

Selective Extraction of Antioxidants by Formation of a Deep Eutectic Mixture through Mechanical Mixing

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ABSTRACT: Extraction of active compounds from natural materials is commonly used to produce a diverse range of ingredients, nutrients, drugs, flavors, and fragrances. While solvent-based approaches are standard for these extraction procedures, large solvent volumes, greenhouse gas emission (due to atmospheric release of volatile organic compounds), and large energy requirements are significant issues. Here, we describe a novel process wherein a mechanical action is applied to a solid-state mixture comprising a quaternary ammonium salt and a biological material containing active molecules to form a deep eutectic mixture. It is first shown that eutectic mixtures can be formed for a wide range of naturally occurring compounds. Selective extraction of these compounds from natural products is then demonstrated using rosemary leaves with three different quaternary ammonium salts. The eutectic mixture formed is purified by liquid/liquid extraction using an oily phase to concentrate the active constituents in the deep eutectic phase. The resulting natural extracts are shown to exhibit high antioxidant activities, which were measured using the conjugated autoxidizable triene (CAT) and the oxygen radical absorbance capacity (ORAC) assays.

KEYWORDS: solid-state extraction, natural product, deep eutectic solvents, antioxidant activity



INTRODUCTION

Sustainable and efficient extraction of active ingredients from natural products is a field of significant interest particularly in the food, pharmaceutical, and personal care industries. Consumer concerns about residues from solvents,¹ along with atmospheric pollution and carbon footprint associated with their use, are significant challenges for these industries.²

Of particular interest for natural product extracts are phenolic compounds which are strong antioxidants.^{3,4} Extraction of phenolics having antioxidant properties is a key sector which was worth nearly \$1.3 bn in 2018 with a 7.2% estimated annual growth from 2019 to 2025.⁵ Alcoholic solvents are commonly used to extract phenolics as well as acetone, ethyl acetate, and other volatile organic solvents. However, efforts have been recently made to shift from these solvents to more sustainable alternatives with less toxicity and better eco-environmental friendliness.^{6,7}

Ionic liquids (ILs) have found application in numerous fields, but their general toxicity, poor biodegradability, and high production cost make them less suitable for natural product extraction.⁸ Deep eutectic solvents (DESs), which consist of binary or ternary mixtures of quaternary ammonium salts with hydrogen bond donors such as alcohols, carboxylic acids, and amines⁹ (i.e., mixtures of Lewis or Brønsted acids and bases) may represent an alternative to ILs. They have been studied for a variety of applications including drug delivery, lubrication, polymer synthesis, metal extraction, electro-

deposition, and desulfurization.¹⁰ Lately, DESs, which are polar, have also been used significantly to extract natural products.^{11–14}

It has been found that more than a hundred DESs can be formed from constituents that occur naturally in plants, mostly known as osmolytes.¹⁵ Expectedly, the solvation ability of these DESs derived from natural products is higher for a range of solutes than that in water.^{13,16,17} For instance, the extraction of phenolic compounds from safflower through H-bond formation using sugar-based DESs (glucose:choline chloride, lactic acid:glucose, and fructose:glucose:sucrose) has been reported.¹⁸ The physical properties of DES such as polarity and viscosity were shown to influence the extraction process. Moreover, DESs can dissolve both polar and nonpolar metabolites.¹⁹ Therefore, they can serve as solvents for the extraction of a variety of natural compounds, depending on the physicochemical properties of each DES.²⁰ Those combining ChCl with various alcohols have been used to extract flavonoids (amentoflavone and myricetin) from *Chamaecyparis obtusa* using many extraction methods. ChCl:1,4-butanediol in

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a 1:5 molar ratio was the best DES for the extraction of amentoflavone and myricetin as bioactive compounds.¹¹ Lee et al. demonstrated the tunability of DESs as designer solvents, which efficiently and selectively extract bioactive compounds.²¹

This study focuses on natural products which are known to contain antioxidant compounds such as phenolic acids, phenolic diterpenes, and flavonoids. We first examined the physical properties of the mixtures obtained between these hydrogen bond donors and a variety of canonical quaternary ammonium salts (QASs) to form deep eutectic mixtures. From this knowledge, we designed specific extraction strategies through a novel mechano-synthetic approach. Finally, a purification process was applied to increase the concentration of active ingredients in the natural deep eutectic mixture.

