DES to extract flavonoids from *Flos sophorae*, actually managed to recover 75% of rutin (major flavonoid extracted) using water as anti-solvent and 92% with a single application of a C_{18} SPE. Jeong and co-workers *(93)* successfully used DESs to extract ginseng saponins from white ginseng. In this work they also managed to easily recover the extracted saponins through application of SPE. Plus, they were able to recycle the DES by freeze-drying the washed solutions from the SPE, making for a "greener" process when it comes to extraction with DESs.

Based on the extended compilation of data so far reported comprising the use of DES as solvent in the extraction of bioactive compounds from biomass, this work aims to evaluate the efficiency and selectivity of the proposed extraction approach using DES, in order to obtain a rich extract in 6-gingerol from ginger biomass. This extraction method will be optimized based on RSM followed by the evaluation of its potential by direct comparison with conventional extraction technologies. Furthermore, these comparisons will allow a discussion about the viability of the studied method. Finally, the antioxidant potential of these extracts will be evaluated in order to perceive their beneficial health effects.

2. Experimental section

In this section, methodologies regarding conventional bioactive compounds extraction from ginger, as well as the development and optimization of an alternative and more efficient methodology using DES are described. Lastly, techniques used to the identification and quantification of 6-gingerol in ginger extract and the respective evaluation of its antioxidant power are also described.

2.1. Materials

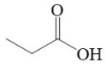
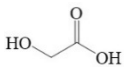
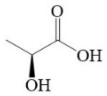
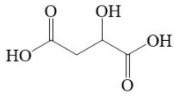
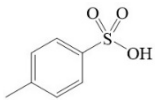
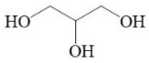
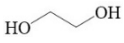
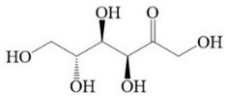
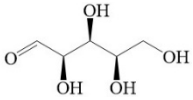
2.1.1. Raw Materials

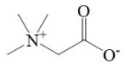
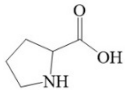
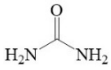
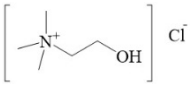
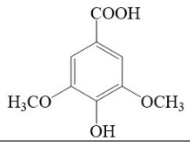
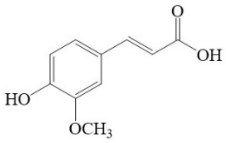
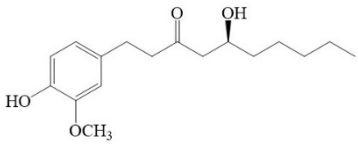
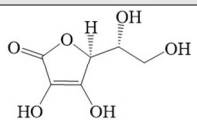
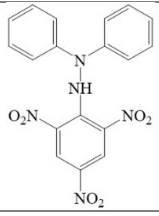
Fresh ginger (*Zingiber officinale*) samples, both harvested in China, were bought from two different supermarkets, Jumbo and Pingo Doce, in Aveiro, Portugal. Every ginger sample was thoroughly washed, sliced and then dried in a freeze dryer. It was then powdered in a coffee grinder to a granulometry lower than 2 mm prior to extraction. Finally, moisture content of each ginger sample was determined in triplicate by the oven-dry weight method. Moisture content was 5.60±0.07 and 6.99±0.05 wt% for Pingo Doce's and Jumbo's grounded ginger samples respectively.

2.1.2. Chemicals

The chemicals used in this work towards the optimization of the proposed methodology are presented in table 3 along with the purity, supplier and structure data.

Table 3. Chemicals used in this work: Name, purity, supplier and structure.

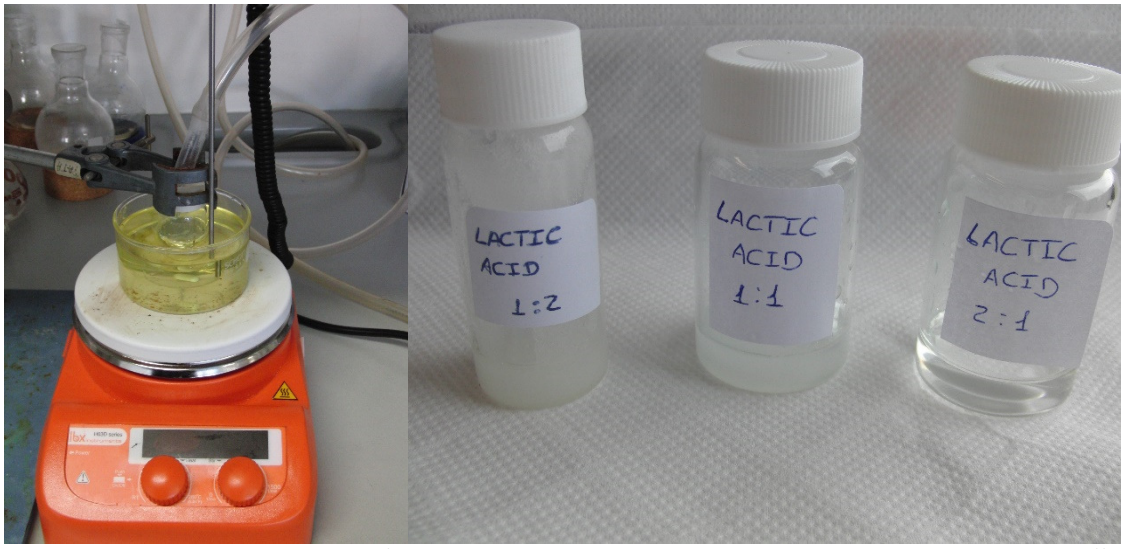
Name	Purity (wt%)	Supplier	Structure
DES Components			
Acetic Acid	>99.5	JMGS	
Formic Acid	>91.0	Panreac	
Propionic Acid	>99.0	Merck	
Glycolic Acid	>99.0	Sigma-Aldrich	
Lactic Acid	88 - 92	Riedel-de-Haen	
Malic Acid	>99.5	Panreac	
p-Toluenesulfonic Acid	>98.5	Sigma-Aldrich	
Glycerol	>99.5	Sigma-Aldrich	
Ethylene Glycol	>99.5	Sigma-Aldrich	
Fructose	>98.0	Panreac	
Xylose	>99.0	Sigma-Aldrich	

Name	Purity (wt%)	Supplier	Structure
DES Components			
Betaine	>99.0	Sigma-Aldrich	
Proline	99	Acros Organics	
Urea	>99.6	Panreac	
Choline Chloride	>98.0	Sigma-Aldrich	
Solubility Model Compounds			
Syringic Acid	>98.0	Acros Organics	
Ferulic Acid	>99.0	Acros Organics	
Target Compound			
6 – Gingerol	>98.0	Sigma-Aldrich	
Antioxidant Activity			
Ascorbic Acid	>99.7	Analar	
DPPH		Sigma-Aldrich	

2.2. Experimental procedure

2.2.1. DES Preparation

For the preparation of the DESs, both the hydrogen bond donor and acceptor counterparts were added, gravimetrically within ± 10^{-4} g, to a closed glass vial and homogeneously mixed and heated in an oil bath (Figure 7) until the formation of a clear, colourless, liquid. After the formation of a clear liquid, the mixture remained at the final temperature for one hour and then was cooled down.



i. ii.

Figure 7. (i) Setup used for the preparation of the DES; (ii) three of the DES mixtures involving lactic acid and [Ch]Cl, prepared in this work. Molar ratios from left to right: 1:2, 1:1 and 2:1 (n_{acid}:$n_{[Ch]Cl}$).

2.2.2. pH and Water Content Measurements

The pH of the DES aqueous solutions were measured at 25 ± 1 ºC using a Mettler Toledo S47 SevenMulti™ dual meter pH/conductivity equipment. Chemical's water content were measured using a Metrohm 831 Karl Fischer coulometer, in order to guarantee the correct molar proportion in the preparation of the DESs.

2.2.3. Solubility Test

Solubility tests were performed with syringic and ferulic acids in different aqueous solutions of DES at temperatures ranging from 30±1 ºC to 50±1 ºC. The concentration of the DES solutions ranged from 0 wt% (pure water) to 100 wt% (pure DES). These tests were

carried out in closed vials with 2.0±0.1 g of DES aqueous solution continuously stirred with the help of a magnetic stirrer. The compound to be tested was constantly added, 2.0±0.5 mg at a time, until the detection of a cloudy mixture. Once saturation was achieved, all samples were filtrated to separate the macroscopic solid and liquid phases and the liquid phases were put into an air oven at the same temperature used in equilibrium assays during 2h. Then, the liquid phases were carefully collected and diluted in ultra-pure water, and the amount of syringic and ferulic acids were quantified by UV-Vis spectroscopy using a Shimadzu UV1700, Pharma-Spec Spectrometer at a wavelength of 265 and 314 nm, respectively, using calibration curves previously stablished (Appendix A- figure A1 and A2).

2.2.4. Ginger Conventional Extraction

2.2.4.1. Soxhlet Extraction

About 2 g of each dried ginger sample were submitted to Soxhlet extraction for 6 h with 150 mL of absolute ethanol in order to extract ginger bioactive components. The extraction temperature was constant, at the normal boiling point of ethanol. The resulting solution was then concentrated to an oleoresin using a rotary evaporator at 323 K. The experiments were conducted in quadruplicate, the oleoresin yield was calculated as percentage weight per weight and the respective standard deviations were calculated.

2.2.4.2. Maceration

Maceration was performed at room temperature and with the help of magnetic stirring for 16 h. About 2 g of each dried ginger sample were weighed into a 500 mL extraction flask and 200 mL of ethanol absolute was added. The flask was covered in order to prevent evaporation of the solvent. The resulting solution was filtered and then concentrated to an oleoresin in a tared round-bottom 50 mL flask using a rotary evaporator at 323 K. The experiments were conducted in quadruplicate. Due to positive results in the solubility tests another two experiments were conducted with Pingo Doce's ginger samples using propionic acid instead of ethanol absolute as extraction solvent.

2.2.5. Ginger Alternative Extraction with DES

Initially, ginger extraction using DES was performed similarly to the conventional maceration extraction, with the exception that an oil bath was used for temperature control.

0.5 g of dried ginger were mixed with 15 mL of DES aqueous solution for each extraction with the aid of a magnetic stirrer. Based on the results obtained from the solubility tests of models compounds, an appropriate DES and concentration in aqueous solution was chosen for this extraction. The experimental conditions were further optimized through SRM. After each extraction, the resulting mixture of DES, extract and ginger was centrifuged for 5 minutes at 3500 rpm to ensure the separation of solid extracted ginger from the liquid fraction (DES and dissolved extract). This solution was then filtered, and diluted at a 1:1 (v/v) ratio in methanol for further analysis of 6-gingerol concentration by HPLC.

2.2.6. Surface Response Methodology

In order to optimize the extraction conditions a surface response was performed. The SRM is a collection of statistical and mathematical techniques used to explore the relationship between several variables, using a sequence of designed experiments to obtain an optimal response. Its objective is to simultaneously optimize the levels of these variables to attain the best system performance, generating a large amount of information from a small number of experiments, thus reducing avoidable costs *(109)*. A central composite design, first introduced by Box and Wilson was used *(110)*. The number of runs of each experiment is given by the following equation:

$$N = 2^k + 2k + Cp, \text{ (Equation 3)}$$

where k is the factor number (number of independent variables), being 2^k the number of factorial runs, $2k$ the number of axial runs and Cp is the replicate number of the central point. Several runs for the central point are important in order to know the residual plot and, consequently, the standard deviation and the repeatability quality of the experiment. On the other hand, the axial points are added to adjust the experiment. The axial points are calculated according to α-values. The α-values depend on the number of variables and can be calculated by:

$$\alpha = 2^{\frac{k-p}{4}} \text{ (Equation 4)}$$

For two, three, and four variables, α-values are respectively, 1.41, 1.68, and 2.00. Figure 8 illustrates a full central composite design for the optimization of three variables.

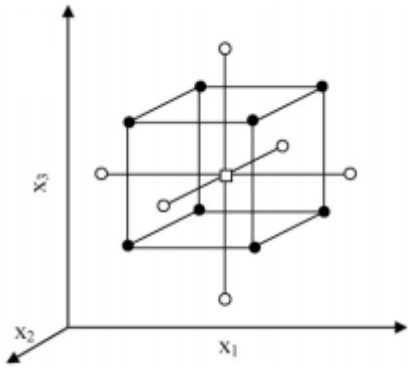


Figure 8. Central composite designs for the optimization of three variables (α= 1.68). (●) Points of factorial design, (○) axial points and (□) central point.*(109)*

For the 6-gingerol extraction from ginger using DES optimization study, three independent variables were selected: extraction time, extraction temperature and the concentration of DES in the aqueous solution. Looking back to equation 3, and thus considering *k*=3, a total of 20 extractions were planned, with 8 extractions for the factorial points, 6 for axial points, and 6 repetitions of the central point, with an α-value of 1.68.

In Table 4 the central point and the calculated factorial and axial points are identified.

Table 4. Identification of central (0), factorial (± 1), and axial (± 1.68) points of a surface response with three independent variables.

