Mechanosynthesis of Therapeutic Deep Eutectic Solvents

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Chemistry

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Resumo

Uma mistura homogénea com um ponto de fusão inferior ao dos seus componentes, normalmente resultante do estabelecimento de interações não covalentes, e numa certa relação molar, é denominada sistema eutético. Os solventes eutéticos profundos (DES – do inglês deep eutectic solvents) que compreendem ou atuam como solventes de ingredientes farmacêuticos ativos (APIs) surgiram recentemente como alternativas promissoras para melhorar a eficiência terapêutica dos APIs. Além disso, como não estão envolvidos solventes orgânicos nem reações químicas na sua produção, evitando a produção de resíduos, podendo assim substituir os solventes convencionais, os DES são considerados "solventes verdes".

A mecanoquímica é uma tecnologia emergente em síntese química, e, dadas as suas inúmeras vantagens, é de especial interesse para o desenvolvimento de uma indústria farmacêutica mais sustentável. Neste trabalho, explorou-se a síntese alternativa de novos DES terapêuticos (THEDES – do inglês therapeutic DES), preparados por moagem automática, usando um moinho de bolas planetário, os quais foram comparados com os produzidos pela via clássica, usando aquecimento. Dos inúmeros THEDES preparados, foram selecionados três, para um estudo mais aprofundado: flavona-timol, flavanona-timol e chalcona-timol. Foram determinados experimentalmente, usando DSC e turbidimetria, os diagramas de equilíbrio sólido-líquido (SLE) e comparados com os diagramas SLE ideais. Foi igualmente avaliada a sua citotoxicidade. Os novos THEDES possuem uma diminuição muito significativa do ponto eutético, sendo alguns deles líquidos à temperatura ambiente numa larga gama de composições, validando não só a mecanoquímica como uma via alternativa de síntese, mas demostrando igualmente o potencial dos flavonoides como uma nova classe de THEDES.

Palavras-chave: Solventes Alternativos, Solventes Eutéticos Profundos, Mecanoquímica, Ingredientes Farmacêuticos Ativos, Flavonoides

Abstract

A homogeneous mixture with a melting point lower than its components, typically using noncovalent interactions, at a certain molar ratio, is called an eutectic system. Deep eutectic solvents (DES), comprising or acting as solvents of active pharmaceutical ingredients, have recently emerged as promising alternatives to improve therapeutic efficiency due to their low toxicity. In addition, no solvents and no chemical reaction are required in their formation which implies better yields and less waste production, turning DES attractive “green solvents”. Due to the numerous advantages of mechanochemistry, especial in sustainable pharmaceutical industry, in this work we explored the formation of new therapeutic DES (THEDES) prepared by ball milling. To validate the mechanochemical synthetic route, these THEDES were compared with those prepared by conventional heating. In order to access the full range of available compositions, the solid-liquid equilibrium (SLE) diagrams of three new THEDES, flavone:thymol, flavanone:thymol and chalcone:thymol, were studied. The experimental SLE phase diagrams were measured using visual turbidimetry and DSC, and the results were compared with the ideal phase diagrams. Toxicity data was also determined. The results showed a deep decrease in the melting temperature of the eutectic point compared to the ideal mixture description. Overall, this work validated mechanochemistry as an alternative synthetic route for DES preparation and demonstrated the potential of flavonoids an interesting new class of THEDES.

Keywords: Green Solvents, Therapeutic Deep Eutectic Solvents, Mechanochemistry, Active Pharmaceutical Ingredients, Flavonoids

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Abbreviations and Symbols

API	Active pharmaceutical ingredient
$CDCl_3$	Deuterated chloroform
ChCl	Chlorine chloride
C_p	Heat capacity
DEIL	Deep eutectic ionic liquids
DES	Deep eutectic solvent
DMSO-*d6*	Deuterated dimethyl sulfoxide
DSC	Differential scanning calorimetry
EPA	Environmental protection agency
FT-IR	Fourier-transform infrared spectroscopy
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond acceptor
HDES	Hydrophobic deep eutectic ionic solvents
IL	Ionic liquids
kJ	Kilojoule
LMM	Low melting point mixtures
LTTM	Low transition temperature mixtures
NADES	Natural deep eutectic solvents
NMR	Nuclear magnetic resonance
p_c	Critical pressure
R	Gas constant
SCB	Biopharmaceutical classification system
SLE	Solid-liquid equilibrium
T	Temperature
T_c	Crystallization temperature
T_g	Glass transition temperature
THEDES	Therapeutic deep eutectic solvents
T_m	Melting temperature
v_c	Critical volume
VOC	Volatile organic compound
ΔH^0_{fus}	Fusion enthalpy

I.Introduction

In 2007, the world demand for solvents was estimated to be around 20 million tons, being 28 million 5 years later, and even higher annual growth rates are expected in future. The pharmaceutical industry is one the biggest consumers of organic solvents, not only in the production process but also in the highly demanding purification steps that are required to fulfil strict guidelines regarding purity and contaminants levels of any active pharmaceutical principle (API). Globally, solvents represent between 80 to 90% of the non-aqueous mass used to manufacture any API.[1] So the correct choice of a solvent has a high impact not only in the product final form but also in the whole chemical process sustainability.

The concept of Sustainable Chemistry emerged in the 1990's, as a philosophy focused on the design of chemical products and processes that involves the reduction or elimination of substances that are harmful to humans or the environment. In fact, Sustainable Chemistry arose from the idea that economic development could lead to a deterioration in the quality of life, rather than an improvement. Hence, the definition of Sustainable Development provided by the Brundtland report of the United Nations Commission on Environment and Development (1987) is the "Development capable of meeting current needs without compromising the ability of future generations to meet their own needs".[2]

Green Chemistry is one of the multiple facets of Sustainable Chemistry as it promotes innovative chemical technologies that reduce or eliminate the use or generation of harmful substances in the design, manufacture and use of chemical products. The Environmental Protection Agency (EPA), together with the American Chemical Society, further encouraged and developed the Green Chemistry concept, which was followed by the establishment of the twelve principles by Paul T. Anastas and John C. Warner in 1998.[3]

Commonly used solvents, such as toluene, chloroform, dimethylsulfoxide, hexane, all come from petroleum, and thus their future use is unsustainable. Moreover, all of them are volatile, flammable and in many cases, toxic and/or not biodegradable and so they have a strongly negative environmental impact. On the other hand, the carbon footprint of these common volatile compounds (VOCs) is enormous, if we consider their manufacturing process, recycling and final destruction. In theory, an ideal solvent should be safe, both for humans and for the environment, and its use and production should also be truly sustainable.[4] Therefore, the need to use new sustainable

solvents, and concomitantly the development of neoteric solvents, is a priority in industry.[5]

Water could be considered the ideal solvent, given its price, toxicity, flammability and its environmental impact.[6] But its limitations in terms of the solubility of organic compounds and expensive purification makes its use limited. Although supercritical fluids,[7] especially carbon dioxide, are currently being used in the agri-food industry, the high cost of the associated equipment, the low solubility of organic polar reagents hinders its widespread use. Fluorinated[8] solvents have also been proposed as alternative solvents, but their high price and their persistence in the earth ecosystem led to their low use.

Another possibility in terms of solvent is ionic liquids (ILs).[9] Ionic liquids are organic compounds generally formed by an ammonium cation, such as imidazolium or pyridinium, and an anion with a low nucleophilicity. These solvents were originally believed to be fully sustainable, as they are thermally very stable, have almost zero vapor pressure at room temperature, are generally non-flammable, are miscible with many typical organic solvents. However, different drawbacks, such as their high price, stability of some ILs in water, intrinsic toxicity, low biodegradability, and low efficiency in synthesis and purification, have been hindering their widespread use.

At the beginning of twenty first century, the Abbott group published a few articles that sparked research in the field of Deep Eutectic Solvents (DES). They examined the properties of these systems and pointed out the possibility of their use as solvents due to their interesting characteristics. Other terms and names have also been used, which may or may not denote the substances that we now consider DES: low transition temperature mixtures (LTTM), low melting point mixtures (LMM) or deep eutectic ionic liquids (DEIL).

Perhaps due to their novelty, DES are mainly limited to chemical, electrochemical and materials applications. Still, their recently discovered biocompatibility with biomaterials (e.g., nucleic acids, enzymes, and drugs) and beneficial applications in organic catalysis, biotransformations, and molecular extractions have attracted interest from the pharmaceutical and biotechnology industries. If we combine this with the growing interest of the chemical industry to develop not only products, but also new synthesis processes that overcome the restrictions that currently condition their use, DES become highly relevant. This new class of solvents has been developing compounds with a broad range of properties. In particular, hydrophobic DES (HDES) were proposed few years ago and have notably showed increased interest of the scientific community, as shown in Figure 1.

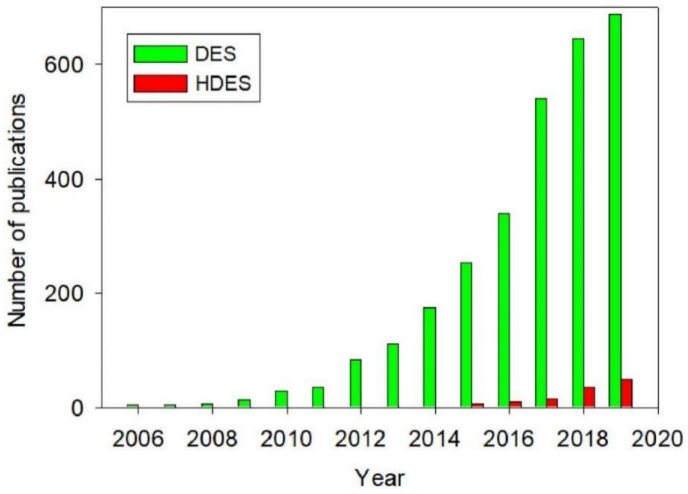


Figure 1. Number of scientific articles published during 2006–2019 on deep eutectic solvents (DES) and hydrophobic deep eutectic solvents (HDES), based on the *Scopus*® database.

Recent progress in synthetic organic chemistry, molecular modelling, and high-throughput screening has led to the production of many new chemical entities with specific pharmacological activity, as well as the discovery of therapeutic properties for existing molecules. However, in order to formulate these new APIs, it is necessary to select an adequate solvent, that will both preserve its integrity and enables the compatibility with the requirements for administration to human patients. Many molecules that are revealed to have high specificity for pharmacological targets have low water solubility, that limits both their formulation and their bioavailability. Nevertheless, traditional organic solvents are not acceptable for pharmaceutical applications and present considerable constraints for manufacturing purposes. At the same time, alternative routes of administration avoiding intravenous injection, such as oral and topical routes, are attracting more and more interest because of their ease of use and better acceptability. This brings new challenges to formulation and a need for suitable additives.

The Biopharmaceutical Classification System (SCB) allows drugs to be classified according to their permeability and solubility, that is, their biopharmaceutical characteristics, as depicted in Figure 2. These two parameters, together with the dissolution rate in the intestinal lumen, govern the oral absorption process.[10] In addition, it raises, as an objective, the possibility of establishing *in vitro-in vivo* correlations in immediate release formulations that allow replacing human trials with in vitro dissolution tests. *In vitro* bioequivalence studies are based on the comparative analysis of two drugs, where the amount or percentage of the active ingredient dissolved is determined as a function of time under controlled and validated conditions.[11]

Figure 2. Classification of drugs based on their permeability and solubility according to the SCB[10].