MATERIALS AND METHODS

Chemicals. All materials and reagents employed in this work have been used as received. Ascorbic acid (99%), rosmarinic acid (96%), caffeic acid ($\geq 98\%$), chlorogenic acid ($\geq 95\%$), choline chloride ($\geq 98\%$), benzyltriethylammonium chloride (BTEAC) (99%), benzyltrimethylammonium chloride (BTMAC) (98%), (lauryldimethylammonio) acetate ($\geq 95\%$), and 1-butyl-3-methylimidazolium chloride (BMIMCl) ($\geq 98\%$) were purchased from Sigma-Aldrich (Saint Quentin, France). L-Carnitine hydrochloride ($>97\%$) was purchased from Axo Industry International (Wavre, Belgium), while DL- α -tocopherols (75%), tocotrienols (90), resveratrol (98%), quercetin (95%), hesperidin (95%), carnosic acid (75%), ellagic acid (80%), and enoxolone (80%) were from Naturex (Avignon, France).

Differential Scanning Calorimetry (DSC). For the deep eutectic mixtures that are liquids at room temperature, melting and glass transition points were measured using a Mettler Toledo differential scanning calorimeter. The samples were sealed in an aluminum pan where they were cooled from 25 to $-125\text{ }^{\circ}\text{C}$ and subsequently heated from -125 to $125\text{ }^{\circ}\text{C}$, and eventually they were cooled back to $25\text{ }^{\circ}\text{C}$ at a heating rate of $5.0\text{ K}\cdot\text{min}^{-1}$. For this process, a flow of nitrogen was used.

The depression of the freezing point (ΔT_f) was determined for the produced eutectic mixtures using DSC and eq 1.

$$\Delta T_f \left[\frac{(\chi_{(\text{HBD})} MP_{(\text{HBD})} + \chi_{(\text{HBA})} MP_{(\text{HBA})})}{\chi_{\text{total}}} \right] - MP_{(\text{mixture})} \quad (1)$$

where $\chi_{(\text{HBD})}$ and $\chi_{(\text{HBA})}$ are numbers of moles of hydrogen bond donor and hydrogen bond acceptor, respectively, $MP_{(\text{HBD})}$ and $MP_{(\text{HBA})}$ are melting points of hydrogen bond donor and hydrogen bond acceptor separately, and χ_{total} is the total number of moles in the mixture.

Water content was estimated using thermogravimetry using a Mettler Toledo TGA/DSC1, with a resolution of $\pm 1\text{ }\mu\text{g}$ and maximum temperature of $1100\text{ }^{\circ}\text{C}$. The controlling software was STARe (version 12.10). Samples of ca. 5 mg were placed in 75 μL alumina crucibles with no lid and measured using a Maximum Resolution (MaxRes) program from 25 to $400\text{ }^{\circ}\text{C}$ at a heating rate varying between 0.15 to 15 K/min.

Fourier Transform Infrared (FTIR). The Fourier transform infrared (FTIR) spectroscopy employed was a PerkinElmer Spectrum One with universal ATR sampling accessories for the solid phase or with a NaCl disc for the liquid one. This machine was used to show that hydrogen bonds form between the two components and compared to the O–H stretching frequencies of the pure HBD.

Liquid Chromatography—Mass Spectrometry (LC-MS). The extracts were analyzed by LC-MS using an Eclipse plus C18 column, 150 mm long \times 2.1 mm (od) and 1.7 μm (id) at a temperature of $40\text{ }^{\circ}\text{C}$. The injection volume of the analyte was 2 μL , and the mobile phase A was acetonitrile with 0.1% formic acid and mobile phase B was water with 0.1% formic acid. The flow rate was 0.3 mL/min, and the MS detection mode was both positive and negative.