	Axial Point **-1.68**	Factorial Point **-1**	Central Point **0**	Factorial Point **1**	Axial Point **1.68**
Temperature (K)	296	303	313	323	330
Time (min)	59	90	135	180	211
DES concentration (% w_{DES})	41.5	50.0	62.5	75.0	83.5

Once defined all points to be tested, and considering the design matrix (Table 5) result of an established mathematical model *(109)*, the applied conditions of extraction were found for all three variables in the 20 extractions performed (Table 5).

Table 5. Design Matrix and decoded conditions for the surface response design for a 2^3 experiment.

Run	Design Matrix			Decoded Conditions		
	Temperature (ºC)	Time (min)	DES concentration (% w_{DES})	Temperature (K)	Time (min)	DES concentration (% w_{DES})
1	-1	-1	-1	303	90	50
2	1	-1	-1	323	90	50
3	-1	1	-1	303	180	50
4	1	1	-1	323	180	50
5	-1	-1	1	303	90	75
6	1	-1	1	323	90	75
7	-1	1	1	303	180	75
8	1	1	1	323	180	75
9	-1.68	0	0	296	135	62.5
10	1.68	0	0	330	135	62.5
11	0	-1.68	0	313	59	62.5
12	0	1.68	0	313	211	62.5
13	0	0	-1.68	313	135	41.5
14	0	0	1.68	313	135	83.5
15	0	0	0	313	135	62.5
16	0	0	0	313	135	62.5
17	0	0	0	313	135	62.5
18	0	0	0	313	135	62.5
19	0	0	0	313	135	62.5
20	0	0	0	313	135	62.5

The analysis of the surface response results was made using the software STATISTICA 8.0 of Statsoft©.

2.2.7. SPE

In extractions using DESs as alternative solvent, the recovery of extracted bioactive compounds from the ginger extracts is fairly challenging due to the DES negligible vapor pressure. SPE is a procedure that makes this recovery simple. Recovery of ginger extract from liquid fraction was performed using reversed phase StrataTM-X 33 μm Polymeric sorbent cartridges (200 mg, 3 mL) provided by Technocroma (Caldas da Rainha, Portugal). First of all, the cartridge was activated with methanol (5 x 1 mL) and pre-conditioned with deionized water (5 x 1 mL), afterwards 3 mL of liquid fraction (mixture of DES aqueous

solution and ginger extract) was loaded onto the cartridge. DES components were removed with deionized water (5 x 1 mL) and the retained ginger extract, that included the target 6-gingerol, was eluted in ethanol (5 x 1 mL). The resulting ethanolic solution was then concentrated to an oleoresin at 323 K using a rotary evaporator, completing the recovery of the extracted compound. Finally, 6-gingerol concentration was quantified in each oleoresin by HPLC technique.

2.2.8. HPLC

For the determination of 6-gingerol concentration, HPLC analysis was performed on a Shimadzu HPLC (model PROMINENCE) unit equipped with a Maisch Reprosil 5 µm C18 column (250 x 4.60 mm). Methanol/H_2O 50:50 was used as mobile phase at a flow of 0.7 mL min^{-1} and the detection was done at 201 nm. The column oven and autosampler temperatures were kept at 303.15 K. The injection volume was 10 µL. A calibration curve was established for 6-gingerol by plotting the nominal concentrations of standard solutions versus peak areas (Appendix A- figure A3). Typical chromatograms of standard 6-gingerol and maceration, Soxhlet and DES extraction are presented in Appendix B – Figures B1-B4. All oleoresin samples were solubilized in methanol. Both oleoresin and DES extract solutions were filtered over a 0.45 µm syringe filter before HPLC analysis.

2.2.9. Antioxidant Activity

The antioxidant activity of the extracts was determined by the DPPH• radical scavenging methodology *(111)*. The method is based on the color decrease of the DPPH free radical solution as it is reduced by antioxidant compounds. The radical has a strong absorption band at 515-528 nm, which gives him a deep violet color. As the reaction progresses the compound will end up with a pale yellow color. This means that this reaction can be easily monitored by a spectrophotometer. The final results are expressed as IC_{50} values, which correspond to the compound or extract concentrations needed to reduce in 50 % the initial DPPH• concentration.

In test tubes, 1 ml of 0.1 mM DPPH• solution in ethanol was added to accurately weighed aliquots of the extracts dissolved in 3 ml of ethanol, corresponding to concentration ranges of extract between 5 and 63 µg mL^{-1} for DES extract, between 10 and 68 µg mL^{-1} for maceration extract, and between 10 and 120 µg mL^{-1} for Soxhlet extract. After mixing, the

samples were maintained in the dark, at room temperature for 30 minutes. The absorbance at 517 nm was measured using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek) and compared with a control without extract. A blank was prepared for each sample using ethanol instead of the DPPH• solution. Ascorbic acid was used as a positive reference, being one the most known commercial antioxidants.

The antioxidant activity is expressed as a percent inhibition of DPPH radical, and calculated by:

$$Antioxidant\ Activity\ (\%) = \frac{A0-A1}{A0} * 100 \quad \text{(Equation 5)}$$

Where A0 is the absorbance from the control and A1 the absorbance from the sample. IC_{50} values were determined from the plotted graphs of antioxidant activity against the concentration of the extracts. All experiments were conducted in triplicate.

3. Results and Discussion

The main goal of this work is the development of an alternative and more efficient extraction process to obtain a 6-gingerol rich extract from ginger using DESs. In this context, the first step of the work was to identify the DES that would have the greatest affinity with 6-gingerol in order to ensure the best extraction conditions. The most promising DES and extraction conditions were further optimized in the second step in order to study the efficiency of this alternative extraction process, comparing with conventional methods. The antioxidant capacity of the 6-gingerol rich extract was further analysed.

3.1. Solubility tests

Aiming at investigating the potential of DESs to selectively extract 6-gingerol from ginger, the solubility of two model phenolic compounds, syringic and ferulic acids, were determined in several DES evaluating diverse parameters such as DES concentration, DES components molar proportions and temperature. The use of these model phenolic compounds can be explained by the high cost of 6-gingerol (44€/mg) *(112)* that makes this type of study very expensive. The solubility of syringic and ferulic acids at 303.15 K in pure water, measured in this work, was 1.28±0.01 and 0.83±0.03 mg g^{-1}, respectively. The chemical structures of the studied model compounds and 6-gingerol is depicted in figure 9. The DES properties depend on the HBD and HBA chemical structures and their molar proportions. In this work several HBD/HBA pairs of DESs were tested using as HBD several organic acids such as formic acid (FA), propionic acid (PA), acetic acid (AcOH), *p*-toluenesulfonic acid (TSA), lactic acid (LA), glycolic acid (GA) and malic acid (MA), polyols as ethylene glycol (ETG) and glycerol (Gly), sugars such as xylose (Xyl), fructose (Fru) and glucose (Glc) and urea (U). As HBA four different molecules were used: betaine (Bet), proline (Pro), [Ch]Cl and U. All the detailed data along with the respective standard deviations are presented in Appendix C.

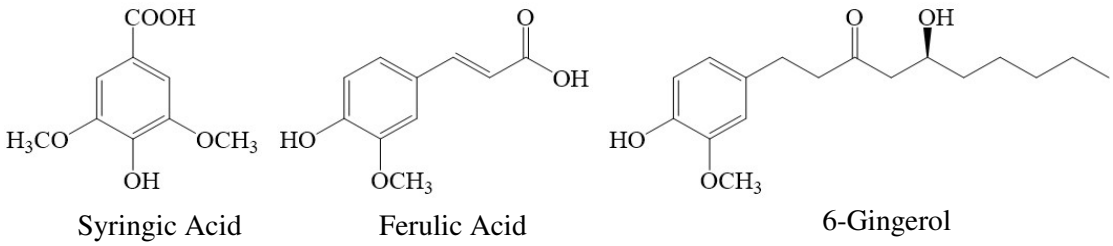


Figure 9. Chemical structures of the model compounds: syringic and ferulic acids, and 6-gingerol.

3.1.1. HBD Effect

In a first approach, the effect of different HBDs, namely, organic acids, polyols, sugars and U, was tested. Based on the well-known DES U:[Ch]Cl, the favourable properties of [Ch]Cl, its biocompatibility, low price and green character overall, this compound was adopted as the HBA for this study*(68)*. The syringic acid solubility in aqueous solutions of different DESs was studied at a concentration of 25 w_{DES}% at 303.15 K. The influence of the HBD in the solubility of syringic acid is depicted in figure 10. Each DES is represented in the generic form of "HBD:HBA" followed by their respective molar proportion. S and S_0 represent the solubility (mg g^{-1}) of each phenolic compound in the aqueous solutions of DES and in pure water, respectively; therefore, S/S_0 represents the solubility enhancement.

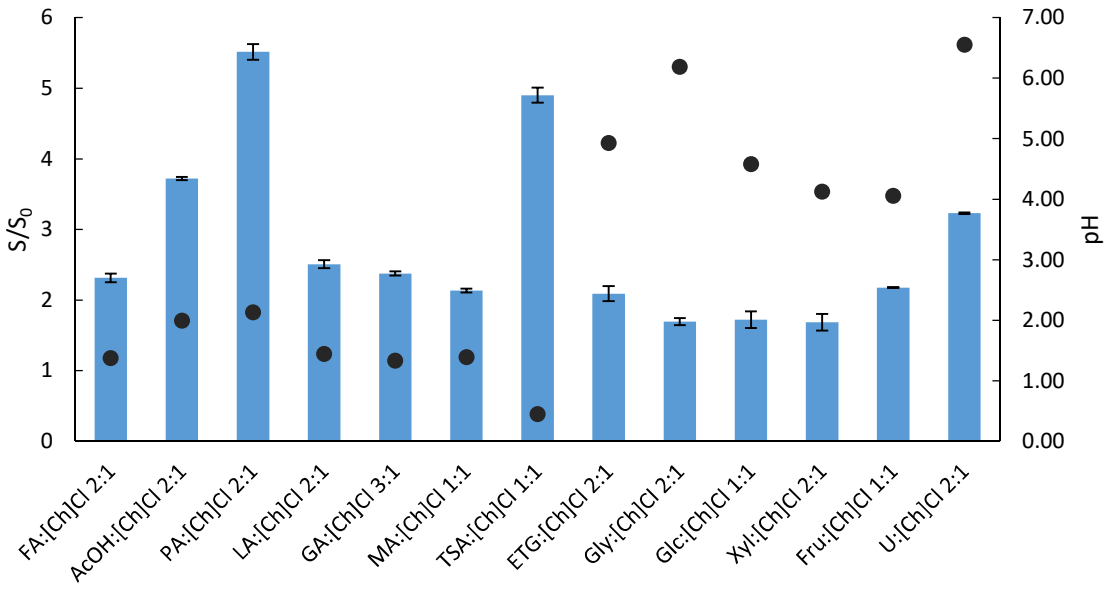


Figure 10. Influence of different HBD of DESs in the solubility of syringic acid in 25 w_{DES} aqueous solutions at 303.15 K. pH of each DES aqueous solution is represented in black dots.

Observing these data it is possible to perceive that the solubility of syringic acid depends on the type of HBD in DES. Sugar and polyols-based DESs exhibited low capacity to solubilize syringic acid, with solubility enhancements of 2-fold. However, the solubility enhancement of carboxylic acids-based DESs were significantly higher than that of other DESs, with solubility enhancements that may reach 5.5-fold. Among the carboxylic acids-

based DESs, the HBDs namely, PA, TSA and AcOH provided the best results (5.5, 4.9 and 3.7-fold, respectively). Furthermore, it is noticeable that a tendency can be seen for the alkyl side chain length of the acid. Comparing the HBDs FA, AcOH and PA it can be observed that an increasing alkyl side chain length results in increased solubility of the solute, probably due to the higher hydrophobicity of the DES. Besides, it seems that hydroxyl groups in the acid alkylic chain have a negative impact in the solubilisation of syringic acid as LA and GA showed lower results than AcOH. Similarly, PA demonstrated to have a higher solubility enhancement than MA. The presence of another carboxyl group can also be the cause of the lower solubilisation performance of MA. Nevertheless, it was also observed that HBDs with a more polar nature such as polyols, sugars and acids such as LA or MA promoted a lower solubility enhancement of the solute. These results suggest that the dispersive interactions between the organic acid alkylic chain and the syringic acid structure are the main responsible for the good performance of PA, instead of hydrogen bond interactions. Furthermore, the pH of DES aqueous solutions seem to have no effect in the syringic acid solubility. Overall PA:[Ch]Cl with a molar proportion of 2:1 presented the best solubility enhancement.

3.1.2. HBA Effect

Considering the advantageous performance of the carboxylic acids-based DES, four different carboxylic acids were combined with four HBAs in order to design the best DES to enhance syringic acid solubility in aqueous solution. Besides the already studied [Ch]Cl, Pro, Bet and U were also used as HBA. Syringic acid solubility in aqueous solutions of different DESs were studied in a concentration of 25 w_{DES}% DES in water at 303.15 K. The influence of different HBAs in the solubility of syringic acid is depicted in figure 11.