The therapeutic effect of a drug depends on its bioavailability and the solubility of the molecules in biological fluids. Therefore, solubility is one of the most important parameters to achieve the desired drug concentration in the systemic circulation to obtain a pharmacological response. There are several strategies to improve the solubility of poorly soluble drugs and, consequently, increase their bioavailability, such as: particle size reduction, crystal engineering, emulsion systems, salt formation and solid dispersions. Liquid drug formulations are much less common and are usually based on eutectic mixtures.[12] The use of an active drug in liquid form can avoid some of the polymorphism problems associated with solid forms. Other similar approaches have been developed with liquid drug formulations prepared as eutectic mixtures,[13] but these can dilute the APIs owing to large quantities of inactive ballast in the formulation. Along these lines, the possibility of solubilizing APIs in biocompatible DES or the formation of API-DES, can provide new perspectives for drug delivery and treatment approaches. From a pharmaceutical industry point of view, the use of liquid salts is very relevant, preferably those having melting points below room temperature.

I.1. Deep Eutectic Solvents

In the last years, the term DES has been used in a broader context to designate any eutectic mixture that is liquid at room temperature, and thus can be used as solvent. The existence of an eutectic point on a mixture cannot be exclusively used to define a DES. They are also often described as hydrogen bond complexes, but the presence of a hydrogen bond between two components of a mixture is not a sufficient definition. DES should be defined as a mixture of pure compounds for which the eutectic point

temperature is far below that of an ideal liquid mixture.[14] The temperature depression should be defined as the difference (ΔT_2) between the ideal ($T_{E,ideal}$) and the real (T_E) eutectic point, and not as the difference (ΔT_1) between the linear combination of the melting points of the pure components and the real eutectic point, as shown in Figure 3.

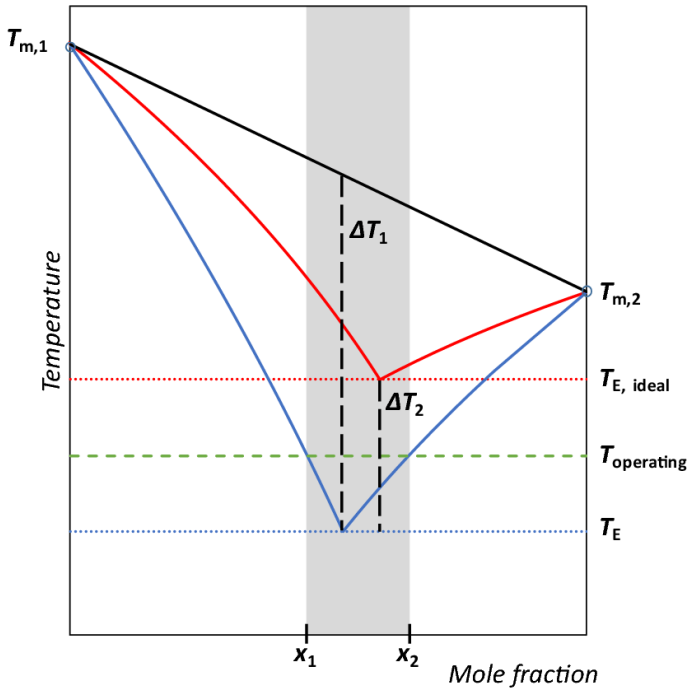


Figure 3. Schematic representation of the comparison of the SLE of a simple ideal eutectic mixture (red line) and a deep eutectic mixture (blue line).[14]

This second approach seems inappropriate since, as discussed above, the presence of a eutectic point in the SLE phase diagram is a characteristic of all mixtures of compounds that are fully, or partly, immiscible in the solid phase. If ΔT_1 is used as definition of a DES then it would cover essentially any mixture of compounds, with exception of those presenting complete solid solutions or forming stable intermediate compounds with melting points comparable to those of the pure precursors. Several authors have proposed different minimum values for ΔT_2, above which the eutectic mixture can be considered a DES, but no agreement has yet been reached.[14]

The eutectic point is an isobaric invariant of the system and represents the composition and the minimum melting temperature along the two intersecting melting curves.[15] Assuming pure solid phase and neglecting the temperature influence on the heat capacities, classical thermodynamics proposes Equation 1 to describe these melting curves,

$$ln\,(x_i\,\gamma_i\,) = \frac{\Delta_m H}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right) + \frac{\Delta_m C_p}{R}\,(\frac{T_m}{T} - ln\frac{T_m}{T} - 1) \qquad (1)$$

where γ_i is activity coefficient of compound *i* at a certain liquid mole fraction composition x_i, *T* is the absolute temperature, T_m and ΔH_m are the melting temperature and enthalpy of melting of the pure compound, respectively, R is the universal gas constant, and $\Delta_m C_p$ is the difference between the molar heat capacity of compound *i* in the liquid and solid phases. When the equilibrium temperature is not far from the melting temperature of the pure compound, the last term of the equation has a negligible value when compared to melting enthalpy term and thus, can be neglected.

To differentiate between the possible DES, four different families have been proposed. Based on the general formula Cat^+X^- zY, where Cat^+ is generally an ammonium, phosphonium or sulfonium, while X is a Lewis base (usually a halide anion). Y represents a Lewis or Brønsted acid and z is the number of Y molecules that interact with the corresponding anion, these families are shown in Figure 4.[16]

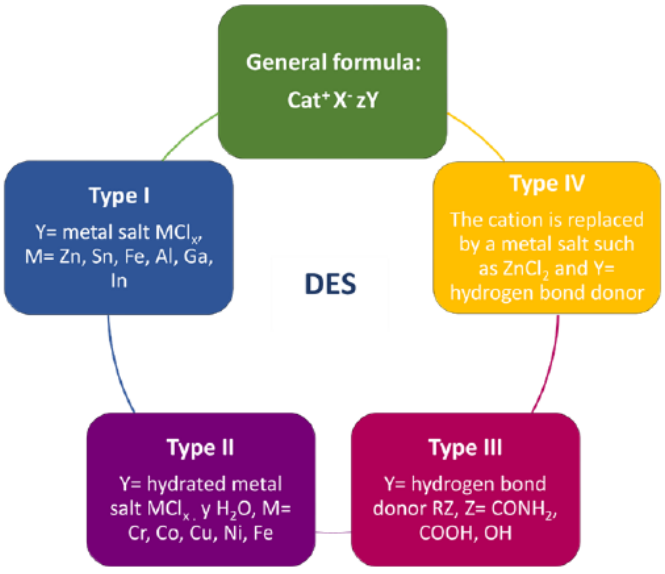


Figure 4. The four types of deep eutectic solvents based on the general formula Cat^+X^- zY.[17]

DES of type III were primarily based on cholinium chloride (ChCl). The hydrogen bond acceptors (HBA) mainly include quaternary ammonium or phosphonium salts, whereas the most common hydrogen bond donors (HBD) are amides, alcohols and carboxylic acids. In addition, compounds like sugars, sugar alcohols and amino acids are also considered for natural DES preparation.[18] More recently, HDES were

introduced, and are based on hydrophobic compounds such as menthol, thymol and fatty acids as hydrogen bond acceptors together with long alkyl chain (above C_{10}) alcohols and carboxylic acids as HBD.[19] Furthermore, DES can be made of active pharmaceutical ingredients like ibuprofen, lidocaine and phenylacetic acid. These DES are named therapeutic deep eutectic solvents (THEDES).[20] Figure 5 shows common HBA and HBD components used in DES preparation.

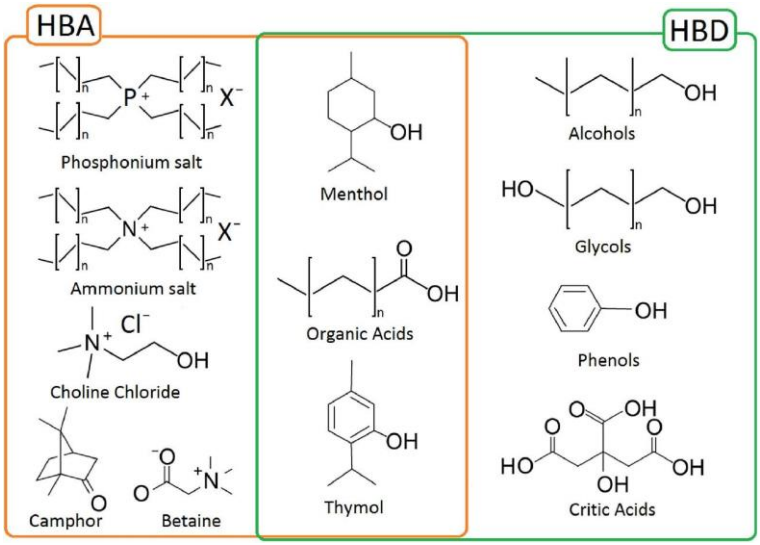


Figure 5. Common HBA and HBD used in DES preparation. [21]

Nevertheless, most of the reported DES do not fall into one of the above-mentioned classes given their versatility and countless combinations. DES of type V, fully composed of non-ionic species, have been recently proposed.[22]

In 2011, it was hypothesized that primary plant metabolites may form a liquid medium, rather than two immiscible liquid phases in plant cells (water and lipids), and that it liquid medium could be involved in certain biosynthetic steps or storage of water insoluble products. A set of 30 novel DES containing natural intracellular compounds including sugars, carboxylic acids, aminoacids, choline, and water were prepared and coined as natural deep eutectic solvents (NADES).[23] Since then, many NADES systems have been reported.[24] Due to their natural origin, NADES are considered as non-toxic and biodegrable and environmentally benign alternatives of ILs and DES.[25]

I.2. DES Preparation

In general, DES are obtained by mixing two or more compounds with capacity to establish hydrogen bonds, thus forming an eutectic mixture at a well-defined molar ratio.

Several methods have been used for DES preparation, which are briefly described below.

In the heating method the components are melted, under constant stirring, until a homogeneous liquid is formed.[26] The temperatures usually range between 50 to 100 °C. However, high temperatures may potentially lead to degradation of the components or chemical reactions (*e.g.,* esterification).[27] Considering the optimization of time and energy consumption, a greener microwave-assisted approach has been proposed, allowing DES preparation within seconds.[28] An ultrasound assisted NADES synthesis was recently introduced.[29]

In the evaporation method the components are dissolved in water, followed by an evaporation at 50 °C, and the resulting liquid is then placed in a desiccator.[30] This is valid only for the preparation of hydrophilic solvents.

Components in aqueous solution may be also used as DES precursors using the freeze-drying method.[31] The obtained solutions are frozen and freeze-dried, resulting in the formation of clear and viscous liquids. However, water was detected in the mixtures because it can strongly interact with DES components, becoming a part of the DES network.[32]

Interestingly, grinding has been also reported, where the components were mixed by crushing with a mortar and pestle at room temperature, until a clear liquid was formed.[33]. Nevertheless, despite its great potential, DES mechanosynthesis is rather unexplored.

I.3. DES Physicochemical Properties

The unique combination of DES physicochemical properties is one of the main reasons behind the rising interest on these solvents. DES are easily tuneable, just by changing the components in the eutectic mixture, meaning they can be easily designed for specific applications. This potential encouraged its investigation as reliable alternatives to conventional solvents.

I.3.1. SLE-Phase Behaviour

As mentioned above, DES are not single components but mixtures of two or more pure components. The knowledge of the solid-liquid equilibria (SLE) behaviour of these mixtures is very important for their application as solvents. A typical SLE diagram is presented in Figure 6, which shows the melting temperature as a function of the mixture

composition. Therefore, if we consider a binary mixture of components A and B, the eutectic point represents the composition and the minimum melting temperature at which the melting curves of both components meet.

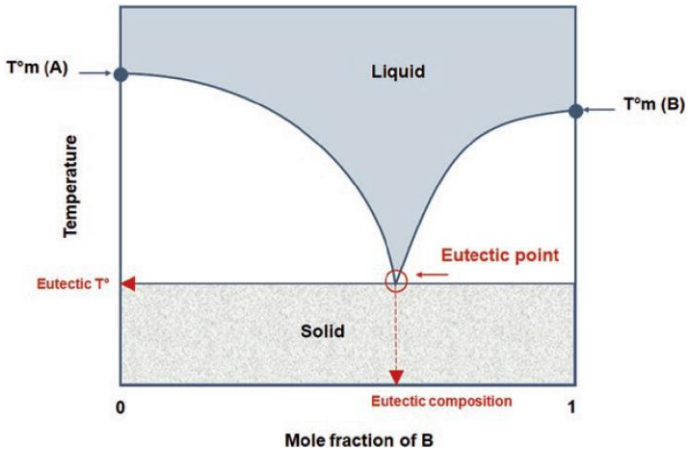


Figure 6. General solid-liquid phase diagram of a binary mixture. T_m(A) and T_m(B) represent the melting temperatures of components A and B, respectively.