Mechano-Synthetic Method to Extract HBD from Biological Materials. A mechano-synthetic approach was used to carry out solid state extraction. The desired amount of rosemary leaves was taken followed by grinding using a mortar and pestle made of porcelain in the presence of liquid nitrogen. The ground powders thus obtained were sieved (Fisherbrand Test Sieve, 1 mm) to fine powders. These biomasses (powders) thus obtained were mixed with different weight ratios of a number of QASs like ChCl, BTEAC, and BMIMCl. The produced mixtures were kept in an oven at $50\text{ }^{\circ}\text{C}$ for 72 h. Then the mixtures were left at room temperature in an open vessel for 10 days to extract as much natural product as possible. Practically viable weight ratios of QASs to dried rosemary were found to be in the range 1:1 to 10:1.

Purification Method to Back Extract the QAS. To 1.0 mL of the rosemary leaf eutectic extract, 0.5 or 0.75 mL of pure oleic acid was added and mixed via magnetic stirrer at $50\text{ }^{\circ}\text{C}$ for 30 min. The resulting mixture was then centrifuged at 5,000 rpm for 15 min to maximize separation of the two layers. The upper layer was then isolated from the lower layer.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The capacity of the raw and purified eutectic extracts to trap peroxyradicals in an aqueous solution was determined using the reference ORAC method.²² Phosphate buffer solutions (pH 7.2) containing the desired concentrations of eutectic extracts or Trolox (reference) were prepared. A total of 50 μL of each solution were transferred by multichannel pipet into a Fluotrac 96-well microplate (Greiner). Each well was then topped up with 100 μL of phosphate buffer solution, pH 7.2, containing 0.126 μM of fluorescein disodium salt. To improve the repeatability, the microplate was preheated at $37\text{ }^{\circ}\text{C}$ under orbital stirring at 1,200 rpm in a temperature-controlled thermoshaker (PHMT series, Grant Instruments Ltd., Shepreth, England) for 20 min. A total of 50 μL of AAPH solution (51 mM) in freshly prepared phosphate buffer solution was then added using a multichannel pipet. Each well contained 200 μL of the final mixture, which was composed of 0.063 μM of fluorescein disodium salt, 12.7 mM of AAPH, and increasing concentrations of eutectic extracts in phosphate buffer solution. A drop in fluorescence at 515 nm (ex: 490 nm) was immediately recorded. Measurements were then taken every minute for 2 h at $37 \pm 0.1\text{ }^{\circ}\text{C}$, with 5 s of stirring before each measurement using a microplate reader. The results were then calculated and expressed in μmol of Trolox equivalent per g of liquid extract (ORAC value) as described in the original method.²³

Conjugated Autoxidizable Triene (CAT) Assay. The antioxidant capacity of the raw and purified eutectic extracts was also measured in an emulsion model using the CAT procedure described by Laguerre et al.²⁴ with slight modifications. Antioxidant solutions were prepared as follows: a methanol solution of eutectic extract or Trolox (reference) was prepared at the desired concentration. The eutectic extract solutions were filtered (0.45 μm) to remove impurities which can interfere in the CAT value measurement. Various volumes of these solutions (25, 50, 75, and 100 μL) were then added to 24.9 mL of phosphate buffer solution (PBS), pH 7.2, and then filled to 25.0 mL with pure methanol (75, 50, 25, and 0 μL , respectively). In this way, all buffered solutions of eutectic extract contained the same methanol volume (100 μL), which minimizes any possible bias among samples. Samples of these solutions (50 μL /well) were transferred using a multichannel micropipet into a UV-Star 96-well microplate (Greiner, Frickenhausen, Germany) (absorbance at 273 nm = 0.03). The microplate was then prewarmed and stirred in a thermostated shaker (PHMT Grant Instruments, Shepreth, England) at $37\text{ }^{\circ}\text{C}$ for 5 min at 1,200 rpm. Twenty-five milliliters of a buffered solution (pH 7.2) containing 34 μM Brij 35 (neutral emulsifier, estimated MW = 1198 g/mol) was added to 5 mg of tung oil (nonstripped) in a brown glass flask. For the next step, this mixture was stirred for 10 s using a Vortex apparatus, before its homogenization in an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at approximately 2,400 rpm for 90 s. Each well was then filled with 100 μL of this tung oil-in-PBS emulsion. To improve the repeatability, the microplate was then immediately prewarmed and shaken and sheltered from light in a thermostated shaker (PHMT Grant Instruments) at $37\text{ }^{\circ}\text{C}$ for 1 min