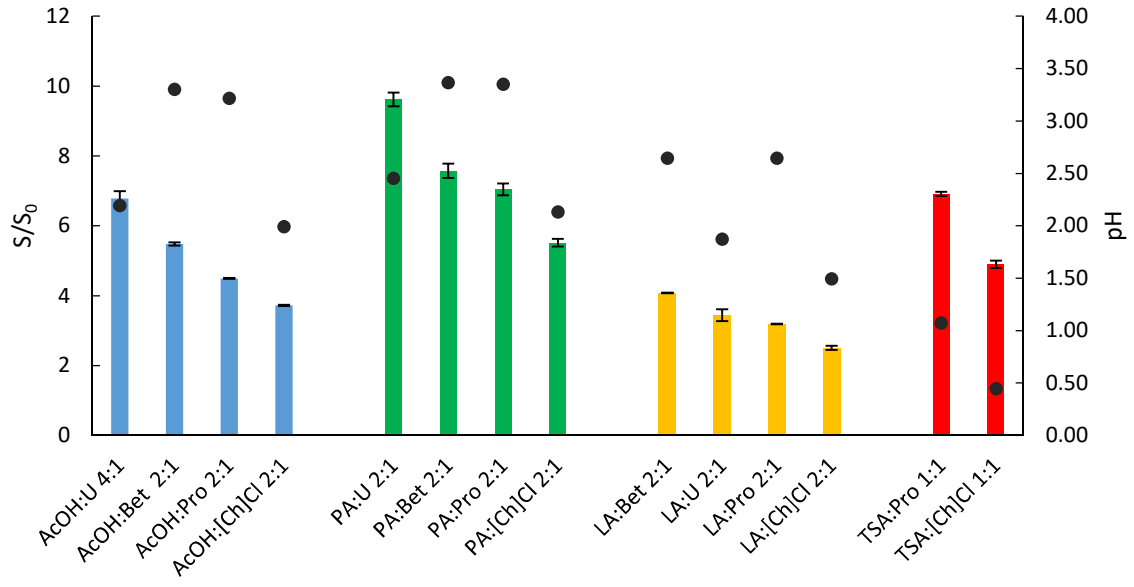


Figure 11. Influence of different HBAs in the solubility of syringic acid in 25 w_{DES}% aqueous solutions at 303.15 K. AcOH-based DESs represented in blue, PA-based DESs in green, LA-based DESs in orange and TSA-based DESs in red. pH of each DES aqueous solution is represented in black dots.

The DES composed by TSA:Bet and TSA:U could not be prepared and therefore were not used on this study. Notwithstanding this fact, the study of the different HBAs was carried on for AcOH, PA and LA. A general trend can be observed in Figure 11, namely the effect of HBAs on the syringic acid solubility enhancement: U>Bet>Pro>[Ch]Cl. This trend suggests that, independently of the carboxylic acids used, the HBAs also have a significant effect on the solubility of syringic acid. However, this trend is not observed when LA is the HBD of the DES. In this case, Bet showed a larger syringic acid solubility enhancement than U as HBA. Despite that fact, in general the DES prepared with PA as HBD demonstrated the best solubility results, being PA:U the best DES in terms of solubility enhancement (about 9.6-fold). Furthermore, the replacement of [Ch]Cl by U lead to an increase of 4-fold on the solubility of syringic acid, explained by the increase of dispersive interactions associated with U.

3.1.3. HBD:HBA Molar Proportion Effect

The molar composition of DES plays an important role on its physicochemical properties. By changing the molar composition it is possible to obtain a solid, a liquid or a

combination of both at room temperature. From the extraction process point of view, only the stable liquid DES at room temperature can be considered. At this point, there was a need to study a phenolic standard compound more similar to our target-compound, 6-gingerol, in order to understand if the trends and results obtained in this study were comparable. Therefore ferulic acid was considered from hereupon. In that sense, syringic and ferulic acids solubility in aqueous solutions of different molar proportions of the most promising DES, PA:U and its counterparts were studied in a concentration of 25 w_{DES}% DES in water at 303.15 K. These results are depicted in figure 12.

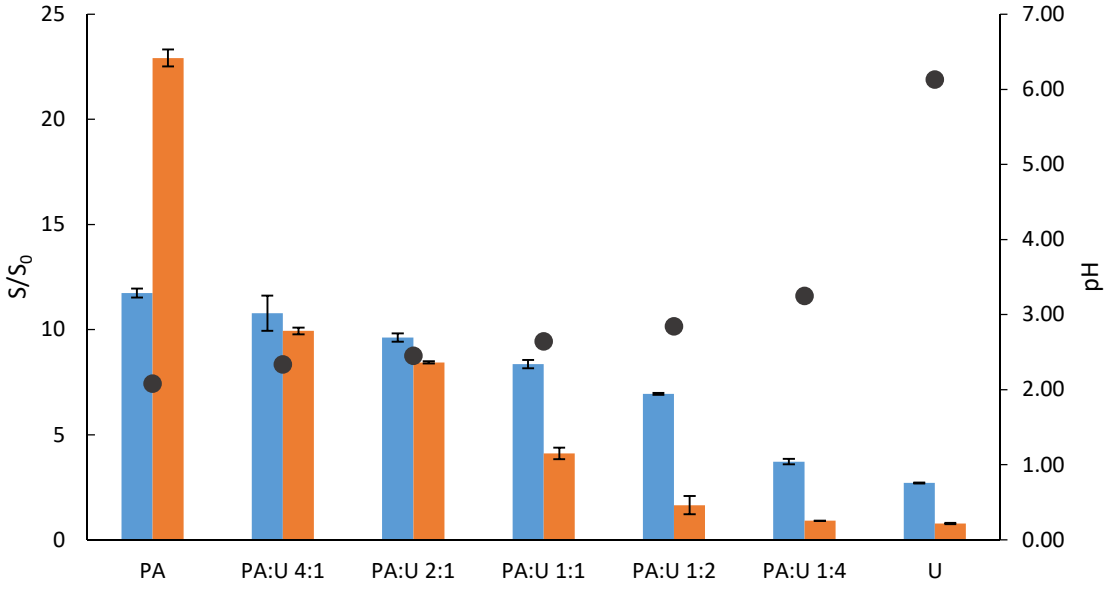


Figure 12. Influence of different molar proportions of the PA:U and its counterparts in the solubility of syringic acid and ferulic acid in 25 w_{DES}% aqueous solutions at 303.15 K. Syringic acid solubility is represented in blue, while ferulic acid solubility is represented in orange. pH of each molar proportion is represented in black dots.

Based on these results, it is possible to apprehend that the molar composition have a significant effect in the solubility enhancement of both solutes (syringic and ferulic acids). Indeed, the solubility enhancement of the solutes increases with the increase of the HBD molar proportion, in other words, the increase of the dispersive interactions between HBD and model compounds promotes the good performance of DES aqueous solutions. Although, the best results were obtained using PA in aqueous solution, this strong acidic environment is not viable for the extraction of bioactive compounds from biomass, as it will be demonstrated further on. Considering the similar solubility enhancement obtained for both

solute using 25 w_{DES}% of PA:U 4:1 and 2:1 aqueous solution at 303.15 K (10.8 and 9.6-fold, respectively), the PA:U 2:1 was the DES selected to study the impact of the temperature and DES concentration upon its ability to enhance the solubility of the phenolic acids investigated.

3.1.4. Temperature and DES Concentration Effects

Syringic and ferulic acids solubilities in aqueous solutions of PA:U 2:1 were studied in the entire concentration range, from pure water to pure DES at three different temperatures 303.15 , 313.15 and 323.15 K. In Figure 13 is represented the syringic acid solubility enhancement results and Figure 14 depicts the results for ferulic acid.

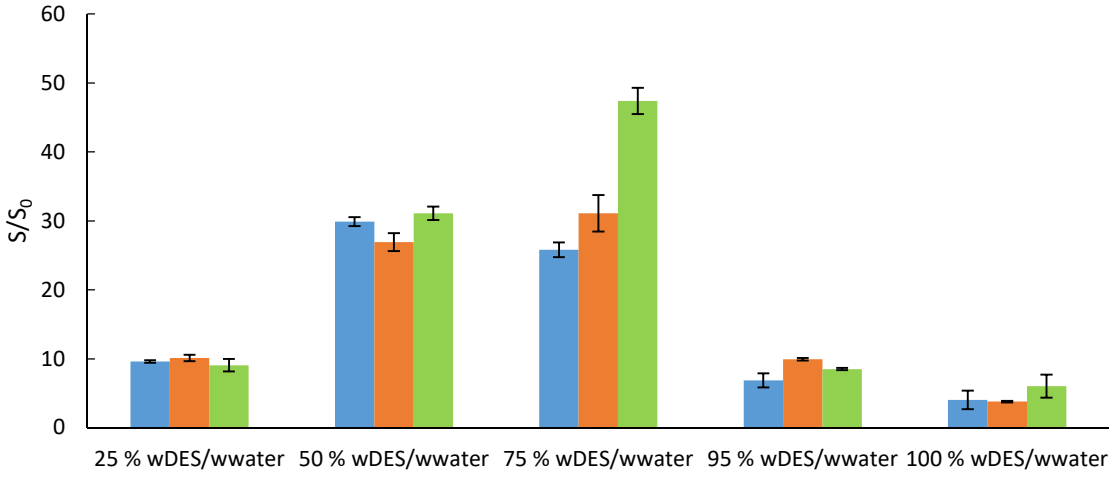


Figure 13. Syringic acid solubility enhancement test results for PA:U 2:1 DES system, at different temperatures and concentration of DES in aqueous solution. Solubility at 303.15 K is represented in blue, at 313.15 K in orange and at 323.15 K in green.

In general, as expected, the solubility of syringic and ferulic acids in both water and DES aqueous solutions increases with the increase of temperature *(113)*. This trend may not be detected in Figure 13 and Figure 14 due to the fact that the graphic is represented in S/S_0 for each temperature but it can be confirmed in the detailed data presented in appendix C – tables C5 and C6. Regarding the DES concentration, 50 w_{DES} % and 75 w_{DES}% seem to allow the best solubility enhancement of syringic acid. In fact, at a temperature of 323.15 K and a concentration of 75 w_{DES} % potentiate the syringic acid solubility by at least 47-fold.

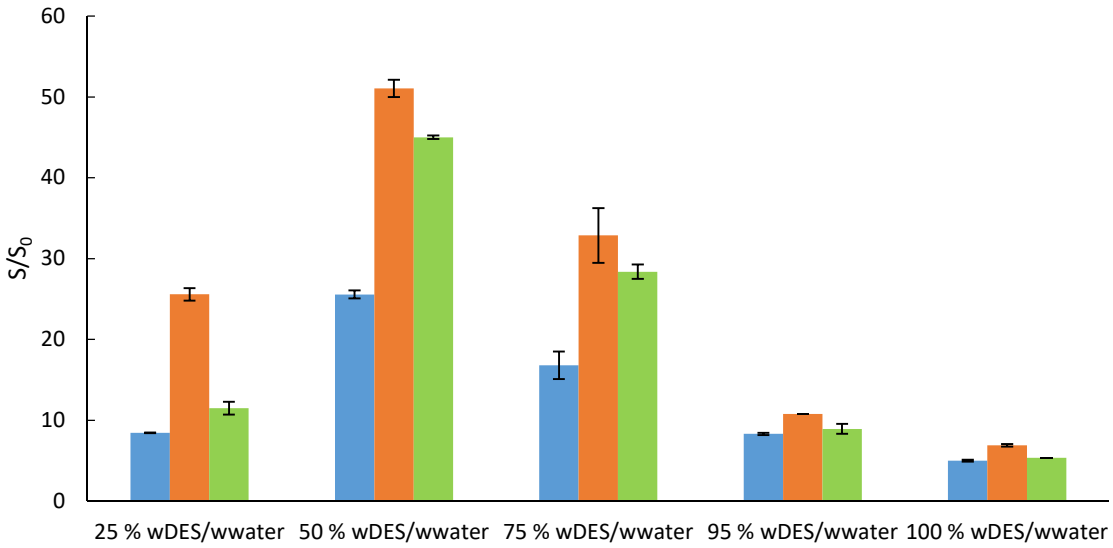


Figure 14. Ferulic acid solubility enhancement test results for PA:U 2:1 DES system, at different temperatures and concentration of DES in aqueous solution. Solubility at 303.15 K is represented in blue, at 313.15 K in orange and at 323.15 K in green.

Similar to syringic acid, the increase of temperature increases ferulic acid solubility. Nevertheless, it also seems to be a tendency for a certain temperature related to solubility enhancement. The temperature of 313.15 K presents in every concentration of the DES the best solubility enhancement of ferulic acid. Also analogous to syringic acid, the best DES concentrations to potentiate ferulic acid solubility seems to be 50 w_{DES}% and 75 w_{DES}%. For ferulic acid, a concentration of 50 w_{DES}% and a temperature of 313.15 K are the conditions that most potentiate ferulic acid solubility. These conditions enhance ferulic acid solubility at least 51 times when compared to water.