The information regarding the melting data of the pure components is essential to determine the ideal solubility curve and thus ascertain if we are in the presence of a DES. There are few reports on the thermodynamic behaviour of DES, being the choice of the HBD,[34] the nature of the organic salt and its anion, and the organic salt/HBD molar ratio[35] done in a trial-and-error approach. However, this information is very important to choose the most appropriate compounds and conditions for specific application.

I.3.2. Density

Density is one of the most important physicochemical parameters of a solvent. The density of DES is a consequence of the molecular organization or packaging of the acceptor (HBA) and donor (HBD) species of hydrogen bonds that constitute them. Similar to what occurs in ILs composed of imidazolium salts, DES contain “vacancies” or “voids”, in such a way that an increase in the radius of these voids leads to a decrease in the density of the DES. On the other hand, the molar ratio of the HBA: HBD species, as well as the temperature, also exert a significant effect on the density of these mixtures.

I.3.3. Viscosity

DES viscosity is a key parameter that determines its applications as a green solvent. Most DES have high viscosities (> 100 cP) at room temperature. This high viscosity is attributed to the presence of multiple hydrogen bonds between the components, which leads to a decrease in the mobility of free species. In general, the viscosity of a DES is affected by the nature and molar ratio of the components, the temperature, the water content, and the free volume. Thus, the use of small cations as HBD species can lead to lower viscosity. Also, the viscosity of binary eutectic mixtures is essentially governed by hydrogen bonds and electrostatic and van der Waals interactions, in such a way that, as occurs in ILs, an increase in temperature decreases viscosity. Consequently, DES prepared from solely neutral compounds are much less viscous than DES prepared with charged compounds, such as salts.

I.3.4. Ionic Conductivity

The conductivity of DES is closely related to their viscosity, and, in general, the conductivity also increases with increasing temperature. However, the high viscosity of most DES means that they present low conductivities at room temperature (below 2 mS cm^{-1}). Furthermore, taking into account that the molar ratio of the HBA:HBD species notably influences the viscosity of DES, this parameter also has a significant effect on their conductivity.

I.3.5. Polarity

Polarity is also a key property since reflects the overall solvation capability of the solvent. Despite its significance, and the usage of DES in extraction processes, the polarity of DES was not addressed until recently. This property is often estimated via solvatochromic parameters which consider the hypsochromic (blue) shift or bathochromic (red) shift of UV-Vis bands for negatively solvatochromic dyes (e.g., Reichardt's betaine dye) or positively solvatochromic dyes (e.g., Nile red), respectively, as a function of the solvent's polarity.[36]

I.3.6. Surface Tension

To date, studies related to the surface tension of DES are very scarce, despite it important in mixing processes.[37] In general, surface tension with temperature is expected

to follow a behaviour like viscosity, since it depends on the strength of the molecular interactions that govern the formation of these mixtures.

I.4. Therapeutic DES

Therapeutic DES, coined as THEDES in 2015, comprise deep eutectic systems that incorporate an API in the mixture or DES that can dissolve an API and improve its properties in terms of bioavailability and toxicity.[38] Many APIs have HBD or HBA groups in their structures, therefore eutectic systems can be prepared using one or more APIs. THEDES can be formed between an API and an excipient or between two APIs. Examples of reported THEDES are ibuprofen–menthol,[39] lidocaine–1,8-octanediol,[40] lidocaine–prilocaine,[41] ranitidine hydrochloride–urea, and aspirin–choline chloride. THEDES have been used to design polymeric drug delivery systems to overcome polymorphism, to enhance the dissolution rate[42], to increase membrane permeability and to improve transdermal delivery. Until now, most of the research has focused on APIs transdermal delivery.

The main application of THEDES relates to formulation of hydrophobic drugs. THEDES not only increase the solubility of these molecules, compared to aqueous media, but may also increase their stability and sometimes, even favour a particular conformation. For example, nonsteroidal anti-inflammatory drugs suffer from low water solubility. The solubility of these drugs in several DES based on choline chloride was studied, but the authors were not able to predict solubility just by the simple analysis of the DES physical properties or at the HBD/HBA chosen ratio.[41]

It is known that many APIs are not stable in aqueous solution. For instance, many ester-containing pharmaceuticals, such as aspirin, undergo hydrolysis upon prolonged storage in water.[43] The hydrolysis of aspirin into salicylic and acetic acids in the ChCl:1,2-propanediol DES was shown to be 8.2 times slower than in aqueous solution.[44] The betaine:urea DES also increased the stability of β-lactam antibiotics, namely imipenem and clavulanic acid, by 7- and 2.5-fold, respectively, when compared with their aqueous solutions.

Most APIs may act as HBDs and/or HBAs, and thus be used as DES phase-forming constituents. However, the proper selection of both the HBA and HBD plays a critical role in THEDES formulation. The introduction of dual function is one of the most promising strategies since allows the incorporation of two active ingredients in the same formulation, thus avoiding possible polymorphism issues.[45]

To achieve the desired therapeutic effect, APIs must efficiently and specifically reach the site of action. However, APIs delivery is often hampered by low solubility in

body fluids, low permeability through biological membranes, chemical instability, and rapid metabolism and elimination.[46] To address these problems, APIs are formulated in different delivery systems such as solid dispersions,[47] cocrystals[48] and polymeric delivery systems.[49] Recently, the potential of limonene-based THEDES was demonstrated in cancer treatment.[50]

Transdermal drug delivery is one of the most popular administration routes for analgesics because it offers several advantages, such as the extension of the duration of activity, minimization of pain, reduction of side effects, and controlled drug release. This approach often faces problems related to permeation through the membrane, and thus eutectic formulations have been used both as absorption promoters[51] or permeation enhancers.[52]

I.5. DES Mechanosynthesis

Mechanochemistry is based on the energy transfer from a mechanical force to chemical or structural transformations. The first major development of mechanochemistry was the introduction of automated equipment. The improvement in the control of parameters, such as the frequency and milling time, resulted in an increase of the reactions efficiency and reproducibility.

Research in mechanosynthesis has recently intensified due to the remarkable advantages of this solventless method. Also, the associated energy efficiency and consumption are highly relevant for industrial applications, being considerably smaller than in conventional or microwave heating.[53] Although mechanosynthesis is usually performed in batch mode, continuous manufacture using twin screw extrusion has been already achieved,[54] even for a kg-scale.[55] A dramatic decrease in reaction time is observed by this method, avoiding thermal degradation, and the methodology may be applicable to other systems, such as ionic liquids.[56]

I.6. Flavonoids and Chalcones

The use of flavonoids and chalcones as DES components is almost inexistent, and to the best of our knowledge only one report was found in literature, using chalcone to prepare a ternary DES.[57] However, these classes of compounds, abundant and cheap, have a high potential to be incorporated in novel eutectic systems. The chemical structures of representative compounds of this two families, flavone, and flavanone (two simple flavonoids) and 2-hydroxychalcone, are shown in Figure 7.

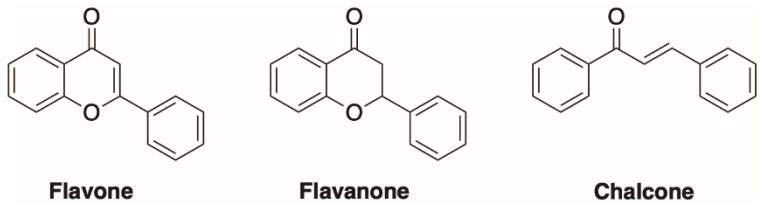


Figure 7. Chemical structures of flavone and flavanone (flavonoids) and 2-hydroxychalcone.

Flavonoids are natural compounds, synthesized by plants. They display a high binding affinity to enzymes, and heavy metal ions as well, and can catalyse electron transport and sequester free radicals. Flavonoids present different pharmacological activities, depending on their structure, such as relaxation of the smooth muscles,[58] reduction of inflammation[59] and antioxidant activity. Protective effects have been described in pathologies such as diabetes mellitus, heart disease, viral infections and cancer. Flavonoids that have been identified as modulators in the carcinogenesis process, showing antimutagenic and anticarcinogenic effects. Several studies demonstrated the antiproliferative and anti-carcinogenic action, as well as the chemopreventive role of flavonoids.[60]

Regarding chalcones, the presence of hydroxyl groups is also responsible for their antioxidant activity.[61] They have antinociceptive potential and have been also evaluated as analgesic and anti-inflammatory agents.[62] Its role as anticonvulsant is also under investigation.

II. Objectives

The main challenges faced by the pharmaceutical industry are the reduction and/or elimination of solvents in industrial processes, and APIs solubility enhancement, as this affects the drug bioavailability. On the other hand, overcoming these challenges could also greatly increase the overall pharmaceutical industry sustainability index, while decreasing APIs cost. The use of DES provides solutions that met these main challenges of the pharmaceutical industry.

The main objectives of this work are:

1. Explore the applicability of mechanochemistry as a feasible synthetic route for the preparation of THEDES.
2. Mechanosynthesis of new THEDES based on thymol, flavonoids and chalcones.
3. Determination of Solid-Liquid Equilibrium (SLE) diagrams for the new THEDES using, DSC and visual turbidimetry.
4. Evaluation of the toxicity of the new THEDES.

III. Experimental Part

III.1. Materials

The reagents used in this study were used as purchased. For some studies, lidocaine hydrochloride and benzocaine hydrochloride, purchased from Aldrich, were converted to the corresponding free base using a standard protocol. The free base was obtained by the addition of sodium hydrogen carbonate followed by extraction with chloroform. Racemic Ibuprofen, flavone, flavanone and 2-hydroxychalcone were purchased from Alfa Aesar.

III.2. Equipment

The NMR experiments (^{1}H and ^{13}C) were performed on a Bruker AVANCE 300 spectrometer, operating at 300 MHz. Spectra were recorded in DMSO-*d6*.

The FT-IR spectra were recorded on Brucker spectrophotometer, using films on NaCl disks, in the interval from 4000 to 400 cm^{-1} using 16 scans.

A NETZSCH Differential Scanning Calorimeter model 200F3 was used in this work.

The mechanosynthesis was performed in a planetary ball mill PM100 (Retsch) using a zirconium oxide reactor and zirconium oxide balls.

III.3. Methods

III.3.1. Differential Scanning Calorimetry (DSC)

DSC is a thermal-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as function of temperature. The glass-transition temperature (T_g), crystallization temperature (T_c) and melting temperature (T_m) can be detected for samples through a heat flow vs temperature plot. Samples of approximately 10-15 mg were weighed into a pan that was closed by a pressing machine.

Once inside the DSC, the pans along with the reference pan were heated and cooled doing two heating-cooling cycles. The first heating-cooling cycle was done at 10 K min^{-1} to clean any impurity inside the sample pan and to clean the sample thermal history. The second cycle was run at 10 K min^{-1}, 2 K min^{-1} and 1 K min^{-1}, in order to check

the influence of the step in the determination of melting events. Just the last cycle was analysed. The nitrogen (N_2) flow rate, at 50 mL min^{-1}, was used to create an inert atmosphere an also to promote a good heat conductivity. The temperature range covered was for each THEDES was -70 ºC to 110 ºC (flavone:thymol), 70 ºC to 90 ºC (flavanone:thymol) and -70 ºC to 70 ºC (chalcone:thymol).