at 1,200 rpm. Fifty microliters of a freshly prepared AAPH solution in PBS (4 mM) was added with a multichannel micropipette. In the end, each well contained 200 μ L of the final mixture consisting of 115 μ M tung oil, 17 μ M Brij 35, 1 mM AAPH, and various concentrations of Trolox (from 0 to 2 μ M) and eutectic extracts in PBS.

The progress of reactions was immediately monitored by recording the decrease in absorbance at 273 nm. Measurements were performed each minute for 5 h at 37 ± 0.1 °C, with 5 s of stirring before each measurement, using a Saffire 2 microplate reader (Tecan, Groedig, Austria) equipped with Magellan software. Each experiment was performed in triplicate (three wells), and results were expressed as the mean of the CAT value (see below) \pm Sd. Similar to the ORAC assay, the antioxidant value of a sample was calculated through the difference between the area under the curve of this sample and that of the blank (without eutectic extract). The result of this operation gave the net area under the curve (AUC) which was then plotted on a graph as a function of the extract concentration. Only the linear part of the curve was taken into account to calculate the slope, which was then divided by the slope of the Trolox (standard) calculated in the same conditions and analyzed on the same microplate. As such, CAT values were expressed as μ mol Trolox equivalent/g eutectic extract.

RESULTS AND DISCUSSION

Formation of DESs. Table 1 shows the depression of the freezing point at the approximate eutectic points for the mixtures of a variety of common antioxidant compounds with a variety of QASs shown in Table 2. First, it is interesting to note that the eutectic point is different for each mixture and the T_g values are all appreciably below room temperature.

Table 1. Hydrogen Bond Donors (HBDs) Containing Hydroxyl Groups Capable of Forming Hydrogen Bonds with Different Quaternary Ammonium Salts (QASs)

Hydrogen bond donors	Salt	HBD:salt	T_g , °C (mix)	ΔT_f , °C
Ascorbic acid (1)	Choline chloride	1:2	−65	329
	Chlorocholine Cl	1:2	−55	277
	Benzyltrimethyl ammonium chloride	1:2	−43	259
	L-Carnitine hydrochloride	1:2	−40	200
	(Lauryldimethylammonio) acetate (8)	1:2	−25	157
Rosmarinic acid (2)	Choline chloride	1:2	−70	327
	L-Carnitine hydrochloride (9)	1:2	−34	188
	Lauryldimethylammonio) acetate	1:2	−20	128
Resveratrol (3)	Choline chloride	1:2	−90	375
	Chloro choline chloride	1:2	−28	274
	Benzyltrimethyl ammonium chloride	1:2	−35	272
	L-Carnitine hydrochloride	1:2	−59	241
Quercetin (4)	Benzyltriethyl ammonium chloride	1:2	−26	291
	L-Carnitine hydrochloride	1:2	−52	254
Caffeic acid (5)	Choline chloride	1:2	−92	367
	Benzyltriethyl ammonium chloride	1:2	−40	275
	Benzyltrimethyl ammonium chloride	1:2	−41	269
DL- α -tocopherol (6)	Benzyltriethyl ammonium chloride	2:1	−65	147
Tocotrienols (7)	Choline chloride	2:1	−33	154
	Benzyltriethyl ammonium chloride	2:1	−29	130

For HBDs which contain one hydroxyl group such as tocotrienols and DL- α -tocopherol, the eutectic composition occurs at a molar ratio of 1 (QAS):2 (HBD). Therefore, two molecules of HBDs are required to form the eutectic to complex with QASs through the chloride ion. This is the same as was observed for preparation of DESs from aspirin, paracetamol, phenol, 2-methyl phenol, 3,4-dimethyl phenol, and benzoic acid with ChCl as QAS.^{25,26}