3.2. Conventional Extraction of 6-gingerol From Ginger

Conventional methodologies were used as benchmark to evaluate the performance of the alternative extraction using DESs. Despite the fact that Soxhlet extraction is the most common in the literature when it comes to 6-gingerol extraction from ginger as it can be seen in Table 6, maceration is the traditional extraction technique used to extract phenolic compounds*(51,52)*. Soxhlet extraction requires extraction temperatures at the boiling point of the used solvent, which in this case are not compatible with the compound aimed for extraction. It has already been pointed out that 6-gingerol is thermo-labile *(10)*. As ethanol

has a boiling point of 351 K and 6-gingerol starts to show decomposition at temperatures above 333 K, Soxhlet extraction may not ensure optimal results. Therefore, two different techniques were studied in this work. Soxhlet extraction conditions were based in Azian and co-workers optimization works*(63)*, while a 16h period of extraction was chosen for maceration in order to safeguard maximum extraction results.

Table 6 presents the oleoresin yield and the 6-gingerol content in the extracts for both methodologies of extraction using ethanol as solvent and ginger from Pingo Doce and Jumbo. It also presents the results when a maceration was performed with PA as solvent of extraction. This test was implemented due to the promising results of PA in the solubility tests as can be seen in Figure 12.

Table 6. Extraction yields and 6-gingerol content of ginger extracts from Pingo Doce and Jumbo with the use of two extraction methodologies.

Solvent	Methodology	Origin	Oleoresin yield (wt%)	6-gingerol content in oleoresin (wt%)	6-gingerol content extracted from ginger (wt%)
Ethanol	Soxhlet	Pingo Doce	21.41 ± 1.83	3.02 ± 0.2	0.54 ± 0.08
		Jumbo	22.76 ± 3.46	2.11 ± 0.27	0.37 ± 0.05
	Maceration	Pingo Doce	13.75 ± 2.72	5.07 ± 0.43	0.71 ± 0.15
		Jumbo	14.07 ± 1.57	3.79 ± 0.34	0.48 ± 0.03
PA	Maceration	Pingo Doce	33.88 ± 2.46	-	-

Interestingly the oleoresin yield is similar for both samples when comparing within the same methodology. In terms of oleoresin yield, the Soxhlet extraction outperformed maceration achieving a greater extraction power. However, when the 6-gingerol content of these oleoresins were analysed, maceration extraction produced oleoresins richer in 6-gingerol than Soxhlet extraction. Not only, but when the 6-gingerol levels extracted from ginger is compared, a similar tendency is shown as maceration extracts more 6-gingerol than Soxhlet extraction. As mentioned before, the 6-gingerol's thermolability could explain these results, as the maceration is performed at room temperature. Solid-liquid extraction using PA as solvent demonstrates a promising performance when it comes to oleoresin extraction, on the other hand it was not detected any 6-gingerol content in this oleoresin meaning that it is not a process meant for 6-gingerol extraction.

3.3. Alternative Extraction of 6-gingerol from Ginger

According to the results previously discussed in the solubility tests, PA:U with a molar proportion of 2 to 1 should be one of the most suitable DESs for 6-gingerol extraction from ginger. Looking towards the optimization of the rest of operational conditions, a RSM was applied using this DES. This methodology allows the investigation of different conditions at the same time and the determination of the relationship between the 6-gingerol concentration extracted from ginger (response variable) and the operational conditions of interest for the DES extraction process (independent variables). For the RSM, a 2^3 factorial planning study (3 factors and 2 levels) was executed (Appendix D: Table D1). Temperature, extraction time and DES concentration were the conditions optimized, being the experimental points used, the model equations, the 6-gingerol's concentration experimentally and theoretically defined using the correlation coefficients obtained in the statistical treatment, as well as all the statistical analyses. Previously, a study of the proper solid-liquid ratio for the extraction of ginger using DES was performed. It was found that a minimum of a solid-liquid ratio of 1:30 (g:mL) was needed in order to obtain a proper extraction of ginger, as a greater solid-liquid ratio led to a soggy mix that could not be properly agitated. Ginger powder's low density was apparently the cause to this. The solubility tests were also determinant to choose the range of the studied conditions. Temperature was studied between factorial points of 303 and 323 K. As previously mentioned, higher temperatures could be harmful for the purpose of this work due to 6-gingerol's thermolability *(10)*. The range of DES concentration in the aqueous solution was chosen based also on the solubility test results. As seen in Figure 13 and Figure 14, the optimum concentration range should be between 50 to 75 w_{DES}%. A previous RSM study with similar studied ranges of concentration of DES and temperature, but testing lower times of extraction ranging from 10 to 110 min indicated the need for longer times (response surface plots and countor plots of the previous RSM studied are presented in Appendix E). The studied ranges from the three studied components are presented in Table 4. Figure 15 depicts the effect of the three variables studied in the concentration of 6-gingerol extracted from ginger in percentage.

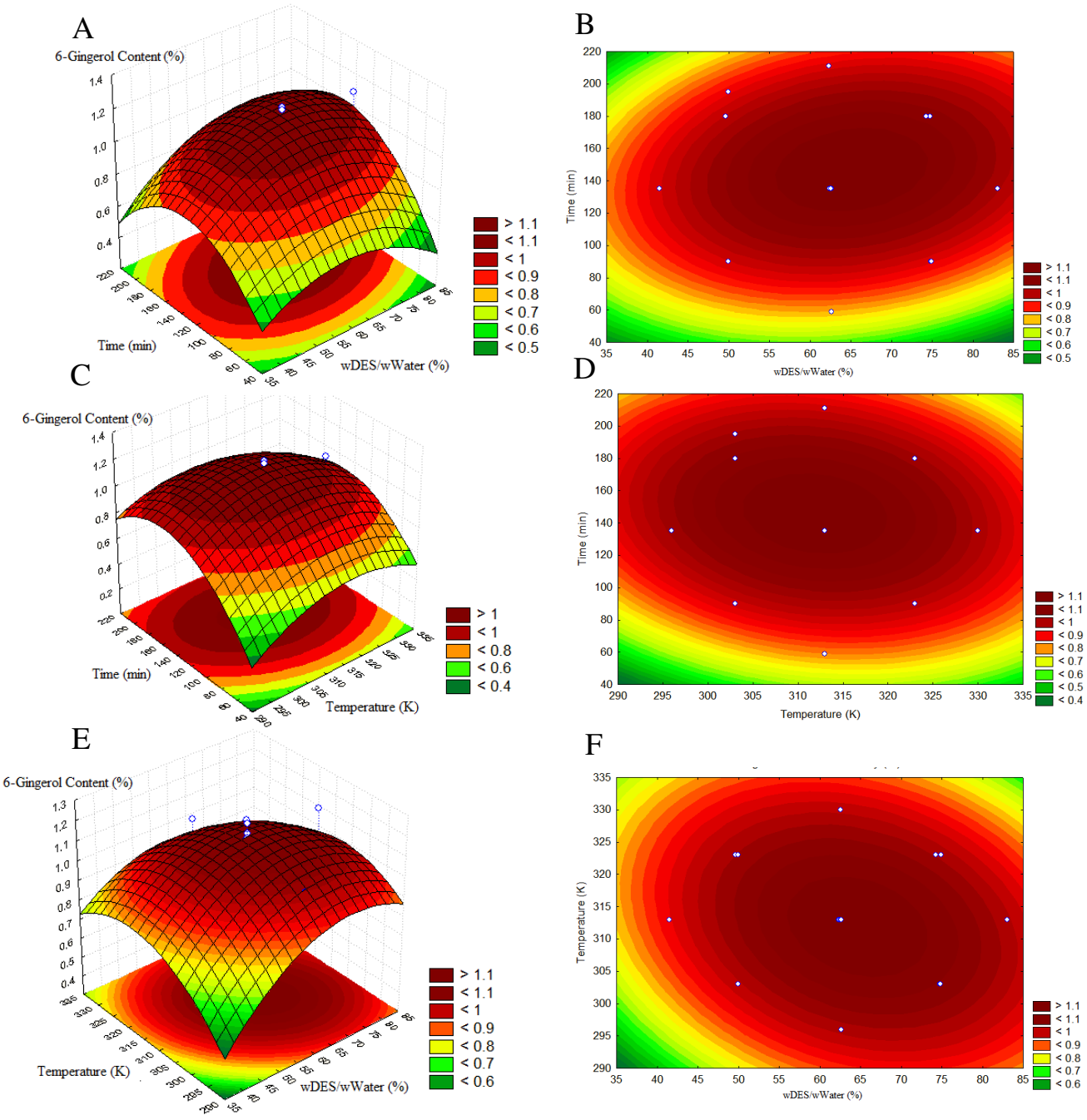


Figure 15. Response surface plots (left) and contour plots (right) on the 6-Gingerol content extracted from ginger (wt%) with the combined effects of (A and B) time (min) and concentration of DES in the aqueous solution (wDES %) (C and D) time (min) and temperature (K); and (E and F) temperature (K) and concentration of DES in the aqueous solutions (wDES %).

The accuracy and the precision of the model equations can be validated by comparing the experimental and the predicted values of extraction yield under the chosen conditions (Appendix D). The results presented in figure 15 C-F seem to confirm the suspicion that high temperatures do not favor high 6-gingerol levels, once that these temperatures may degrade the target compound during the extraction. The graphics indicate that optimum temperature of extraction should be between 310 and 315 K. The analysis of the concentration of DES in water indicates that it has a theoretical maximum near 65.7 wDES % in agreement with the results presented in the solubility tests. Regarding the extraction time, it seems that this is the most significant condition studied. Extractions below 80 minutes are not sufficient for a significant 6-gingerol extraction, while longer times of extraction, higher than 200 minutes seem to cause a somewhat negative impact in 6-gingerol extraction. This could be explained by the fact that extraction may have reached its maximum and then the exposure of the 6-gingerol to the low pH of the acidic DES at high temperatures may cause degradation of the target compound *(10)*. These results are also patent on the Pareto's Diagram, represented in Figure 16.

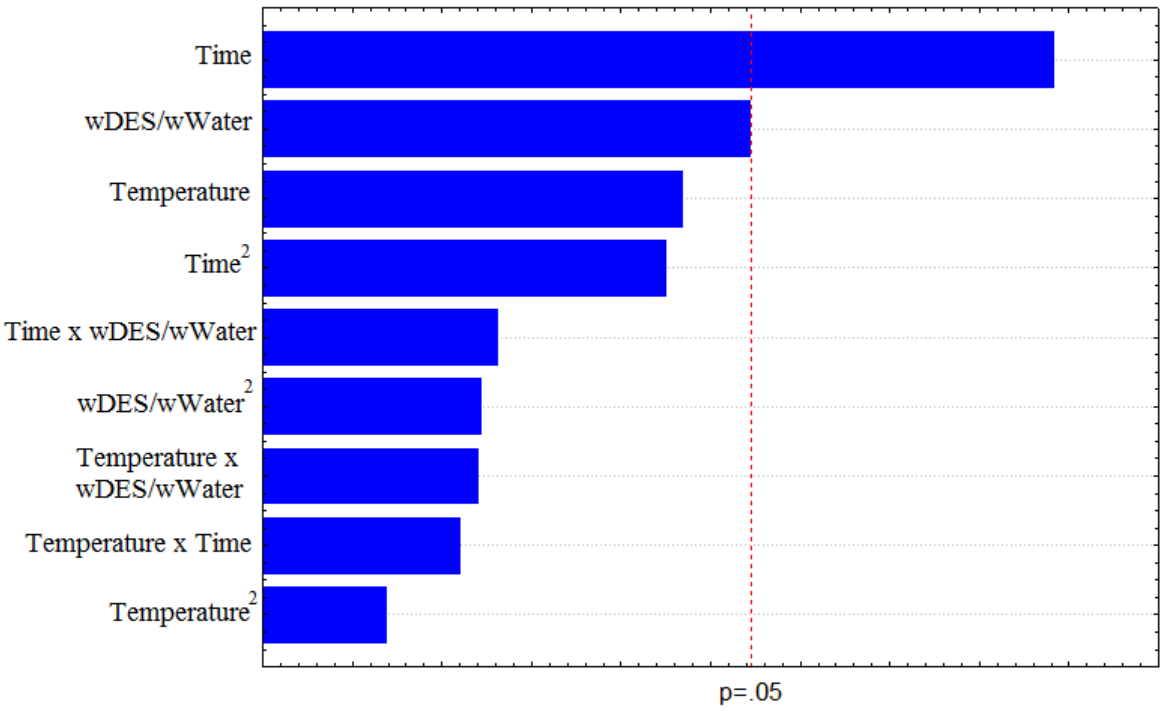


Figure 16. Pareto chart of standardized effects using a factorial design of 2^3, being 6-gingerol content in ginger (wt%) variable.

3.4. Experimental validation of the optimum extraction time value

The surface response design previously analyzed has drawn the attention for the importance of the extraction time optimization. Accordingly, a study of the time effect on the 6-gingerol extraction, and its confrontation with the theoretical maximum given by the surface response design and the method validation is depicted in Figure 17.