III.3.2. Construction of SLE Ideal Phase Diagrams

This method is very simple and provides valuable information. According to Equation 2, the whole SLE phase diagram depends on both the melting properties (melting enthalpy and melting temperature) of the pure components and their activity coefficients in solution. When the solution is assumed as ideal (γ_i^L=1), the SLE phase diagram can be modelled based on the melting properties of the pure components, denote as *i*.

$$ln\, x_i^L \gamma_i^L = -\frac{\Delta_m H_i}{RT}\left(1 - \frac{T}{T_{m,i}}\right) \quad (2)$$

III.3.3. Joback's Method

This method allows the estimation of phase transition temperatures (either boiling or melting) at standard pressure, critical properties (p_c, v_c, T_c,), enthalpies of formation, Gibb's energies of formation and thermal capacities of liquids at constant pressure (C_p). For each case, there is a single equation, which contains one or more terms, where the sum of the partial contributions of the groups involved is collected. Equation 3 can be used to estimate the enthalpy of melting of pure compounds.[63]

$$\Delta_m H^0 \left(\frac{kJ}{mol}\right) = -0.88 + \sum_i N_i H_{fus,i} \quad (3)$$

where N_i is the number of *i* groups in the molecule. The present group contribution method is very useful in the case of compounds for which experimental values are not available. Once the molecular structure of the compound is known, the constituent groups are known and therefore, first and second-order groups can be identified and the $\Delta_m H^0$ can be estimate using the above equation and tables. This method can only be applied to organic compounds.

III.4. THEDES synthesis

III.4.1 Conventional heating

For control experiments, some THEDEs were synthesised using the conventional heating protocol. The components were weighed (*ca.* 200 mg scale), mixed in a vial, and allowed to melt (using a silicone bath) under stirring, until a homogeneous liquid was formed. The heating was then turned off and the mixture allowed to slowly reach room temperature.

III.4.2 Mechanosynthesis

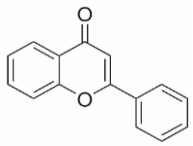
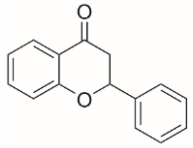
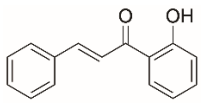
The mechanosynthesis (*ca.* 200 mg scale) was performed in a planetary ball mil using a 50 mL zirconium oxide reactor and 50 zirconium oxide balls of 5 mm. The optimized parameters for the mechanosynthesis of THEDES were between 5 minutes and 1 hour of reaction at 500 rpm, with rotation inversion cycles of 5 min (5 secs pause between inversion cycles). In this work, a wide range of compositions was prepared for the mechanosynthesis of THEDES, both for control experiments (Table 1) and to explore new systems using flavonoids and chalcones (Table 2).

Table 1. Summary of THEDES prepared by mechanosynthesis for control experiments.

API	HBD	API Molar Composition	Milling Frequency and Time	Aspect and temperature[a]
Lidocaine	DL-Menthol	0.77	500 rpm 1 h	Liquid 30 ºC
Lidocaine	DL-Camphor	0.61	500 rpm 5 min	Liquid 26 ºC
Lidocaine	Thymol	0.61	500 rpm 5 min	Liquid 30 ºC
Lidocaine	Lawsone	0,67	500rpm 10min	Liquid 30 ºC
Benzocaine	Thymol	0.52	500 rpm 10 min	Liquid 30 ºC
Ibuprofen	DL-Menthol	0.57	500 rpm 5 min	Liquid 30 ºC
Ibuprofen	DL-Camphor	0.58	500 rpm 1 h	Liquid 30 ºC
Ibuprofen	Thymol	0.67	500 rpm 30min	Liquid 30 ºC
Flavone	DL-Menthol	0.59	500 rpm 30min	Solid 30 ºC

[a]Temperature inside the reactor after the reaction. Measured using a digital laser thermometer.

Table 2. Summary of new THEDES prepared by mechanosynthesis containing flavone, flavanone and 2-hydroxychalcone.

THEDES	Molar fraction	Abbreviation	Aspect[a]
Flavone:Thymol	0.1	fl:th (0.1)	Solid
 Flavone	0.2	fl:th (0.2)	**Liquid**
	0.3	fl:th (0.3)	**Liquid**
	0.4	fl:th (0.4)	**Liquid**
	0.67	fl:th (0.67)	Solid
	0.8	fl:th (0.8)	Solid
Flavanone:Thymol	0.1	flavanone:th (0.1)	Solid
 Flavanone	0.2	flavanone:th (0.2)	Solid
	0.3	flavanone:th (0.3)	**Liquid**
	0.4	flavanone:th (0.4)	**Liquid**
	0.5	flavanone:th (0.5)	Solid
	0.67	flavanone:th (0.67)	Solid
	0.8	flavanone:th (0.8)	Solid
Chalcone:Thymol	0.1	ch:th (0.1)	**Liquid**
	0.25	ch:th (0.25)	**Liquid**
	0.3	ch:th (0.3)	Solid
	0.4	ch:th (0.4)	Solid
	0.57	ch:th (0.57)	Solid
	0.8	ch:th (0.8)	Solid

[a]At room temperature.

III.5. THEDES Cytotoxicity Assays

The toxicity of selected THEDES was evaluated by a resazurin based assay using PrestoBlue™, PB (Invitrogen, Carlsbad, CA, USA) reagent. The cell line L929 was purchased from the European Collection of Authenticated Cell Cultures. The cells were seeded in 96-well flat-bottomed polystyrene plates with a density of 1x104 per well and left overnight in a CO_2 incubator (5%) at 37 °C. Next, the medium was discarded and replaced with a solution of the eutectic solvent, previously prepared in complete culture medium, and the cells were incubated for 24 h. The following steps were performed according to the PrestoBlue™ reagent kit protocol. The resorufin conversion was monitored by measuring the fluorescence intensity (excitation at 530 nm, emission at 590 nm) in a microplate reader (BMG Labtech, Polar Star Optima) at 37 ºC.

IV. Results and Discussion

IV.1. THEDES Mechanosynthesis

In this work we synthesised, for the first time, a series of THEDES using mechanochemistry (see annexes). To validate the methodology, we first selected a few THEDES, well described in literature, to be used as a reference. These THEDES were prepared following the reported protocols, in different compositions, using conventional heating.[64]

Next, the mechanosynthesis of the selected THEDES was investigated at <1 g scale (*ca.* 200 mg) using a planetary ball milling and a zirconium oxide reactor. The zirconium oxide reactor was selected to avoid possible iron contamination that is common to occur when stainless steel reactors are used. The number of zirconium oxide balls and the time of milling was investigated for the different systems. The initial number of balls (200) was decreased to 50 since allowed an easier product recovery from the reactor, without compromising the synthesis.

IV.1.1. Lidocaine based THEDES

To prepare the lidocaine based THEDES we started the reactions using lidocaine hydrochloride. After several attempts, without success, both using conventional heating and mechanosynthesis, we hypothesised that the hydrochloride salt could be interfering with the formation of hydrogen bonds and thus preventing THEDES formation. Therefore, using a standard protocol the lidocaine free base was obtained and used in the next experiments. After the successful formation of lidocaine-DL-menthol THEDES by conventional heating, confirming lidocaine free base as a key component, lidocaine THEDES were obtained by mechanosynthesis using DL-menthol, DL-camphor, thymol and lawsone (see Table 1).

To better understand the interactions between the components and compare the THEDES obtained by conventional heating and mechanochemistry, the products were analysed by FT-IR and NMR. The FT-IR spectra of lidocaine:DL-menthol (1:1) THEDES obtained by mechanosynthesis, presented in Figure 8, show a new band, with strong intensity at 1675 cm^{-1}, which may be attributed to the carbonyl of lidocaine after hydrogen bonding (1665 cm^{-1}, pure lidocaine).

Similarly, the FT-IR spectra of lidocaine:thymol (1:1) THEDES obtained by mechanosynthesis, presented in Figure 9, also show new bands, with strong intensity at

1688 and 1743 cm^{-1}. Like in the case of lidocaine-DL-menthol (1:1) THEDES, the bands at 1688 cm^{-1} and 1743 cm^{-1}may be attributed to vibrations of the carbonyl of lidocaine after hydrogen bonding (1665 cm^{-1}, pure lidocaine).

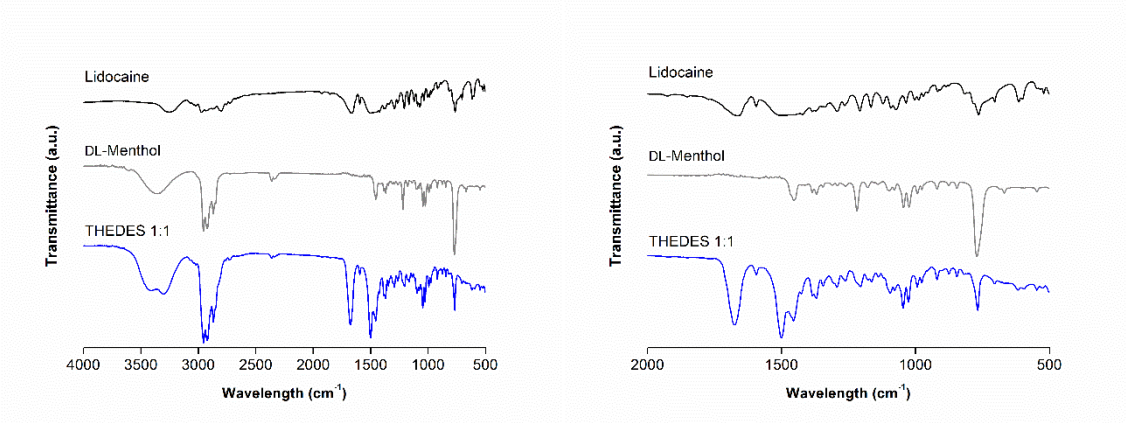


Figure 8. FT-IR spectra of lidocaine:DL-menthol (1:1) THEDES obtained by mechanosynthesis (full spectra, left, and zoom, right). The spectra of lidocaine and DL-menthol are also shown.

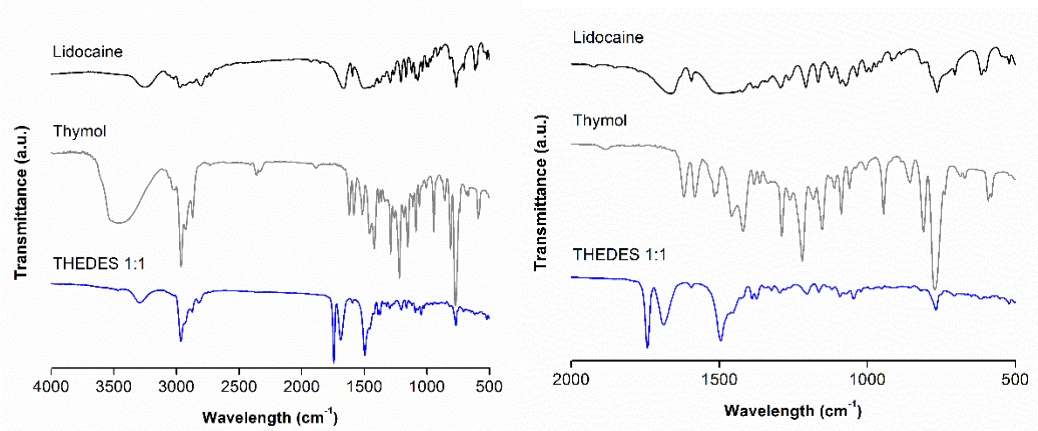


Figure 9. FT-IR spectra of lidocaine:thymol (1:1) THEDES obtained by mechanosynthesis (full spectra, left, and zoom, right). The spectra of lidocaine and thymol are also shown.

The establishment of hydrogen bonding between each parent species was then assessed by using NMR spectroscopy. NMR is commonly used to elucidate the types of interactions, as well as the atoms of each counterpart involved, allowing to get insights into the hydrogen bonding network.