The eutectic composition occurs at 2 (QAS):1 (HBD) for the HBDs including more than two hydroxyl groups, namely, ascorbic acid, resveratrol, caffeic acid, quercetin, and rosmarinic acid. Therefore, one molecule of HBD is required to form the eutectic mixture to complex with two molecules of QAS. This is in agreement with a previous work on DESs prepared from tricarboxylic acids such as tricarballic and citric acid, which have eutectic compositions in the range of 30–35 mol % acid.²⁷

DESs formed from polyacids or polyols always tend to form viscous liquids which freeze to form glasses, i.e., they are almost never crystalline. This could be due to their asymmetry and their ability to hydrogen bond in a variety of orientations.

Table 1 shows the depression of the freezing point (ΔT_f) for the different DESs. In principle, the depression is related to the deviation from ideality of the mixture. The greater the interaction between QAS and HBD, the larger the depression of the freezing point. Most of the prepared DESs are viscous liquids at room temperature as shown in Figure 1.

In this study, the extraction of antioxidants from natural products depends on the formation of hydrogen bonds between the antioxidant and the species in the DES, most probably the chloride, as the strongest base. There are many factors controlling the hydrogen bonding of a substance such as its chemical structure and composition, the intermolecular forces, and the conformational degrees of freedom. The shape of the molecule and position of the functional group may preclude the formation of DESs. For example, carnosic acid, hesperidin, and enoxolone have steric hindrance around the OH groups which could explain the difficulty for hydrogen bond formation. This may explain some of the selectivity in antioxidant extraction.²⁸ Interactions between the chloride anion and the HBD should be viewed as equilibria in which the exchange kinetics between species are relatively fast. A single HBD does not stay bound to a chloride for a significant length of time.

Besides knowing precisely which antioxidant compounds form eutectic mixtures, it is also useful to know which ones do not. A variety of antioxidant HBDs, namely, carnosic acid, hesperidin, asiaticoside, ellagic acid, chlorogenic acid, and enoxolone, were mixed with a wide range of QASs such as ChCl, BTMAC, BTEAC, and L-carnitine hydrochloride over a range of compositions. None of these formed liquids close to ambient temperature (Table S1, Supporting Information). Analysis of the shape of the molecule and position of the functional group may elucidate steric and structural factors which are important in the formation of DESs. Carnosic acid, hesperidin, and enoxolone have steric hindrance around the OH groups which could explain the difficulty for hydrogen bond formation. This is in agreement with some of the compounds shown previously.²³ In the hesperidin case, however, apart from the phenolic hydroxyls, the sugar moieties can H-bond with chloride ion. DESs have already been formed from ChCl with a variety of sugars.²⁹ It must therefore be the planar phenolic moieties and high melting point which

Table 2. Structures of the DES Components Listed in Table 1

Ascorbic acid (1)	Rosmarinic acid (2)	Resveratrol (3)
Quercetin (4)	Caffeic acid (5)	DL-Alpha-Tocopherol (6)
Tocotrienols (7)	(Lauryldimethylammonio) acetate (8)	L-Carnitine hydrochloride (9)

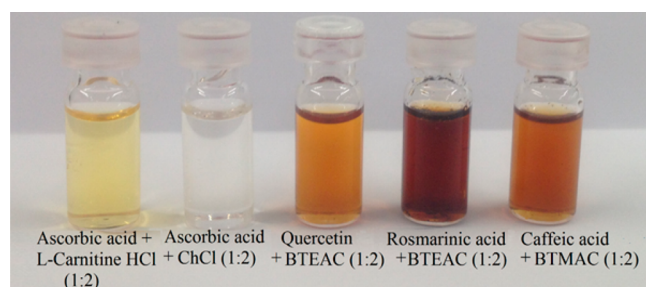


Figure 1. Prepared DESs containing mixtures of different HBDs with ChCl, L-carnitine HCl, BTMAC, and BTEAC separately.