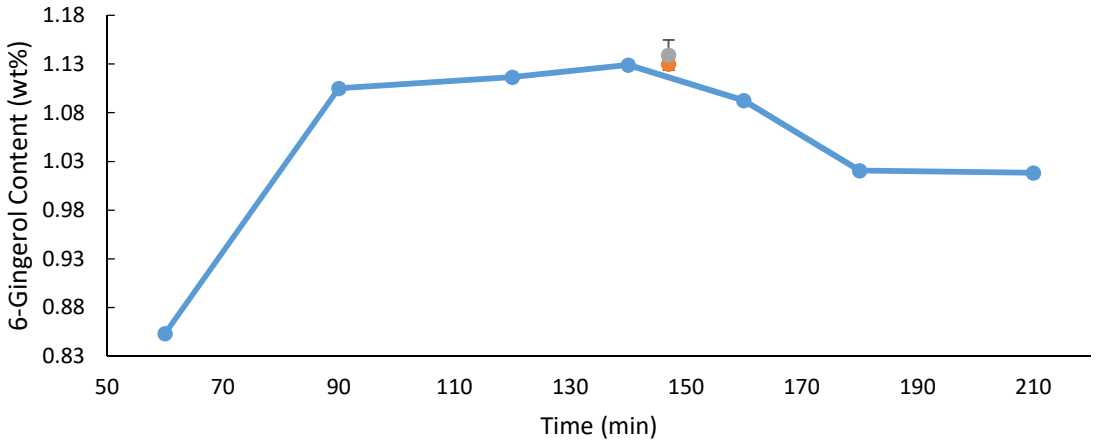


Figure 17. Effect of the extraction time in the 6-gingerol extraction: tested time values (blue), theoretical maximum by the SRM (orange) and experimental validation of theoretical data (grey).

The blue points present in this figure are tested time values at the optimum conditions of temperature and concentration of DES in the aqueous solution representing an extended tendency. Apparently 6-gingerol content levels reach its maximum for times between 120 and 160 minutes of extraction. As previously said, lower extraction times seem to be insufficient and longer extraction times seem deleterious to the target compound.

Coming back to the analysis of the surface response, the optimum conditions correspond to an extraction time of 148 minutes, a temperature of 311.1 K and a concentration of DES of 65.7 w_{DES} % leading to a theoretical maximum extraction of 1.13 wt% of 6-gingerol content in ginger (point in orange in Figure 17). To claim the validity of the method for the optimal extraction point given by SRM, the theoretical maximum was performed in triplicate (point in grey in Figure 17). The experimental validation of the theoretical data resulted in an extraction of 1.14±0.02 wt% which is very close to the theoretical maximum, ensuring the success of the assay.

3.5. Evaluation of optimized conditions

After the optimization of several parameters of the proposed alternative extraction for Pingo Doce's ginger, it was made a triplicate experiment in optimal conditions for Jumbo's ginger. This allowed to make the final comparison between conventional and alternative methodologies and for both gingers. Figure 18 depicts that comparison. For a better assessment, conventional methodologies extraction yield were calculated in content of 6-gingerol per ginger in percentage.

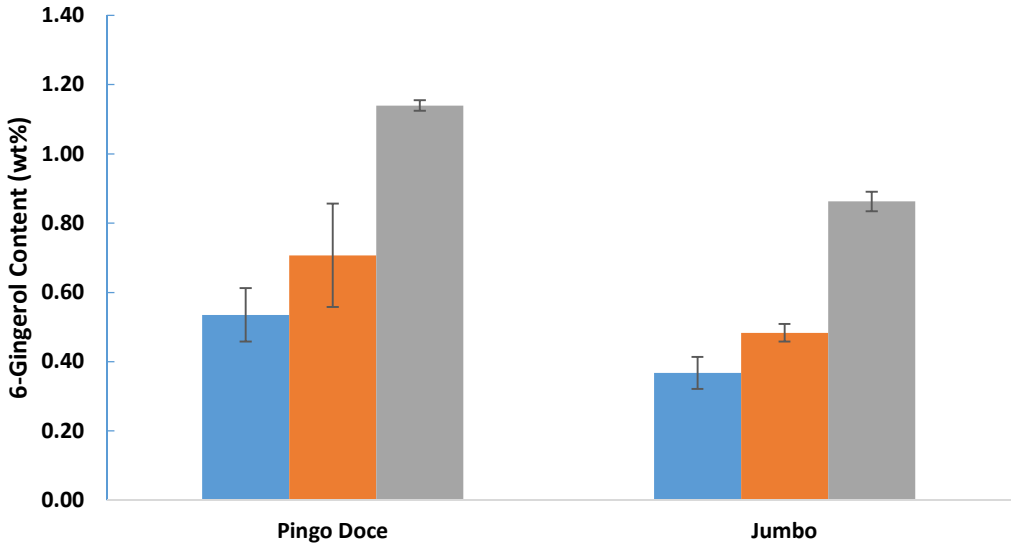


Figure 18. Comparison of the 6-gingerol content (wt%) extracted from both ginger samples and for the three methodologies tested. Soxhlet in blue, maceration in orange and PA:U DES extraction in grey.

The experimental data in Figure 18 shows the better performance of DES to extract 6-gingerol from ginger, increasing the extraction by at least 60% when compared to the best conditions of the conventional extraction, proving the success of the methodology.

3.6. Antioxidant activity

In order to determine the antioxidant activity, a SPE procedure was applied to a DES extract solution obtained from Pingo Doce's ginger. Although there was a successful separation of extract from the aqueous DES extract solution, there is still a need for an

optimization of this step of the procedure as a low level of recovery (≈40 wt%) of 6-gingerol was obtained. 6-gingerol level of recovery was calculated through the following equation:

$$6-gingerol\ recovery\ (wt\%) = \frac{C1\ (mg\ g^{-1})\ x\ m\ (g)}{C0(mg\ mL^{-1})x\ 3\ mL},$$

(Equation 6)

Where, *C1* represents the concentration of 6-gingerol in extract, *m* the final mass of the extract obtained by SPE and *C0* represents the original concentration of 6-gingerol in the aqueous DES solution. 3 mL corresponds to the volume used in the SPE column. One reason for such a low result might be the low capacity of the SPE column used. This could be enhanced through the use of a bigger column or by applying less quantity of DES solution. Nevertheless this technique allowed to obtain an extract with a concentration of 6-gingerol of 6.88 wt%, higher than those presented by extracts obtained from conventional methodologies (table 6).

Table 7 presents the antioxidant activity of the studied extracts obtained from Pingo Doce's ginger, expressed in terms of the amount of extract required to reduce into 50% the DPPH concentration (IC_{50}). The IC_{50} values for ascorbic acid were also obtained for comparative purposes. Detailed data is presented in Appendix F.

Table 7. Antioxidant activity of the extracts of ginger by DPPH radical scavenging, expressed as IC_{50} values, in µg of extract per mL.

	IC_{50} (µg mL^{-1})
Ascorbic acid	11.93 ± 0.08
DES extract	37.69 ± 1.69
Maceration extract	55.08 ± 2.34
Soxhlet extract	90.4 ± 0.87

In general, the obtained IC_{50} values demonstrated that all extracts presented lower antioxidant activity than ascorbic acid. Despite that fact, it seems that 6-gingerol content can be correlated with the antioxidant activity of each extract as it follows the same trend present in Figure 18. DES extract demonstrated to have a higher antioxidant potential when compared with extracts prevenient from conventional methodologies such as maceration and Soxhlet. All in all, DES extraction methodology proved to be a successful technique.

4. Conclusions

In this work, an alternative method for the extraction of 6-gingerol from ginger using a DES was developed. Initially, solubility tests using syringic and ferulic acids as model compounds were carried in order to learn what type of DES and what conditions could work best. The PA:U 2:1 was the DES that most enhanced the model compounds solubility. This DES offered the best solubility enhancement at a concentration between 50 and 75 w_{DES} % and at temperatures of 313 and 323 K for syringic and ferulic acids, respectively. Then, using PA:U, a surface response methodology was performed using a 2^3 factorial design aiming at the simultaneous study the effect of the temperature, time, and the DES concentration used in the extraction. Results of surface response methodology showed that the optimum conditions for the extraction of 6-gingerol correspond to an extraction time of 148 minutes, a temperature of 311.1 K and a concentration of 65.7 w_{DES} % matching a theoretical maximum extraction of 1.13 wt% of 6-gingerol content in ginger. Conventional methodologies, Soxhlet and maceration, were also performed as benchmark. Final results demonstrated that the alternative extraction using PA:U could be up to at least 60% more efficient that conventional extraction.

Finally, a SPE procedure was applied to obtain a recovery of the compound of interest from the DES solution, however it demonstrated to still have low efficiency as only 40% of the 6-gingerol was recovered. This procedure should be aim of optimization. With the extract prevenient from SPE and conventional methodologies, a DPPH assay was made in order to perceive the beneficial effects of the extracts. Among the 3 extract types, the DES extract proved to have the most antioxidant activity.

5. Future Work

Considering the results obtained in the solubility tests regarding acid alkylic chain effect it would be interesting to study DESs composed by acids of larger chains. In order to achieve a maximum extraction yield it would be interesting to study the effect of the solid-liquid ratio as this was the only parameter in this work that was not optimized. It would also be important to carry out several extraction cycles, reusing either the biomass or the aqueous solutions of DES in order to enhance the sustainability of the process.

Given the preliminary results of the SPE 6-gingerol recovery it would be of great importance to optimize this or other methodology in order to obtain an efficient 6-gingerol recovery from the DES solutions.

Taking into account that the DES extract presented the best antioxidant activity amongst the ones tested, cytotoxicity assays should be performed in order to evaluate if there is worth in recovering the extract from the DES aqueous solutions.

Finally, and due to the growing search for a sustainable and improved process and scarce work regarding DES recyclability, a DES recycling methodology should also be explored. Although Jeong and co-workers*(93)* successfully recycled the DES from the aqueous fraction by freeze-drying, this method should not work for the DES PA:U as PA is quite volatile. However, a process of fractional distillation may work for this DES and is worthy of study.

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7. Appendix

Appendix A: Calibration Curves

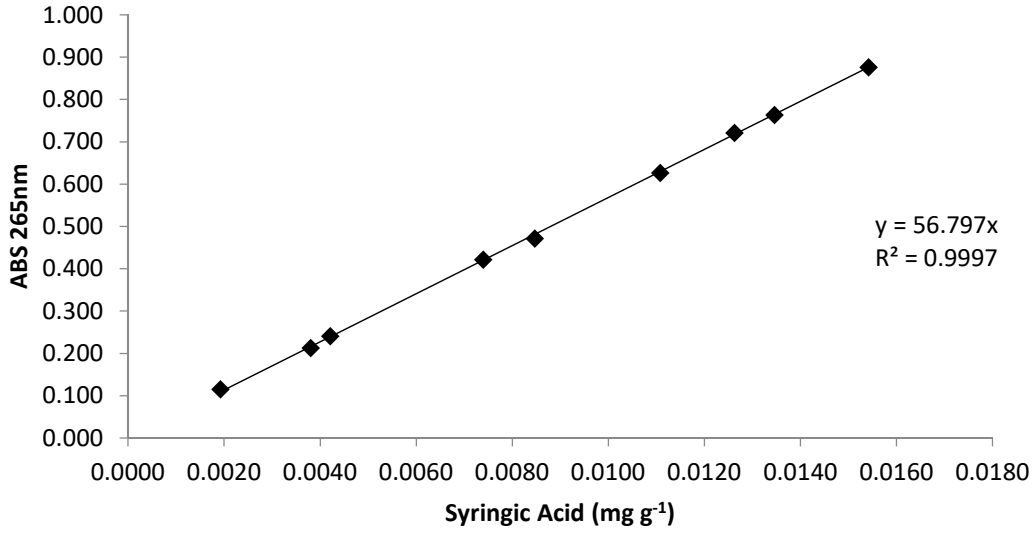

Figure A1. Calibration curve for syringic acid quantification in aqueous solution at 265 nm.

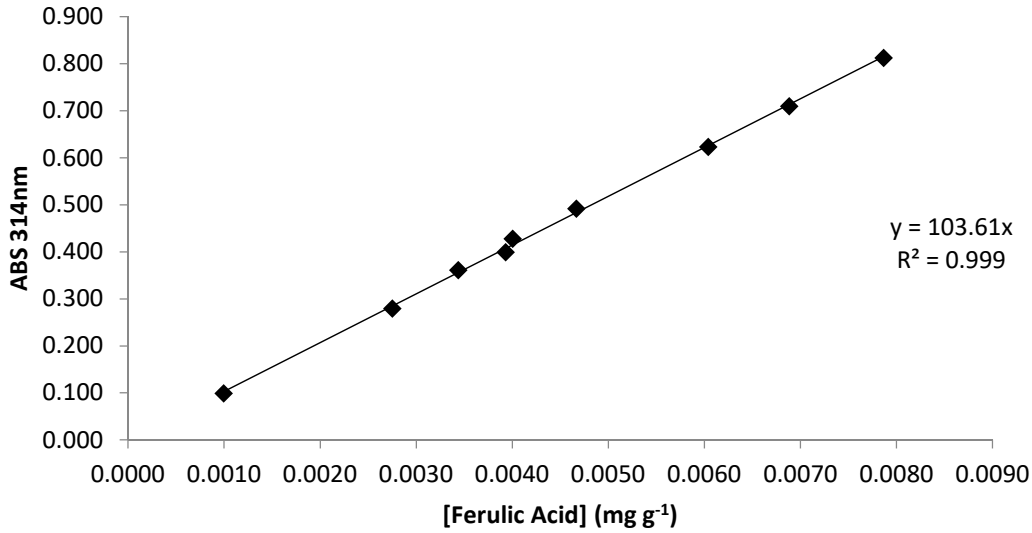

Figure A2. Calibration curve for ferulic acid quantification in aqueous solution at 314 nm.