The ^{1}H-NMR spectrum for lidocaine:DL-menthol (1:1) THEDES obtained by mechanosynthesis is presented in Figure 10. The comparison with the spectra of pure lidocaine and DL-menthol, show a chemical shift for the CH_2 protons of the *N*-ethyl group

of lidocaine (at 3.23 ppm), to 2.61 ppm in THEDES. This upfield shift may be attributed to the presence of DL-menthol in the vicinity of lidocaine after hydrogen bonding between the lidocaine carbonyl and the hydroxyl group from D-menthol. This is also confirmed by the signal changes between THEDES and pure DL-menthol. It presents, a multiplet at 3,23 ppm, as expected. However, in the THEDES ^{1}H-NMR spectra, a well-defined multiplet was found, which further suggests the hydrogen bond interactions between the parent molecules.

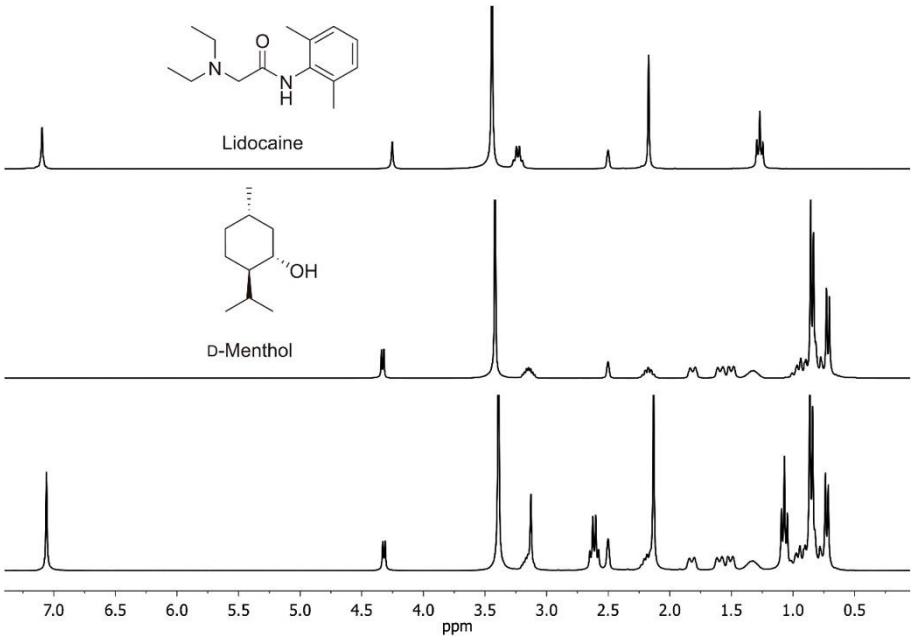


Figure 10. ^{1}H-NMR spectrum of lidocaine:DL-menthol (1:1) THEDES obtained by mechanochemistry in DMSO-*d6*. The spectra of lidocaine and D-menthol are also shown for comparison.

^{13}C NMR was also performed to investigate whether a chemical reaction occurred between THEDES components. From the lidocaine based THEDES studied, both DL-menthol and thymol are unreactive with lidocaine, since the hydroxyl groups present in both molecules are unlikely to react, under the experimental condition, with lidocaine. However, lawsone is a very reactive component, able to participate in 1,4-additions in the present of adequate nucleophiles. Despite the amide group of lidocaine has a low nucleophilic character we did not discarded a possible reaction between lidocaine and lawsone or lawsone decomposition under the milling condition. To exclude possible chemical reactions or the formation of by-products in THEDES mechanosynthesis we recorded the ^{13}C NMR spectrum of lidocaine:lawsone (2:1) THEDES, which is presented in Figure 11. As can be seen by the analysis of the spectra, the THEDES spectrum show both peaks from lidocaine and lawsone, and slight deviations in the chemical shifts are

observed. A special attention was paid to C-3. This carbon which should be highly affected if any reaction occulted as a result from a 1,4-addition, but only a shift from 108.94 to 111.05 ppm was observed, which is probably due to hydrogen bonding interactions.

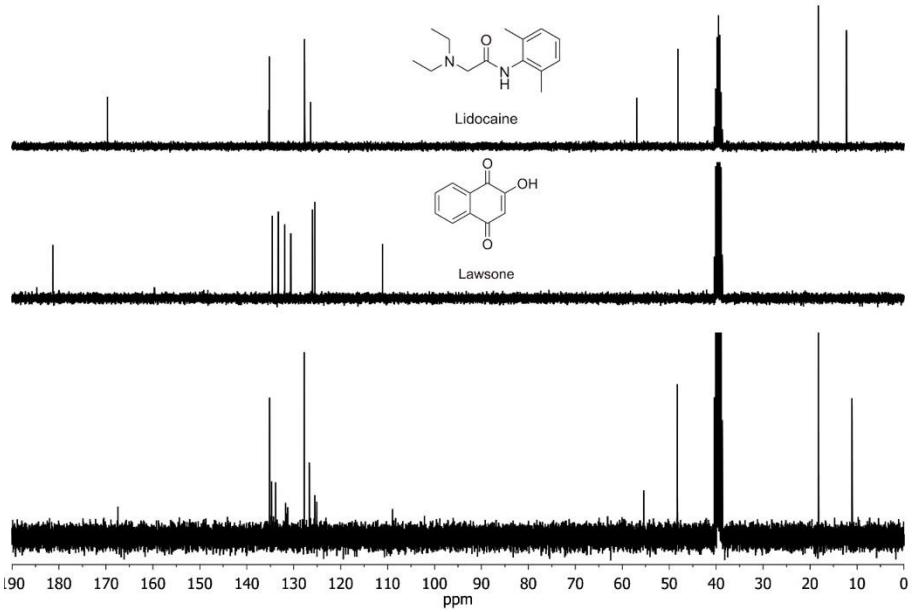


Figure 11. ^{13}C-NMR spectrum of lidocaine:lawsone (2:1) THEDES obtained by mechanochemistry in DMSO-*d6*. The spectra of lidocaine and lawsone are also shown for comparison.

IV.1.2. Ibuprofen based THEDES

Ibuprofen based THEDES were obtained by mechanosynthesis using DL-menthol, DL-camphor, thymol and lawsone (see Table 1). Although all THEDES were successfully formed, there was found a difference between time required for forming THEDES. The fastest was with D-menthol (5min), followed by thymol (30min) and finally camphor (1h). This happened because even if a THEDES is synthetized, the interactions established between DL-menthol and ibuprofen are stronger than those formed by thymol and camphor. This result confirms the need for a better understanding of the mechanism of THEDES formation, as well as the understanding of the interactions established once it has been formed. To better understand the interactions between the components and compare the THEDES obtained by conventional heating and mechanochemistry, the products were analysed by FT-IR and NMR.

FT-IR spectra for ibuprofen based THEDES were also recorded. As can be seen in Figure 12, the spectrum of ibuprofen:DL-menthol (1:1) THEDES present shift in its bands. As expected, the band from the ibuprofen carbonyl is the most shifted, from 1708

cm^{-1} in pure ibuprofen to 1675 cm^{-1} in the THEDES, thus corroborating the participation of the ibuprofen carbonyl in hydrogen bonding with DL-menthol.

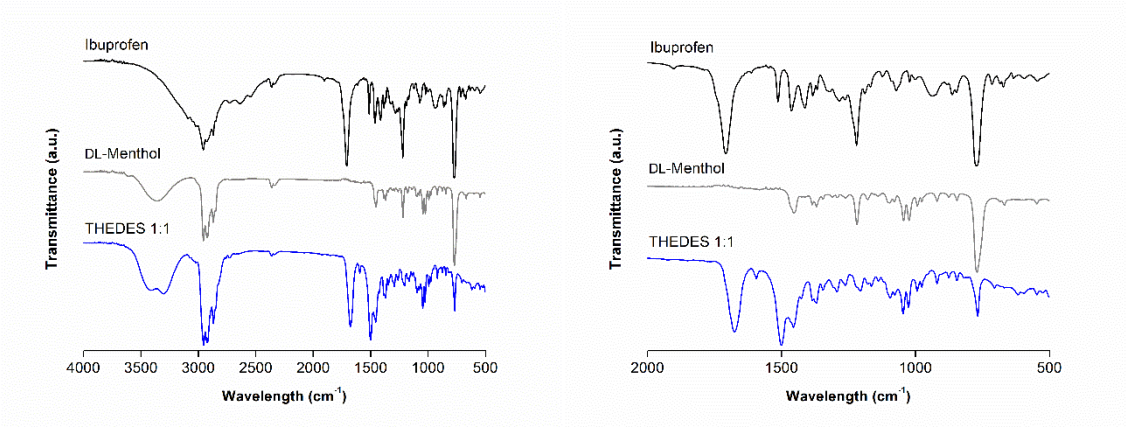


Figure 12. FT-IR spectra of ibuprofen:DL-menthol (1:1) THEDES (full spectra, left, and zoom, right). The spectra of ibuprofen and DL-menthol are also shown.

As in the case of lidocaine:DL-menthol (1:1) THEDES obtained by mechanochemistry, the ^{1}H-NMR spectrum of ibuprofen:DL-menthol (1:1) THEDES also display significative deviations on chemical shifts. Since ibuprofen was recorded in a different deuterated solvent it was not possible to compare the data for this component, but chemical shift deviations are clear for DL-menthol. These chemical shifts may also be attributed to resulting interactions between the carbonyl group of ibuprofen with the hydroxyl group of DL-menthol, as shown in Figure 13.

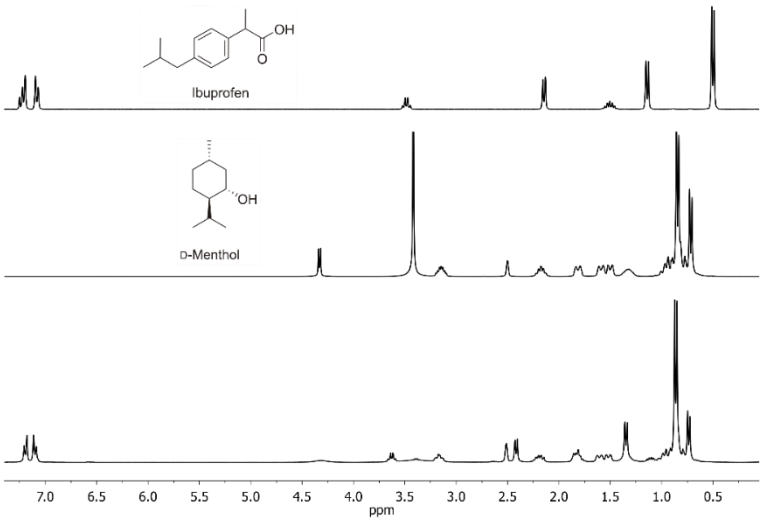


Figure 13. ^{1}H-NMR spectrum of ibuprofen:DL-menthol (1:1) THEDES obtained by mechanochemistry in DMSO-*d6*. The spectra of ibuprofen (in $CDCl_3$) and DL-menthol (in DMSO-*d6*) are also shown for comparison.

IV.1.3. Benzocaine based THEDES

The preparation of THEDES using benzocaine hydrochloride was unsuccessful for most of the studied composition with different components. We attributed these results to the presence of the hydrochloride salt, in analogy with lidocaine hydrochloride which did not produce any THEDES in this form. To overcome this problem, we attempted to obtain the benzocaine free base, but the product was found to be too unstable, leading to decomposition.

Nevertheless, in the case of thymol and benzocaine hydrochloride (1:1) THEDES was obtained. We cannot explain this result, but in general we found that thymol is excellent compound for THEDES preparation.

IV.1.4. Flavonoids and Chalcone based THEDES

Once we validated mechanosynthesis as a reliable and efficient methodology for THEDES preparation, by comparison whit known THEDES prepared by the conventional heating methodology, we investigated the mechanosynthesis of new THEDES.

We selected flavonoids and a chalcone as models from this large family of compounds. Many different mixtures using different compositions with thymol were carried out (Table 2).