preclude DES formation. As for ellagic acid, it is planar and symmetrical, which could prevent DES formation due to π - π stacking. The sugar moieties of asiaticoside would suggest that it should be able to form DESs, but the high molecular weight (959 g/mol) may be a reason why the DES does not form, although high molar mass *per se* does not mean it will not be soluble.²⁸

Phase Behavior. Figure 2 shows the phase diagram for binary mixtures of ascorbic acid as the HBD with two hydrogen bond acceptors, namely, choline chloride and L-carnitine hydrochloride. In this study, only two QASs are tested, as not many are currently acceptable for use in cosmetic applications. QASs are generally considered as irritants because they are hygroscopic; however, it is known that DESs containing QASs are significantly less hygroscopic than the pure QASs tested alone.³⁰ Hence, skin testing is required before they can be used routinely on skin. Absorption of water by a ChCl:glycerol (1:2) mixture was shown to be significantly less than that of the pure components tested separately, thus suggesting that QASs involved in a DES should be less irritating than expected.²⁹

The other component of the studied DESs is ascorbic acid, one of the best-known natural antioxidants, which is found in a

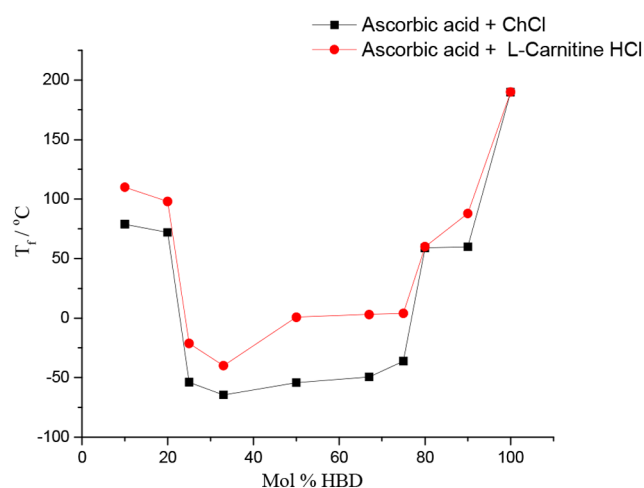


Figure 2. Freezing points of ascorbic acid with (black) choline chloride and (red) L-carnitine hydrochloride as a function of composition.

variety of plants, particularly fruits and vegetables. In its reduced form, ascorbic acid has four hydroxyl groups that can theoretically hydrogen bond to the anions of four QASs. As shown previously,²³ the number of HBD groups tends to control the eutectic composition. Dicarboxylic acids reach eutectic composition at 50 mol % HBD, whereas monocarboxylic acids have a eutectic composition at 67% HBD. It has been previously shown that polyols have an unusual phase behavior and that the eutectic point is not sharp, but instead there are a wide range of compositions which have similar melting points.³¹ The logical explanation for this phase behavior is that the HBD can coordinate to the chloride using 1, 2, or 3 functional groups and the melting point is not overly different when 1, 2, or 3 HBDs are coordinated.

L-Carnitine hydrochloride, which is one of the two QASs tested here, is a salt of a naturally occurring quaternary

ammonium compound found in most mammals, plants, and some bacteria. It is similar in structure to choline chloride but has an additional ethanoate functionality ($((\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CHOH}(\text{CH}_2\text{COOH})\text{Cl}^-)$). Both salts, when mixed with ascorbic acid, form room temperature liquids in the composition range 25 to 75 mol % HBD, which is similar to tricarboxylic acids such as tricarballic and citric acid which form ambient temperature liquids with ChCl in the range 33 to 67 mol % HBD.²⁵ In all compositions, eutectics with L-carnitine hydrochloride have slightly higher melting temperatures than ChCl mixtures due to the extra polar ethanoate functionality.