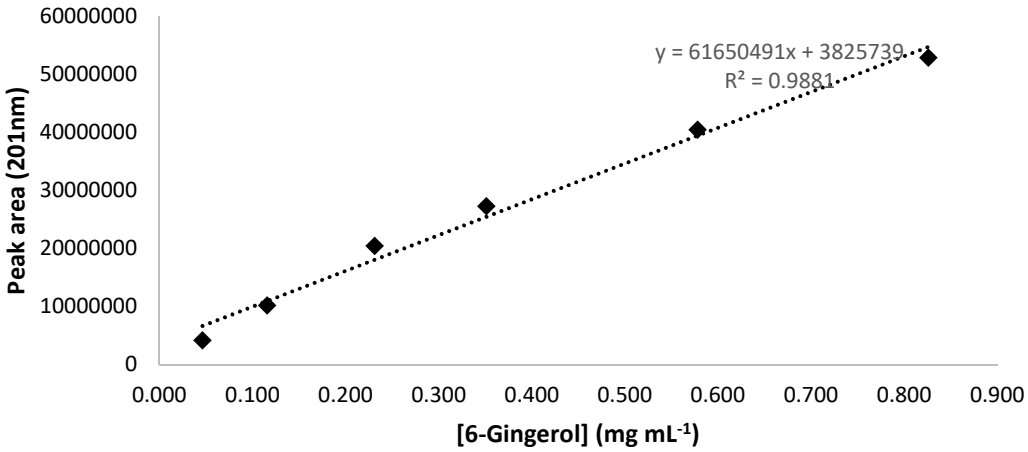

Figure A3. Calibration curve for 6-gingerol quantification in methanolic solution at 201 nm using a HPLC.

Appendix B: Chromatograms

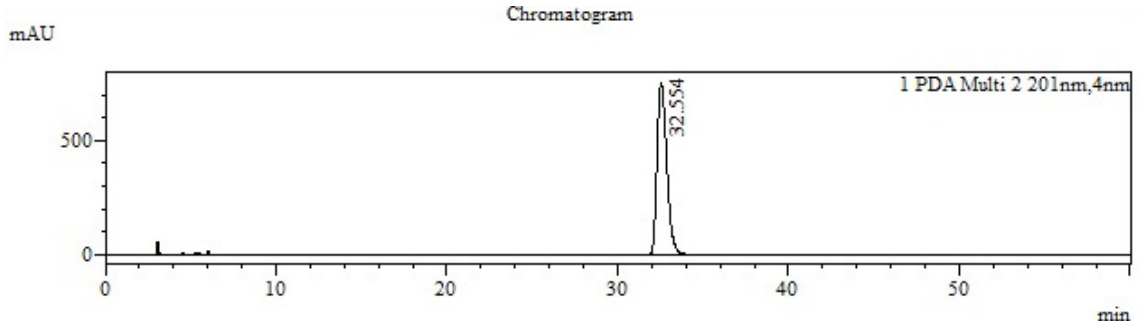

Figure B1. HPLC profile of 6-gingerol standard methanolic solution.

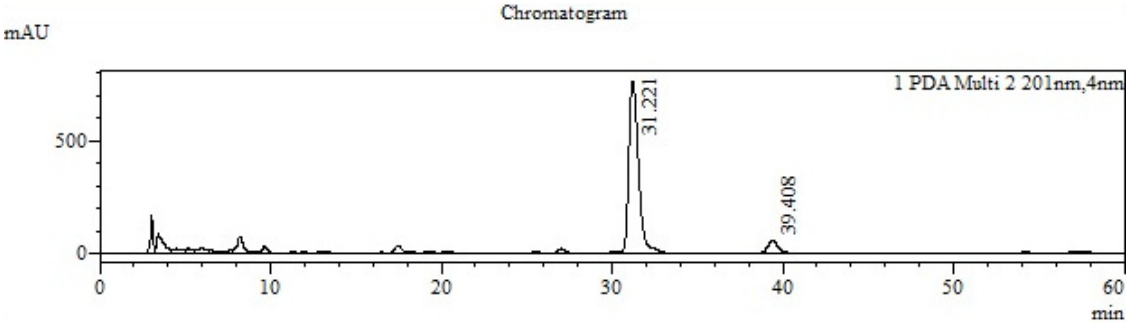

Figure B2. HPLC profile of maceration extract methanolic solution.

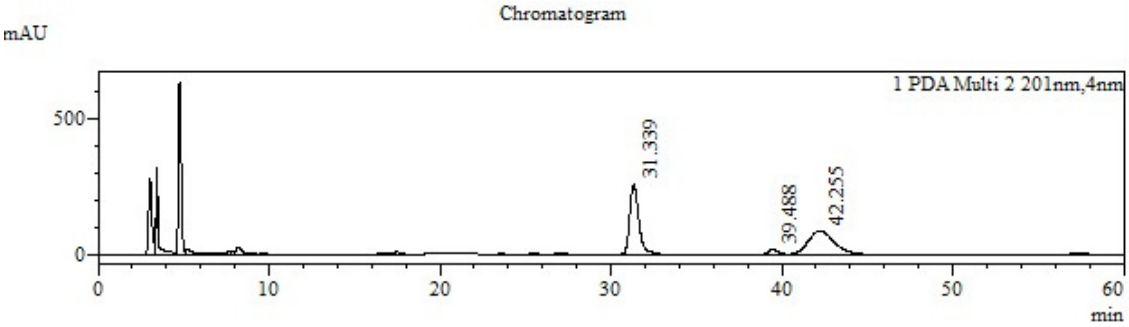

Figure B3. HPLC profile of Soxhlet extract methanolic solution.

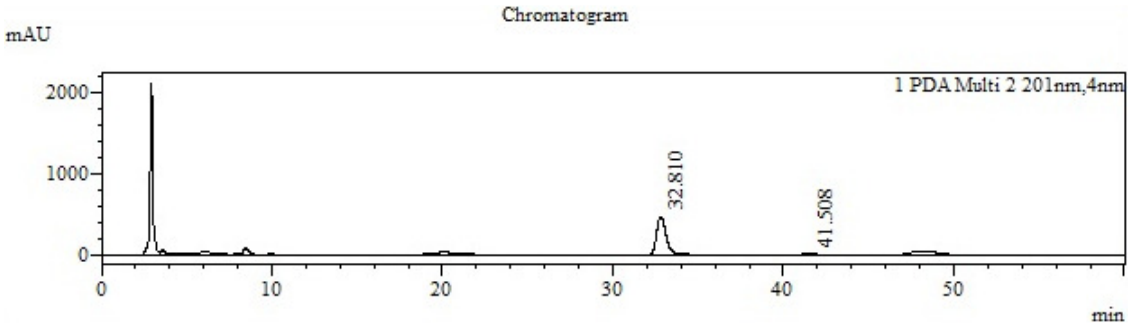

Figure B4. HPLC profile of DES extract methanolic solution.

Appendix C: Solubility Tests Data

Table C1. Solubility data to the HBD effect in syringic acid solubility in 25 wt% aqueous solutions at 303.15 K.

DES	S (mg g^{-1})	S/S_0
PA:[Ch]Cl 2:1	7.07 ± 0.14	5.51 ± 0.11
AcOH:[Ch]Cl 2:1	4.77 ± 0.03	3.72 ± 0.02
FA:[Ch]Cl 2:1	2.97 ± 0.08	2.31 ± 0.06
LA:[Ch]Cl 2:1	3.21 ± 0.07	2.51 ± 0.06
GA:[Ch]Cl 3:1	3.05 ± 0.04	2.38 ± 0.03
MA:[Ch]Cl 1:1	2.73 ± 0.04	2.13 ± 0.03
TSA:[Ch]Cl 1:1	6.28 ± 0.14	4.9 ± 0.11
U:[Ch]Cl 2:1	4.14 ± 0.01	3.23 ± 0.01
ETG:[Ch]Cl 2:1	2.68 ± 0.13	2.09 ± 0.1
Gly:[Ch]Cl 2:1	2.17 ± 0.06	1.7 ± 0.05
Fru:[Ch]Cl 1:1	2.79 ± 0.01	2.18 ± 0.01
Glc:[Ch]Cl 1:1	2.21 ± 0.15	1.72 ± 0.12
Xyl:[Ch]Cl 2:1	2.16 ± 0.15	1.69 ± 0.12
Water	1.28 ± 0.01	-

Table C2. Solubility data to the HBA effect in syringic acid solubility in 25 wt% aqueous solutions at 303.15 K.

	S (mg g^{-1})	S/S_0
AcOH:U 4:1	8.69 ± 0.28	6.78 ± 0.22
AcOH:Bet 2:1	7.02 ± 0.06	5.48 ± 0.04
AcOH:Pro 2:1	5.76 ± 0.02	4.49 ± 0.01
AcOH:[Ch]Cl 2:1	4.77 ± 0.03	3.72 ± 0.02
PA:U 2:1	12.33 ± 0.25	9.62 ± 0.2
PA:Bet 2:1	9.71 ± 0.26	7.57 ± 0.21
PA:Pro 2:1	9.03 ± 0.21	7.04 ± 0.17
PA:[Ch]Cl 2:1	7.07 ± 0.14	5.51 ± 0.11
LA:Bet 2:1	5.23 ± 0.01	4.08 ± 0.01
LA:U 2:1	4.41 ± 0.21	3.44 ± 0.17
LA:Pro 2:1	4.09 ± 0.01	3.19 ± 0.01
LA:[Ch]Cl 2:1	3.21 ± 0.07	2.51 ± 0.06
TSA:Pro 1:1	8.87 ± 0.08	6.92 ± 0.06
TSA:[Ch]Cl 1:1	6.28 ± 0.14	4.9 ± 0.11
Water	1.28 ± 0.01	-

Table C3. Solubility data to the molar proportion effect in syringic acid solubility in 25 wt% aqueous solutions at 303.15 K.

	S (mg g^{-1})	S/S_0
PA	15.05 ± 0.27	11.74 ± 0.21
PA:[Ch]Cl 4:1	8.67 ± 0.2	6.76 ± 0.16
PA:[Ch]Cl 3:1	8.2 ± 0.38	6.4 ± 0.29
PA:[Ch]Cl 2:1	7.07 ± 0.14	5.51 ± 0.11
PA:[Ch]Cl 1:1	4.65 ± 0.07	3.63 ± 0.05
PA:[Ch]Cl 1:2	4.01 ± 0.1	3.13 ± 0.08
PA:[Ch]Cl 1:3	3.72 ± 0.17	2.9 ± 0.13
PA:[Ch]Cl 1:4	3.36 ± 0.18	2.62 ± 0.14
[Ch]Cl	3.07 ± 0.45	2.4 ± 0.35
PA:U 4:1	13.82 ± 1.07	10.78 ± 0.84
PA:U 2:1	12.33 ± 0.25	9.62 ± 0.2
PA:U 1:1	10.72 ± 0.25	8.36 ± 0.2
PA:U 1:2	8.89 ± 0.06	6.94 ± 0.05
PA:U 1:4	4.77 ± 0.16	3.72 ± 0.13
U	3.47 ± 0.03	2.7 ± 0.03
Water	1.28 ± 0.01	-

Table C4. Solubility data to the molar proportion effect in ferulic acid solubility in 25 wt% aqueous solutions at 303.15 K.

	S (mg g^{-1})	S/S_0
PA	18.91 ± 0.33	22.92 ± 0.4
PA:U 4:1	8.2 ± 0.13	9.94 ± 0.16
PA:U 2:1	6.96 ± 0.04	8.44 ± 0.05
PA:U 1:1	3.39 ± 0.23	4.11 ± 0.28
PA:U 1:2	1.36 ± 0.36	1.65 ± 0.44
PA:U 1:4	0.75 ± 0.01	0.91 ± 0.01
U	0.64 ± 0.02	0.78 ± 0.03
Water	0.83 ± 0.03	-

Table C5. Syringic acid solubility enhancement test data for PA:U 2:1 DES system, at different temperatures and concentration of DES in aqueous solution.

	S (mg g^{-1})	**S/S_0**
Temperature	**303 K**	
Water	1.28 ± 0.01	-
25 % w_{DES}	12.33 ± 0.25	9.62 ± 0.2
50 % w_{DES}	38.3 ± 0.82	29.88 ± 0.64
75 % w_{DES}	33.11 ± 1.38	25.82 ± 1.07
95 % w_{DES}	8.81 ± 1.32	6.88 ± 1.03
100 % w_{DES}	5.19 ± 1.75	4.05 ± 1.36
Temperature	**313 K**	
Water	1.6 ± 0.03	-
25 % w_{DES}	16.17 ± 0.74	10.12 ± 0.47
50 % w_{DES}	43.04 ± 2.07	26.92 ± 1.3
75 % w_{DES}	49.71 ± 4.24	31.09 ± 2.65
95 % w_{DES}	15.89 ± 0.32	9.94 ± 0.2
100 % w_{DES}	6.07 ± 0.2	3.8 ± 0.13
Temperature	**323 K**	
Water	1.87 ± 0.08	-
25 % w_{DES}	16.94 ± 1.66	9.08 ± 0.89
50 % w_{DES}	58.02 ± 1.83	31.11 ± 0.98
75 % w_{DES}	88.37 ± 3.55	47.38 ± 1.9
95 % w_{DES}	15.89 ± 0.32	8.52 ± 0.17
100 % w_{DES}	11.27 ± 3.11	6.04 ± 1.67

Table C6. Ferulic acid solubility enhancement test data for PA:U 2:1 DES system, at different temperatures and concentration of DES in aqueous solution.