A curious result is that 7-hydroxyflavone, despite its similarity to flavone and flavanone did not lead to THEDES formation. One possible explanation cloud be the hydroxyl interference in the formation of hydrogen bonds, although we are uncertain if this could be an explanation. To better understand the behaviour of substituted flavonoids, the mechanosynthesis of this type of THEDES should be further investigated in future.

The new THEDES were characterized by FT-IR, as presented in Figure 14. In this system the more shift band was the hydroxyl group from thymol, from 3446 to 3339 cm^{-1}. The flavone carbonyl bands shift suffered a less pronounced shift, from 1647 to 1630 cm^{-1}. This is an indication of the establishment of hydrogen bonding between thymol, that acts as hydrogen bond donor, and flavone, that acts as hydrogen bond acceptor.

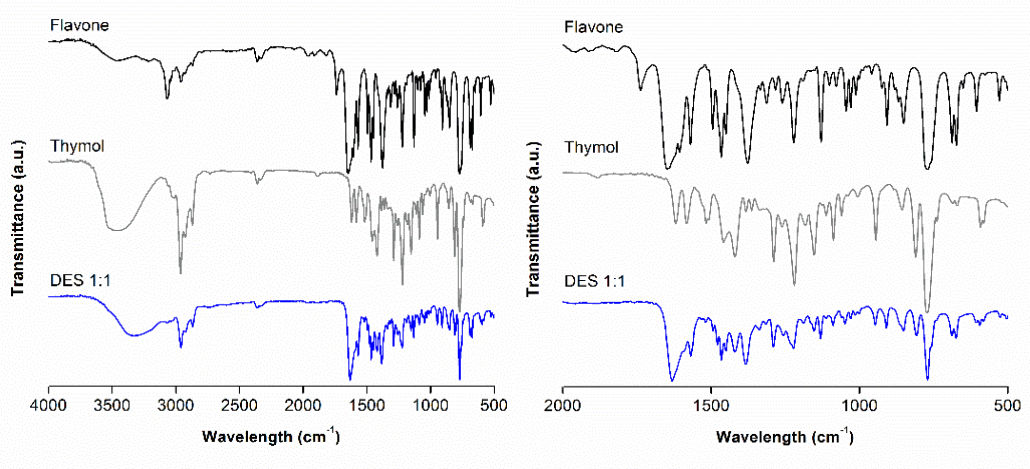


Figure 14. FT-IR spectra of flavone:thymol (1:1) THEDES (full spectra, left, and zoom, right). The spectra of flavone and thymol are also shown.

The successful preparation of the flavone-thymol (1:1) THEDES, either by conventional heating or by mechanosynthesis was further confirmed by ^{1}H-NMR. As shown in Figure 15, the spectra obtained for THEDES prepared with the two methods are identical and no chemical reaction occurred.

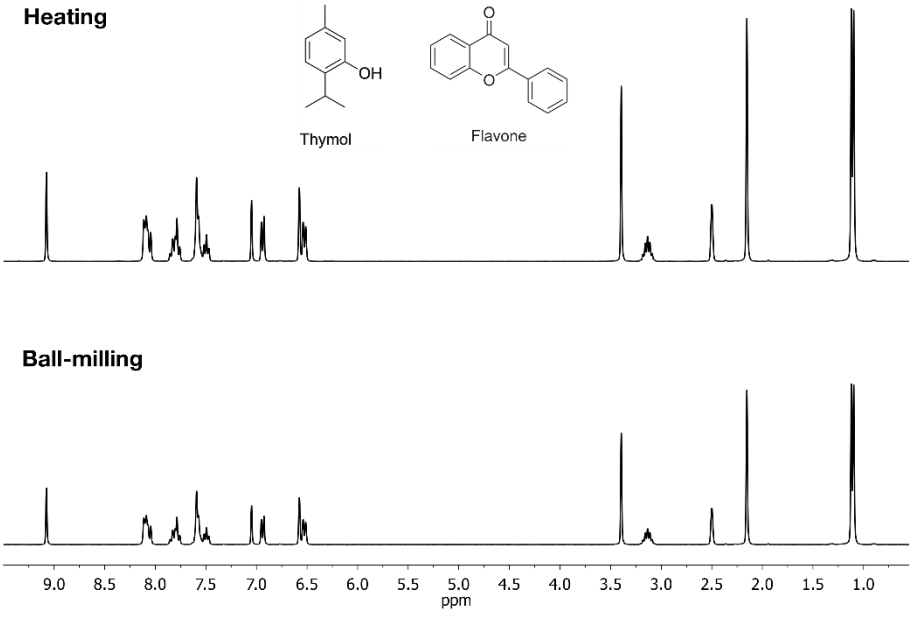


Figure 15. ^{1}H-NMR spectra of flavone:thymol (1:1) THEDES obtained by conventional heating and mechanochemistry in DMSO-*d6*.

Similar results were obtained for flavanone:thymol (1:1) THEDES. The ^{1}H-NMR obtained, presented in Figure 16, is identical for THEDES prepared by heating or by mechanosynthesis. No chemical shifts are observed for this system, comparing the obtained THEDES with the pure components.

For the chalcone:thymol (1:1) THEDES, shown in Figures 17 and 18, similar results were observed, thus indicating that this class of compounds behave in an analogous way.

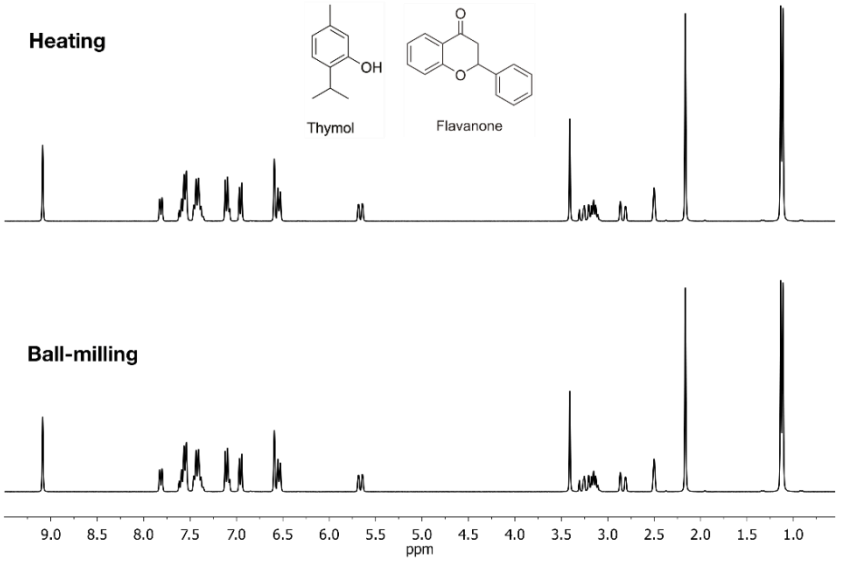


Figure 16. ^{1}H-NMR spectra of flavanone:thymol (1:1) THEDES obtained by conventional heating and mechanochemistry in DMSO-*d6*.

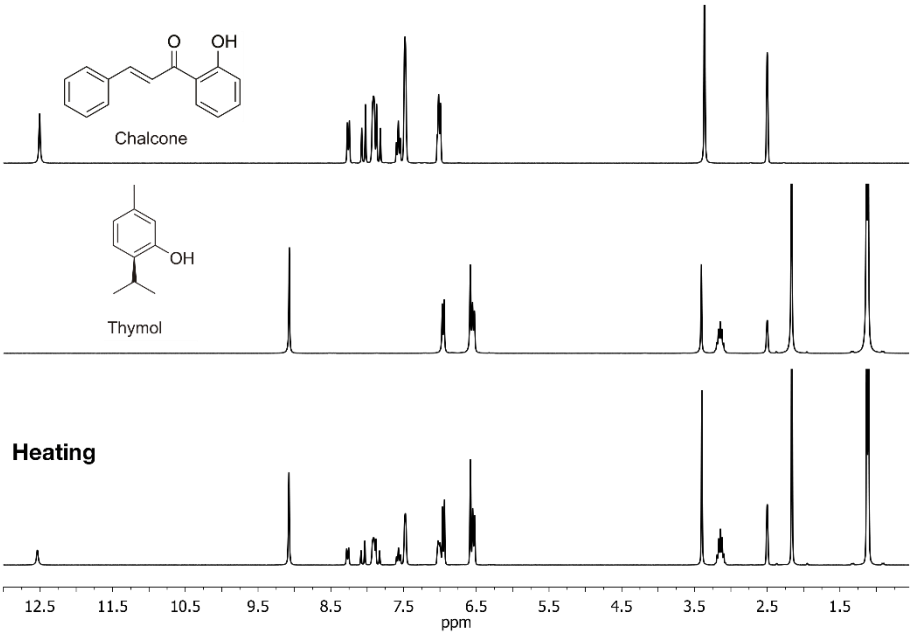


Figure 17. ^{1}H-NMR spectra of chalcone:thymol (1:1) THEDES (full spectrum, top, and zoom, bottom) obtained by conventional heating in DMSO-*d6*.

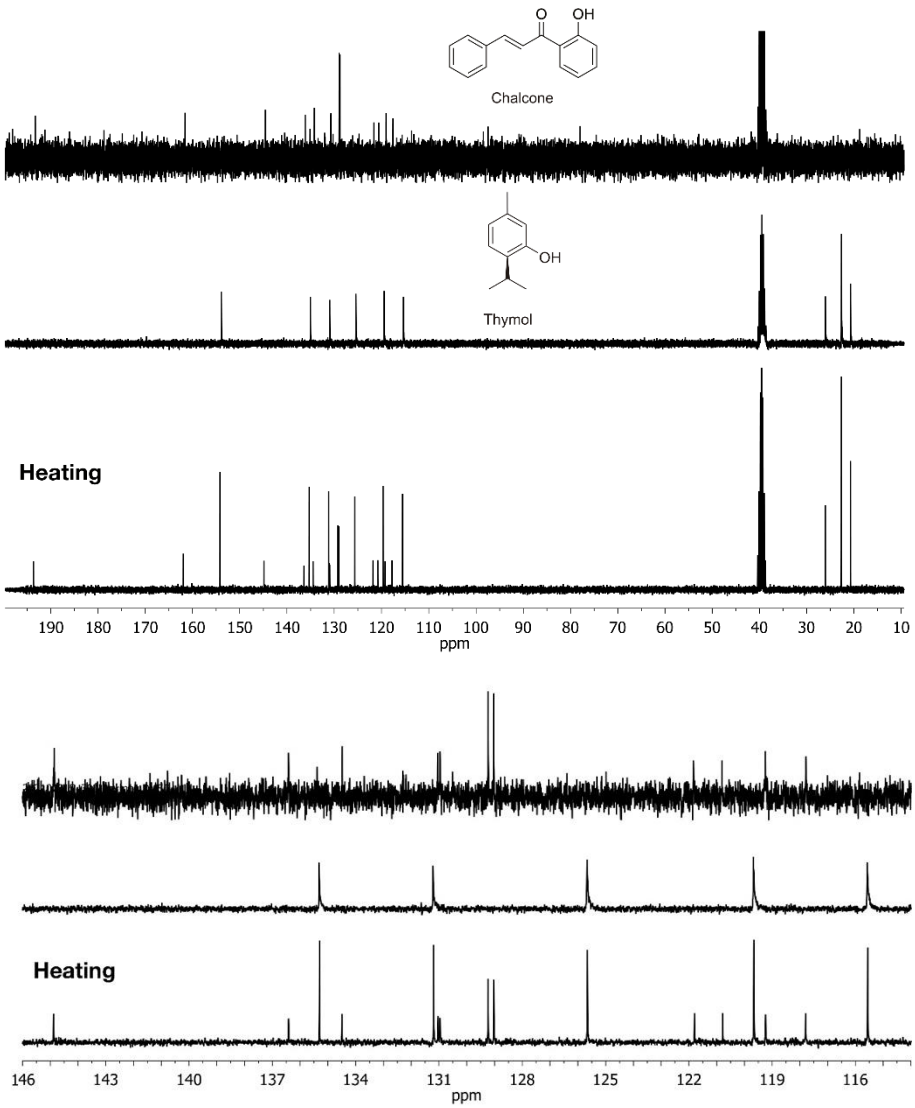


Figure 18. ^{13}C-NMR spectra of chalcone:thymol (1:1) THEDES (full spectrum, top, and zoom, bottom) obtained by conventional heating in DMSO-*d6*.

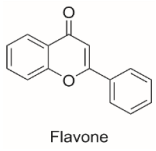
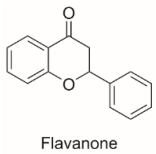
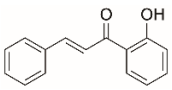
In conclusion, in this work we found that mechanochemistry can be used an alternative methodology to prepare THEDES under very mild conditions (temperatures below 30 ºC). This route is of special importance in the cases where thermal degradation could be an issue.

IV.2. Prediction of Fusion Enthalpy - Jobacks Method

To set the ideal SLE diagram, we needed to estimate the melting enthalpy of flavone, flavanone, and 2-hydroxychalcone since no experimental results were found for

these compounds in literature. The $\Delta_m H^0$ for these compounds was calculated using equation 3, and the results are summarized in Table 3.

Table 3. Estimation of ΔH_{fus} for flavone, flavanone and 2-hydroxychalcone. Joback's contribution groups values.

Compound	Contribution groups	Contribution groups value[65]	Functional groups	ΔH_{fus} (kJ mol^{-1})
 Flavone	−CH_3 (ring)	1.010	10	30.693
	=C< (ring)	2.394	5	
	−O− (ring)	5.789	1	
	=O	3.624	1	
 Flavanone	−CH_3 (ring)	1.010	9	31.022
	=C< (ring)	2.394	4	
	−O− (ring)	5.789	1	
	=O	3.624	1	
	−CH_2− (ring)	0.490	1	
	>CH− (ring)	3.243	1	
	−CH_3 (ring)	1.010	9	29.453
	=C< (ring)	2.394	3	
	>C=O (non-ring)	4.189	1	
	-OH (phenol)	4.490	1	
	=CH− (non-ring)	2.691	2	

IV.3. Thermal Characterization of new THEDES

THEDES design is still a trial-and-error process due to the lack of knowledge on the interactions established between the components of the mixture. THEDES formulations can take advantage of the liquid state at the eutectic point, which corresponds to the mixture with the lowest melting point, and thus the most non ideal mixture, with the strongest interactions between the THEDES components. The analysis of the melting/crystallization events by DSC can point out these mixtures. The thermal behaviour of THEDES is different from the behaviour of the individual components.

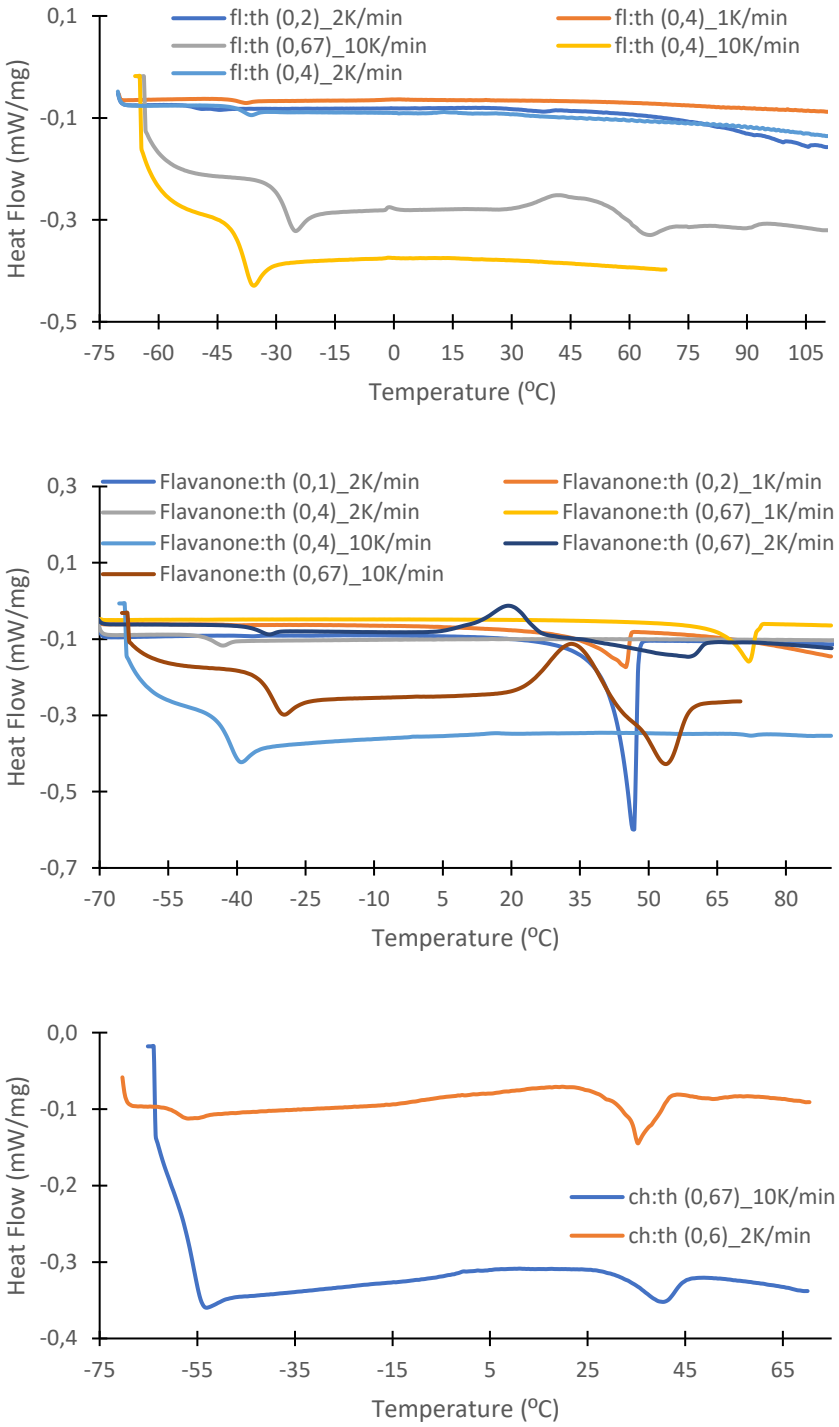


Figure 19. DSC thermograms obtained for flavone:thymol (top), flavanone:thymol (middle) and chalcone:thymol (bottom), using with different molar compositions and different scanning ratios.

Based on literature, thymol showed a melting point of 50 °C[66], while flavone, flavanone and chalcone melting points of 97 ºC, 76 ºC and 55 ºC[67] respectively. In Figure

19, DSC thermograms of different compositions binary THEDES prepared with flavone, flavanone and 2-hydroxychalcone combined with thymol, at different scanning ratios are presented.

Table 4. THEDES thermal properties obtained from the last DSC cycle and the visual turbidimetry data.

THEDES	Visual Turbidimetry Melting Point, ºC	DSC Onset Temperature, ºC
fl:th (0.1)	36	N.A.
fl:th (0.2)	12	N.A.
fl:th (0.3)	7	N.A.
fl:th (0.4)	-10	N.A.
fl:th (0.67)	58	60
fl:th (0.8)	65	N.A.
flavanone:th (0.1)	51	40
flavanone:th (0.2)	37	30
flavanone:th (0.3)	-18	N.A.
flavanone:th (0.4)	-15	N.A.
flavanone:th (0.5)	30	N.A.
flavanone:th (0.67)	47	52
flavanone:th (0.8)	57	N.A.
ch:th (0.1)	8	N.A.
ch:th (0.25)	-4	N.A.
ch:th (0.4)	28	N.A.
ch:th (0.57)	36	36
ch:th (0.8)	46	N.A.

Most of the DSC thermograms do not exhibit melting points, and only glass transition temperatures are observed. The glass transition behaviour of these systems corresponds to transitions between crystalline and amorphous phases depending on the complex molecular assembling that THEDES can adopt. It is interesting to point out that

the glass transition/melting points are expressively lower than the melting point of the starting compounds showing strong interactions exist within THEDES. Another important aspect is the fact that the transitions between crystalline and amorphous phases are dependent on the composition and thus reflect the different complex molecular ordering that each system can adopt.

Table 4 shows the comparison of the DSC melting data with melting temperatures obtained by visual observation. The fist comment is that despite the larger error in the second method, which in our case we estimate to be around ± 1ºC, it is always possible to observe the melting point of THEDES. Using DSC, it is sometimes impossible to observe the melting point of THEDES. On the other hand, we can see that whenever DSC can be of use, there is a good agreement between DSC data and visual observation data, validating the quality of this last one.

For all THEDES, as the mixture becomes closer to the eutectic or in the eutectic point, the more difficult it is to observe the melting peak in DSC, as it can be seen Figure 19, despite of the several scanning ratios used. The fact that some THEDES does not show any crystallization or melting during cooling cycle and the second heating cycle clearly suggests the formation of a stable eutectic system.

The experimental SLE phase diagram of the three studied THEDES measured using DSC and visual turbidimetry are presented in Figure 20. The predictions assuming an ideal mixture are also depicted for comparison.

The flavone:thymol THEDES presents a non-ideal behaviour, with large deviations from an ideal mixture description, especially increasing near the eutectic point. This means that the interactions between the two components at this point are significantly different from those present in the pure compounds, inducing a melting temperature depression. Another important fact is the lack of prediction of the composition of the eutectic point by the ideal mixture model, which typically does not occur. From the experimental SLE diagram obtained, it can be concluded that this mixture is liquid at room temperature between the flavone molar composition 0.2 to 0.5 approximately.

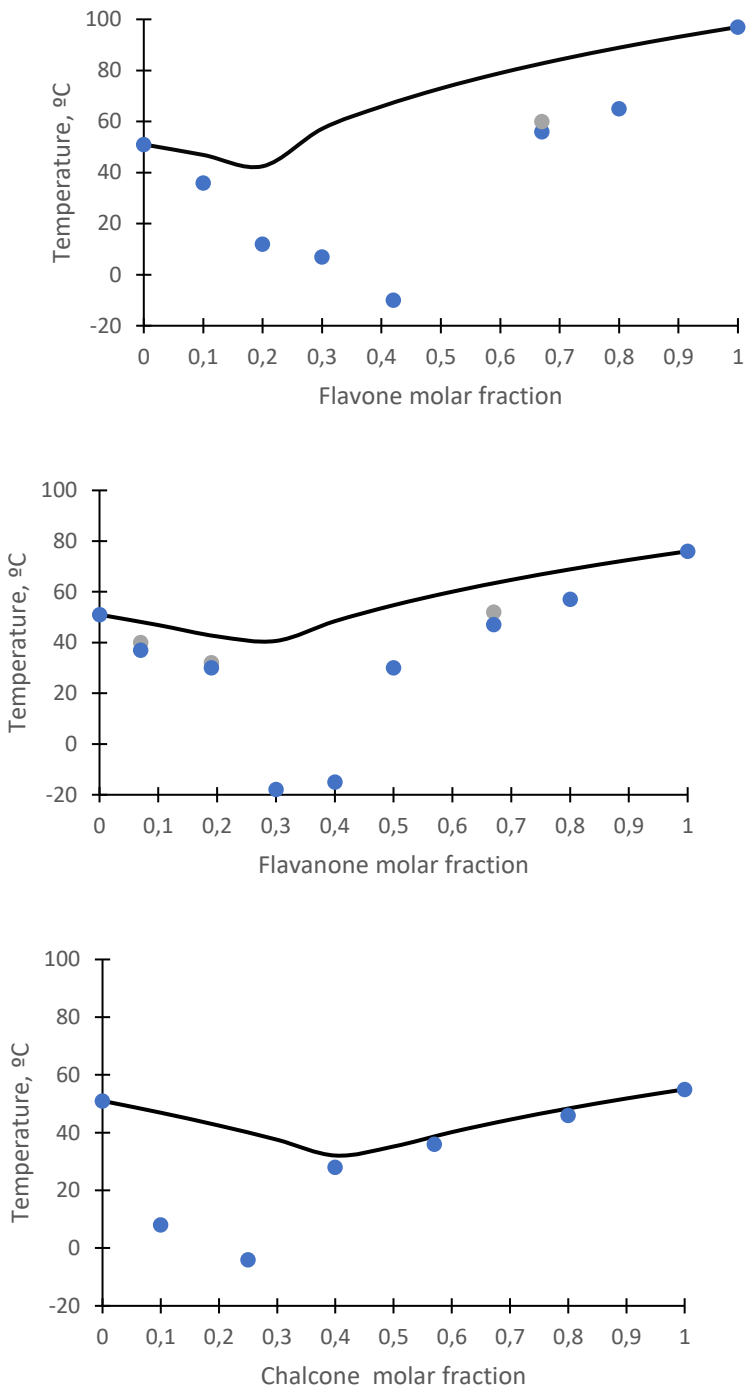


Figure 20. SLE phase diagrams of studied THEDES. Ideal behaviour (black line) and experimental data measured by visual techniques (blue symbols) or DSC (grey symbols). Flavone:thymol (top), flavanone:thymol (middle) and chalcone:thymol (bottom).