	S (mg g^{-1})	S/S_0
Temperature	**303 K**	
Water	0.83 ± 0.03	-
25 % w_{DES}	6.96 ± 0.04	8.44 ± 0.05
50 % w_{DES}	21.09 ± 0.41	25.57 ± 0.49
75 % w_{DES}	13.85 ± 1.4	16.79 ± 1.69
95 % w_{DES}	6.85 ± 0.12	8.31 ± 0.14
100 % w_{DES}	4.13 ± 0.1	5 ± 0.13
Temperature	**313 K**	
Water	0.82 ± 0.03	-
25 % w_{DES}	21.09 ± 0.63	25.58 ± 0.76
50 % w_{DES}	42.1 ± 0.89	51.06 ± 1.08
75 % w_{DES}	27.1 ± 2.79	32.87 ± 3.39
95 % w_{DES}	8.89 ± 0	10.78 ± 0
100 % w_{DES}	5.69 ± 0.14	6.9 ± 0.17
Temperature	**323 K**	
Water	1.53 ± 0.05	-
25 % w_{DES}	17.6 ± 1.2	11.48 ± 0.79
50 % w_{DES}	68.99 ± 0.35	45.02 ± 0.23
75 % w_{DES}	43.5 ± 1.35	28.38 ± 0.88
95 % w_{DES}	13.69 ± 0.93	8.93 ± 0.61
100 % w_{DES}	8.18 ± 0	5.34 ± 0

Appendix D: SRM Extra Data

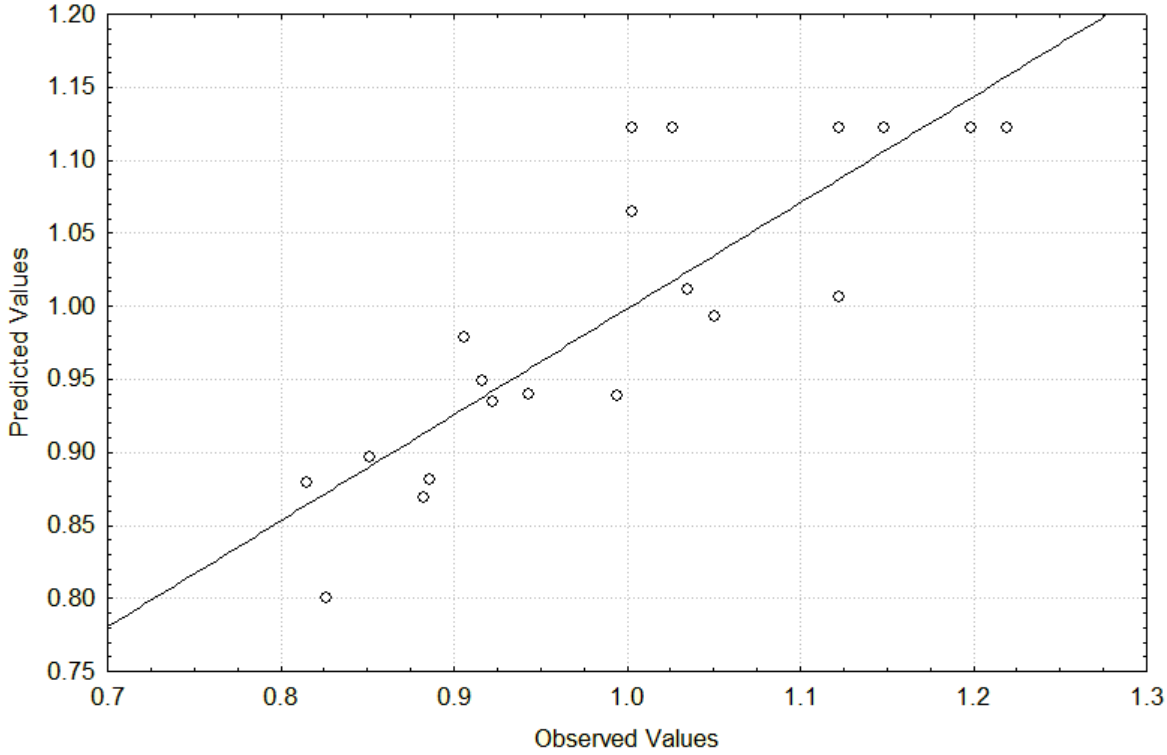

Figure D1. Predicted versus observed values for the factorial design of 2^3.

Table D1. Data attributed to the independent variables (Temperature, time and wt% of DES in the aqueous solution) to define the 2^3 factorial planning for the system under study and respective results of concentration of 6-gingerol extracted experimentally, the theoretical results found for the mathematical model developed and the respective relative deviation.

Run	Temperature (K)	Time (min)	w_{DES} (%)	6-Gingerol Content (%)		Residues
				Experimental Values	Theoretic Values	
1	303	195	50	0.89	0.88	0.003
2	323	180	50	0.92	0.94	-0.013
3	303	180	75	1.00	1.07	-0.063
4	323	180	75	0.91	0.98	-0.074
5	296	135	62.5	1.04	1.01	0.026
6	330	135	62.5	1.05	0.99	0.058
7	313	210	62.5	0.99	0.94	0.056
8	313	135	41.5	0.92	0.95	-0.031
9	313	135	83.5	1.12	1.00	0.118

10	313	135	62.5	1.12	1.12	0.001
11	313	135	62.5	1.00	1.12	-0.119
12	313	135	62.5	1.03	1.12	-0.095
13	313	135	62.5	1.15	1.12	0.026
14	313	135	62.5	1.22	1.12	0.098
15	313	135	62.5	1.20	1.12	0.077
16	303	90	50	0.88	0.87	0.012
17	323	90	50	0.94	0.94	0.002
18	303	90	75	0.85	0.90	-0.046
19	323	90	75	0.82	0.88	-0.065
20	313	60	62.5	0.83	0.80	0.028

Table D2. Regression coefficient of the predicted second-order polynomial model for the 6-gingerol extraction obtained from the RSM design using PA:U 2:1 as solvent.

	Regression Coeffient	**Standard Deviation**	**t-student (10)**	**p-value**
Interception	-47.0242	23.2813	-2.0198	0.0710
Temperature	0.2791	0.1445	1.9313	0.0823
Temperature^2	-0.0004	0.0002	-1.8375	0.0960
Time	0.0226	0.0215	1.0521	0.3175
Time^2	0.0000	0.0000	-3.9144	0.0029
w_{DES}	0.0931	0.0822	1.1325	0.2839
w_{DES}^2	-0.0003	0.0002	-2.2189	0.0508
Temperature x Time	0.0000	0.0001	-0.6022	0.5605
Temperature x w_{DES}	-0.0002	0.0003	-0.7014	0.4991
Time x w_{DES}	0.0000	0.0001	0.8117	0.4358

Table D3. ANOVA data for the extraction of 6-gingerol obtained from the factorial design of 2^3 planning.

	Sum of Squares	**Degrees of Freedom**	**Mean of Squares**	**F_{calc}**	**p-value**
Regression	0.225	9	0.025	3.194	0.034
Error	0.078	10	0.008		
Total	0.282	19	R^2=0.7224		

Appendix E: First RSM Study Data

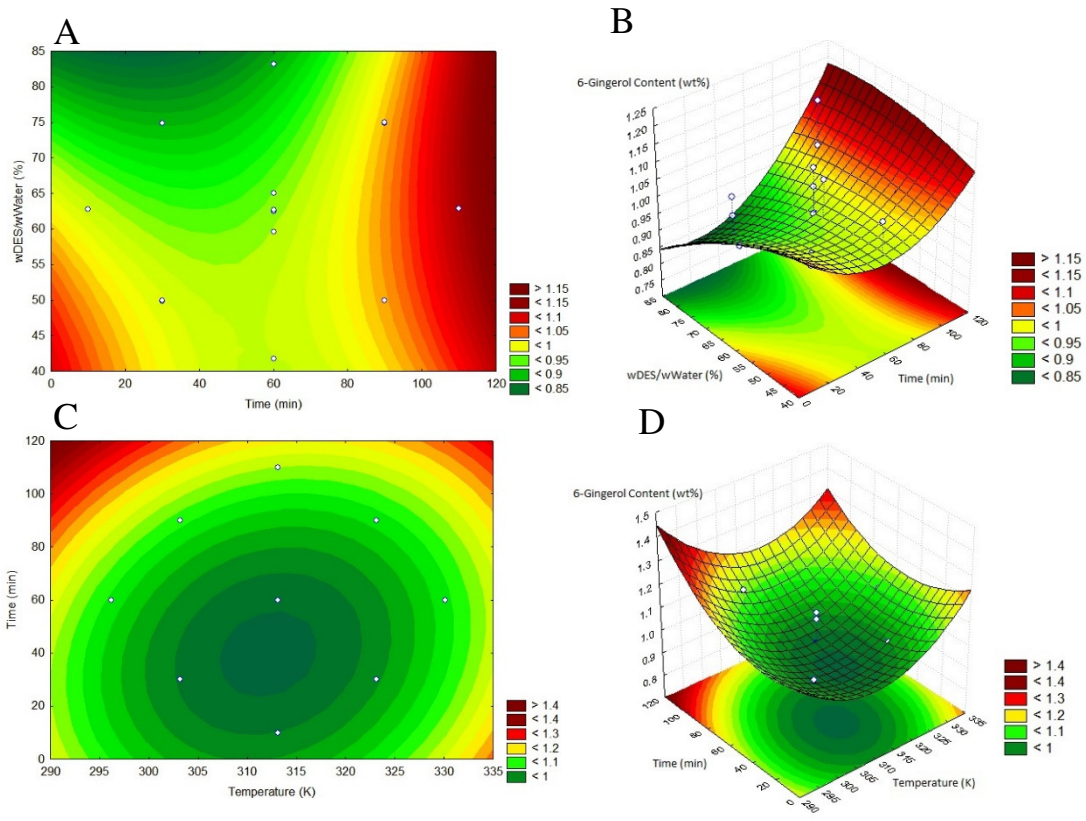

Figure E1. Response surface plots (left) and contour plots (right) on the 6-Gingerol content extracted from ginger (wt%) with the combined effects of (A and B) time (min) and concentration of DES in the aqueous solution (wDES%) (C and D) time (min) and temperature (K).

Appendix F: Antioxidant Activity Extra Data

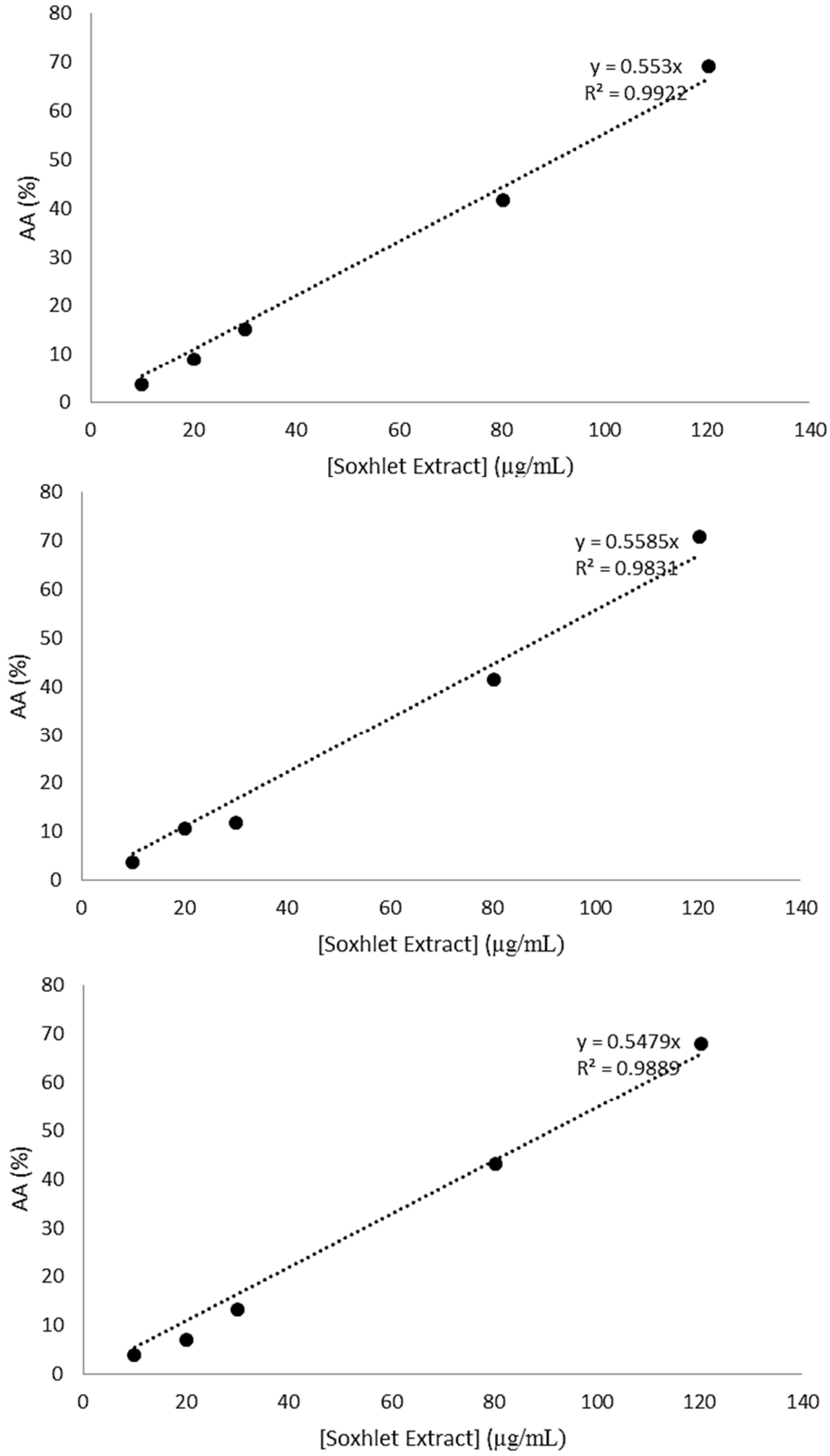

Figure F1. Correlation between Soxhlet extract concentration and their antioxidant activity in DPPH assay performed in triplicate.

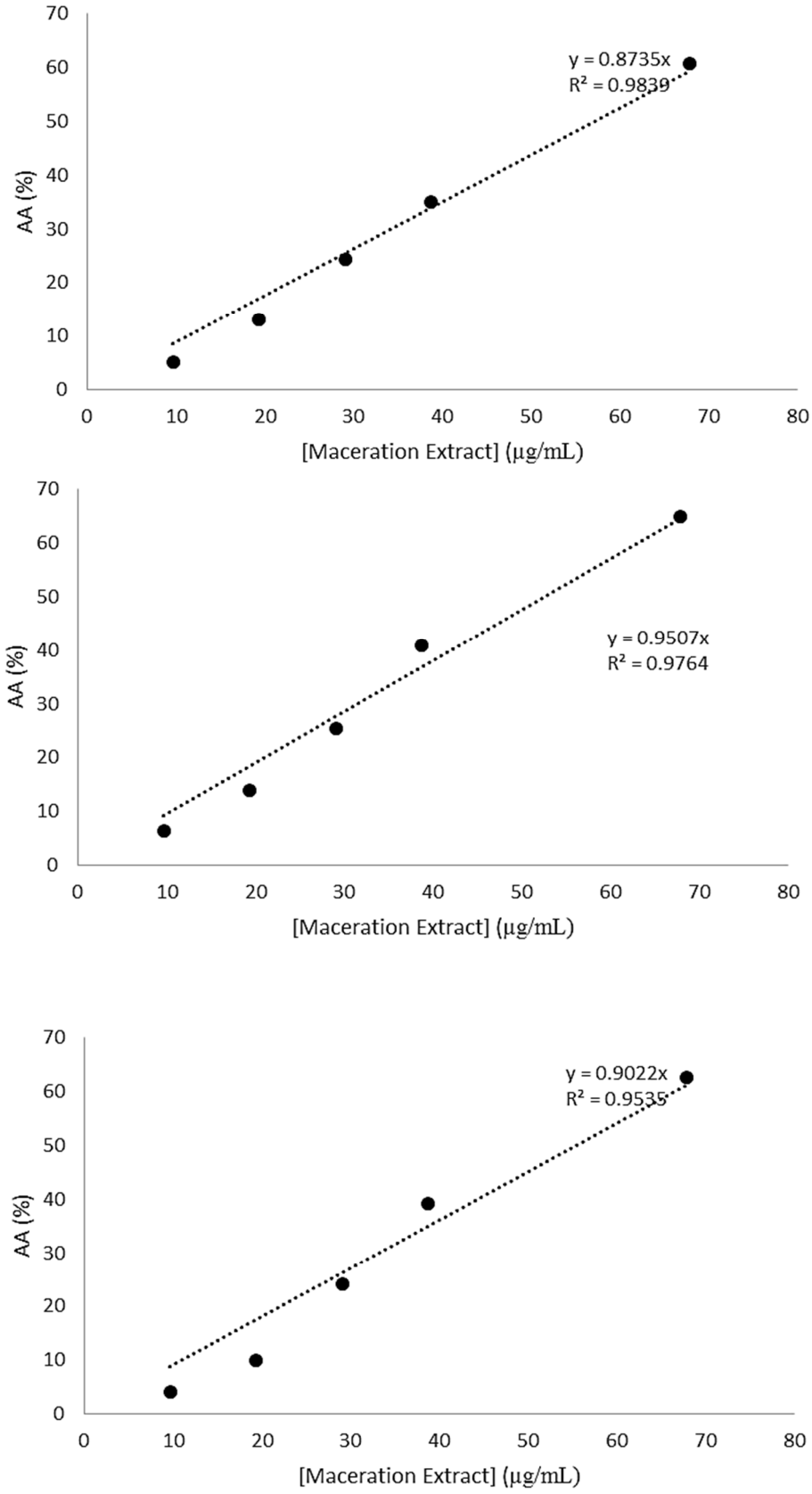

Figure F2. Correlation between maceration extract concentration and their antioxidant activity in DPPH assay performed in triplicate.

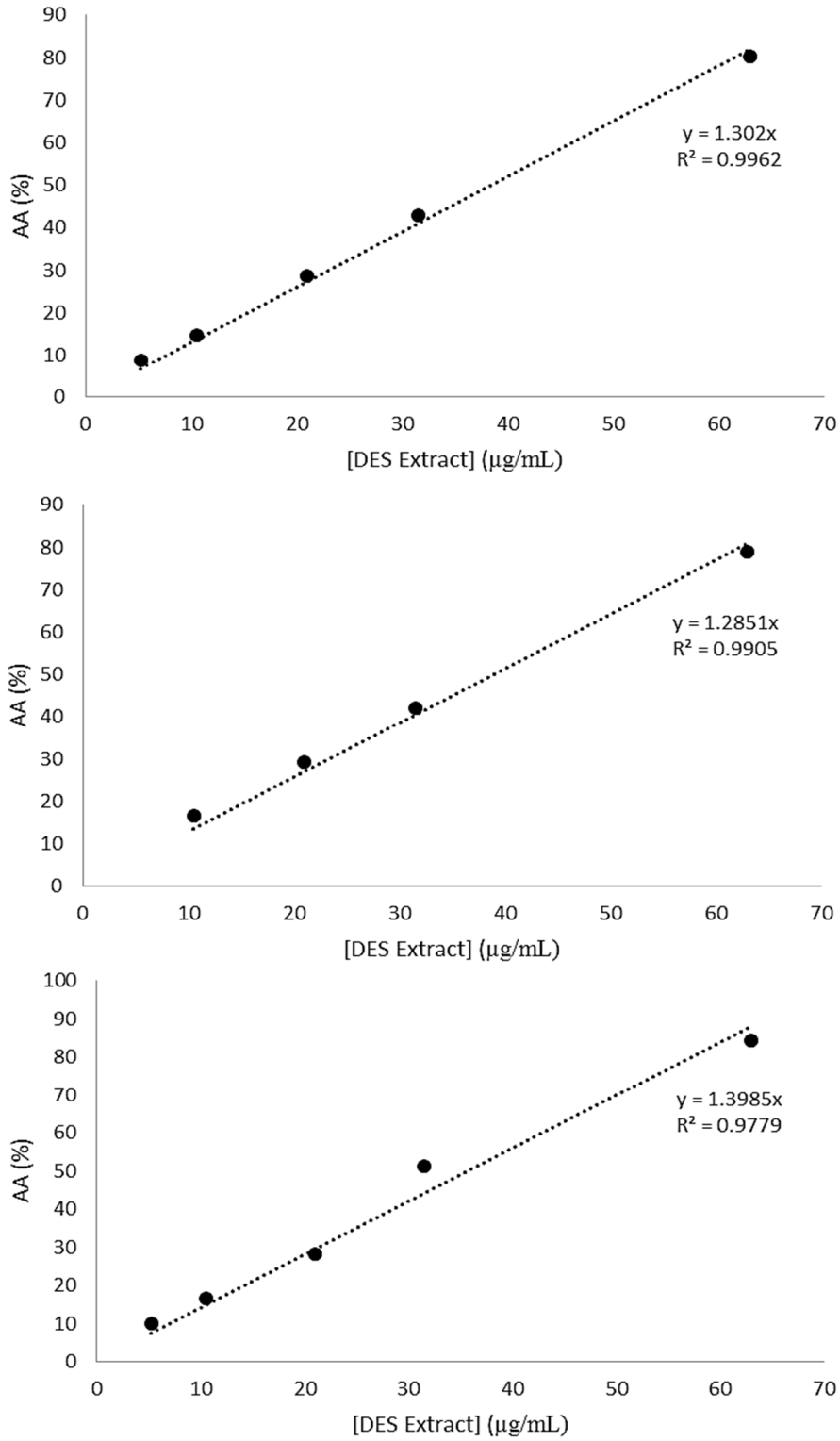

Figure F3. Correlation between DES extract concentration and their antioxidant activity in DPPH assay performed in triplicate.

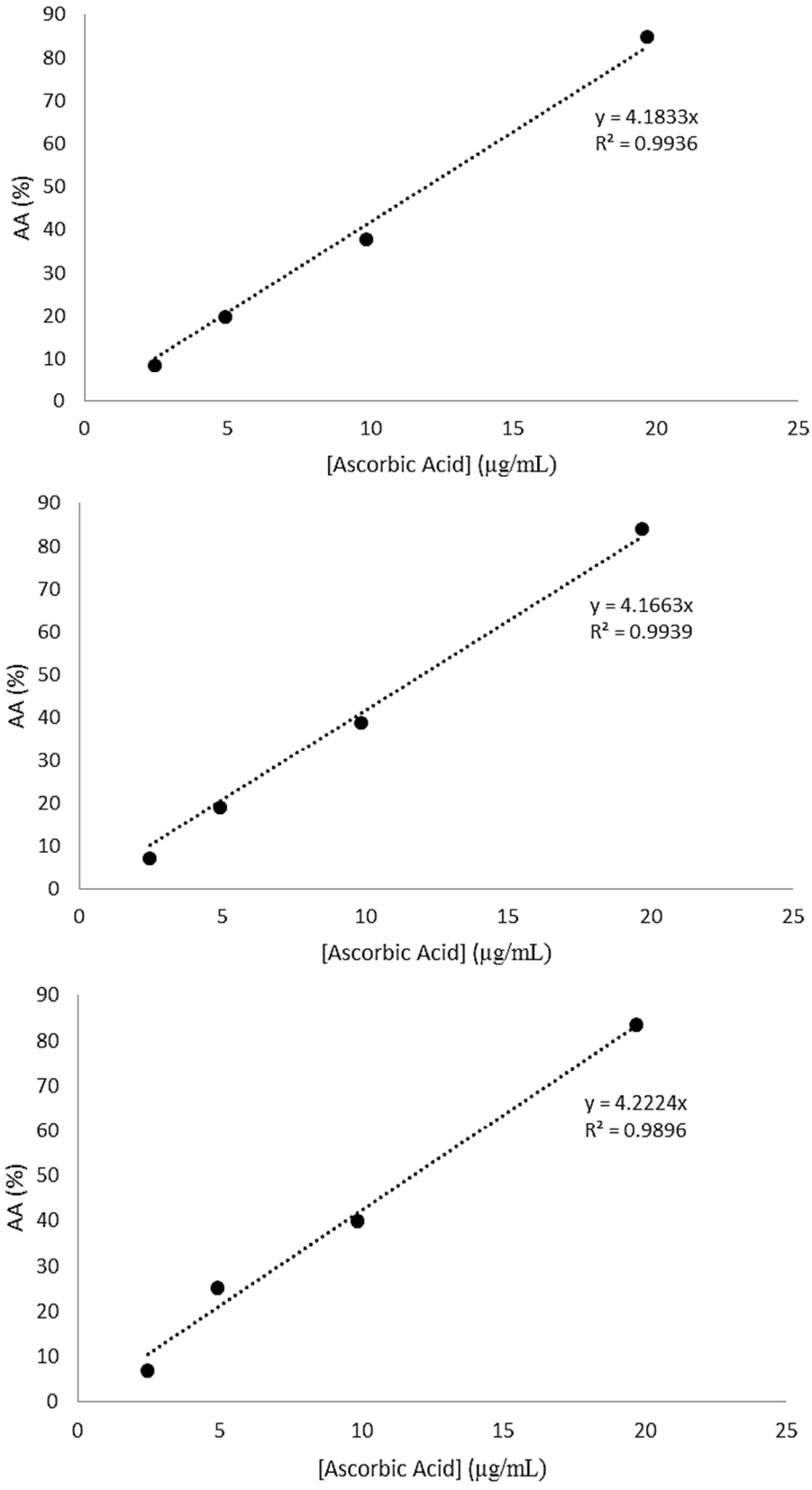

Figure F4. Correlation between ascorbic acid concentration and their antioxidant activity in DPPH assay performed in triplicate.