The non-ideal behaviour of flavanone:thymol THEDES is quite evident, with a large deviation of the melting temperature predicted by the ideal mixture model (40 ºC) and that measured visually by turbidimetry (around -20 ºC). From this observation, it can

be concluded that there is strong favourable interaction between thymol and flavanone, most probably through hydrogen bonding. From experimental SLE diagram obtained we can conclude that this THEDES is liquid at room temperature between the flavone molar composition 0.25 to 0.45, approximately.

Chalcone:thymol THEDES shows a very different behaviour from the expected since at high concentrations of chalcone an almost perfect ideal behaviour is observed, while at low concentrations of chalcone very large deviations from the ideal model are found. This can give us clues about the interaction between the components in THEDES formation. A small quantity of chalcone greatly affects the thymol molecular network of interactions, leading to large deviations from the ideal system, while the presence of thymol does not affect the chalcone network of interactions even in mixtures 1:1. This interesting phenomenon needs to be further explored. From the real SLE diagram obtained, we can conclude that this THEDES is liquid at room temperature between chalcone molar composition 0.1 to 0.3, approximately.

IV.4. THEDES Cytotoxicity Assays

The cell viability was assessed for the THEDES obtained by mechanochemistry and the corresponding components. Figure 21 show the cell viability data for flavonoid based THEDES and Figure 22 show the cell viability data for the lidocaine:lawsone (2:1) THEDES. Dimethyl sulfoxide (DMSO) was used as the solvent to prepare the stock solutions before serial dilutions with PBS. A low percentage (less than 1.5%) was used in the assays to avoid solvent induced toxicity (see Annexes).

Overall, the obtained data show that THEDES are slightly toxic than the single components, considering 80% as an acceptable cell viability threshold. In the case of the chalcone:thymol (1:2) THEDES, the lower toxicity is explained by a lower percentage of 2-hydroxychalcone.

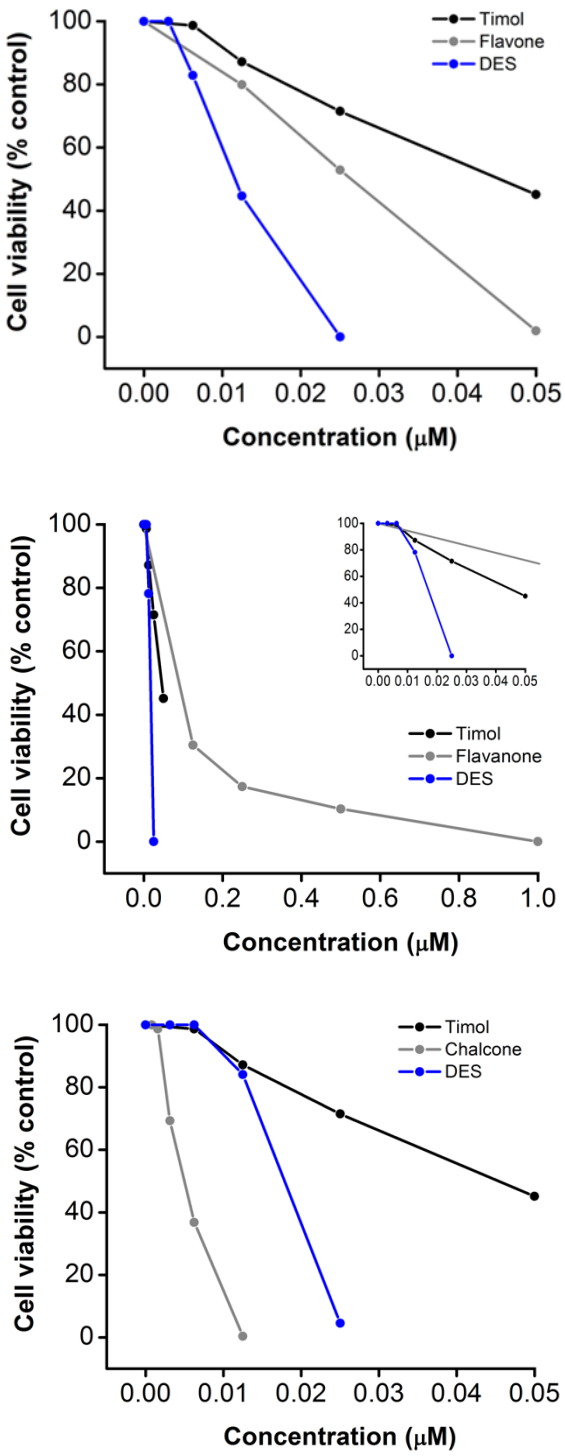


Figure 21. Cell viability for flavone, flavanone, 2-hydroxychalcone, thymol, and the corresponding THEDES, flavone:thymol (1:1) (top), flavanone:thymol (1:1) (middle), chalcone:thymol (1:2) (bottom).

A similar result was found for the lidocaine:lawsone (1:2) THEDES, where lawsone is also present in a lower percentage, thus explaining the overall toxicity.

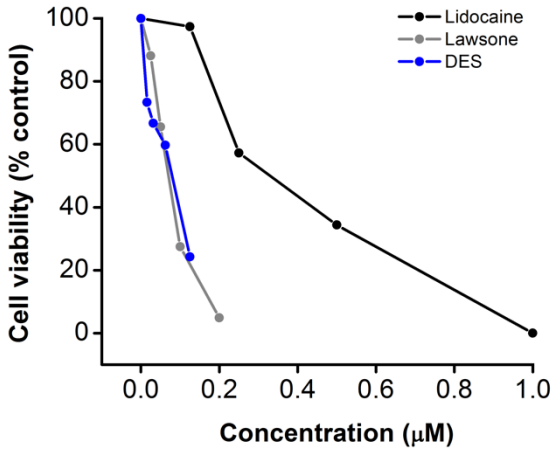


Figure 22. Cell viability for lidocaine and the lidocaine:lawsone (2:1) THEDES.

V. Conclusions and Future Perspectives

Even though many drugs exhibit multiple crystalline forms, the reliance of the pharmaceutical industry on solid-state drugs remains. However, solid-state drugs and their polymorphs have a profound effect on the chemical properties and therapeutic efficacy of a specific drug. Furthermore, solid drugs display lower solubility in water, and thus lower bioavailability, than similar liquid forms. To overcome these drawbacks, novel liquid forms of APIs in the form of THEDES have been proposed and investigated and represent a new approach that must be considered by pharmaceutical industries.

In this work, the use of mechanochemistry to prepare THEDES was tested, and the results obtained compared with those achieved using the melting and mixing preparation method. It was observed that thymol is a good candidate to prepare THEDES through its combination of one API. Flavone, flavanone and 2-hydroxychalcone were chosen to carry out the study. The SLE phase diagram of the 3 selected API-DES was experimentally determined, allowing to conclude that it is possible to prepare liquid et room temperature formulations of these compounds at room temperature. The three THEDES present large deviations from ideal mixture behaviour leading to the conclusion that there is a strong interaction between thymol and the 2nd component. The cytotoxicity of these 3 THEDES at the eutectic point indicate that they are slightly toxic than the single components.

Future studies on the prepared THEDES should contemplate the evaluation of drug stability, solubility, octanol-water partition coefficient, skin permeation, bioavailability and therapeutic action. Even though THEDES hold great promise to be accepted by the pharmaceutical industry and commercialized as novel formulations of APIs, more complete and thorough studies are still required.

VI. Annexes

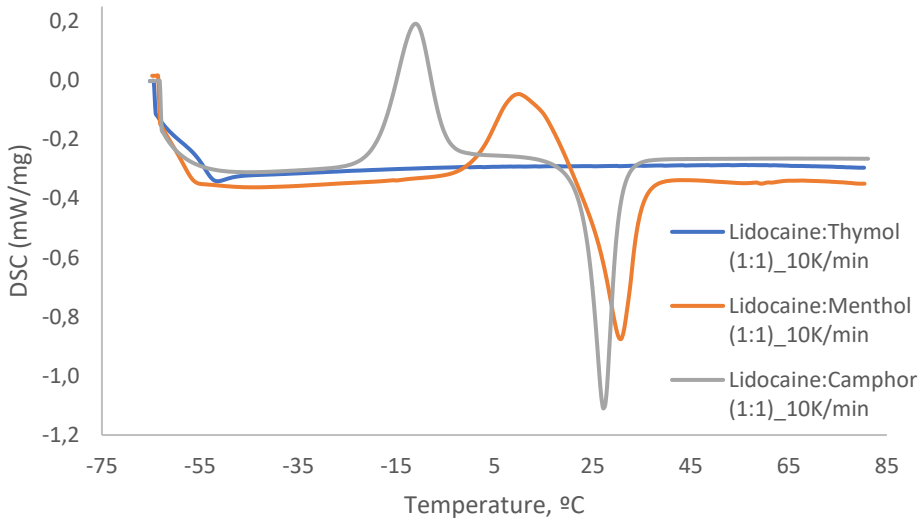


Figure A1. DSC thermograms obtained for lidocaine:DL-menthol (1:1), lidocaine:thymol (1:1) and lidocaine:camphor (1:1).

Figure A2. THEDES mechanosynthesis. Photos of the reactor at the end of the reaction in the case of flavone:thymol (1:1) (top) and lidocaine:lawsone (1:2) (bottom).

Figure A3. THEDES mechanosynthesis. Photos of the reactor at the end of the reaction of an unsuccessful reaction, benzocaine:thymol (1:1).

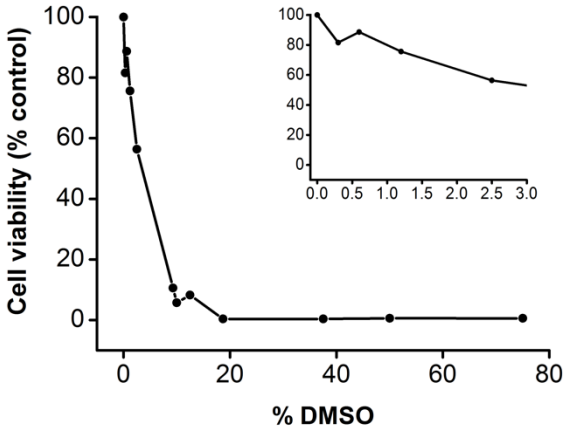


Figure A4. Effect of dimethyl sulfoxide (DMSO) concentration on cell viability.

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