Extraction of Active Antioxidant Substances from Rosemary Leaves. The industrial procedure for natural product extraction and isolation usually uses a volatile organic solvent, typically an alcohol. While this may appear at first sight as a simple process, it often requires many sequential extractions that can be energy intensive. As for the extraction of a natural product into an ionic liquid or a DES, it is not generally appropriate due to the difficulty of extracting the product from the mixture at the end due to the lack of an appreciable vapor pressure to easily evaporate the ionic liquid or DES. An alternative approach has been developed to extract natural products into a nontoxic DES which remains with the extract. A DES made from betaine and lactic acid was shown for instance to extract more natural product than the standard alcohol-based process, and that extract has a higher antioxidant power.¹⁷

Active natural antioxidants can be found in most parts of a plant including leaves, fruits, seeds, roots, peels, and barks. In most cases, the extract is a solid at ambient temperature, but it can be dissolved in an aqueous phase in fruit juices and plant saps. In this study, rosemary (*Rosmarinus officinalis*) was used as an example to apply a novel extraction approach, the novelty of which is that a solid QAS is used with the aim of forming a deep eutectic mixture *in situ* with the endogenous HBDs present in the natural material. The same process has been carried out using fruits, barks, and roots with a similar effect.³²

The mixtures of these biomasses and QAS were kept in an oven at 50 °C for 72 h. The ideal scenario would have been that the natural products would spontaneously form a liquid deep eutectic mixture with the QAS. This was not the case as solid-state interactions are slow. Absorption of atmospheric moisture did occur when the solids were stored at room temperature in an open vessel on the benchtop for 10 days. The liquid leached out of the mixture and the presence of the natural product extract was confirmed using Fourier transform infrared spectroscopy and LC-MS (see Figures S1 and S2). This is, in practice, similar to the salting out effect used in food preservation to draw out liquid components with NaCl .

In this study, a new concept is attempted which is to use a QAS mixed with the natural product in the solid state to see if a DES forms from the HBD from the plant and the salt. Indeed, the salt should draw the moisture out of the material and, at the same time, the natural product. The DES can then be concentrated by evaporating off the excess moisture or back extracting the excess QAS.

Dried rosemary leaves were chosen with the aim of targeting active ingredients such as rosmarinic acid and luteolin-3-glucuronide, two relatively polar antioxidant compounds. ChCl , BTEAC, and BMIMCl were tested as QASs at different weight ratios with rosemary to form the deep eutectic mixture and to extract components that are able to hydrogen bond with

the QASs tested. The dried biomass was mixed with QASs using a glass stirring rod for 5 min and left at room temperature in an open vessel, unstirred for 10 days to extract as much natural product as possible. Practically viable weight ratios of QASs to dried rosemary were found to be in the range 1:1 to 10:1.

At the end of this process, a dark colored liquid had formed due to the hygroscopic nature of the QASs. Remarkably, however, the liquid did not have any odor and there was no characteristic smell of rosemary. To some extent, this is not surprising as the odoriferous compounds are mostly esters and ketones (e.g., camphor and verbenone) which are known not to partition into DESs due to their poor hydrogen bonding ability.²³ This shows that the extraction is more selective for hydrogen bonding molecules, especially HBDs. The liquid was analyzed using LC-MS and found to contain a variety of natural products including azelaic acid, hesperidin, rosmarinic acid, luteolin-3-glucuronide, and rosmanol (Table 3). A more detailed list of components is shown in Table 3, and the rosmarinic acid and luteolin-3-glucuronide contents obtained in the eutectic extracts are shown in Figure 3.

Table 3. Names and Formulas of Some of the Components Extracted from Dried Rosemary Leaves Using ChCl (1:8 mass ratio) Identified Using LC-MS Comparing the Data to Library Standards and the Deviation in Mass Error (m/z) in ppm (See Figure S2)

Possible identification	Formula found	$[M - H]^-$	$\Delta\text{ppm } +/ -$
Nepitrin	$\text{C}_{22}\text{H}_{22}\text{O}_{12}$	477.1025	4.77
Azelaic acid	$\text{C}_9\text{H}_{16}\text{O}_4$	187.0972	1.33
Hesperidin	$\text{C}_{28}\text{H}_{34}\text{O}_{15}$	609.1792	-5.87
Rosmarinic acid	$\text{C}_{18}\text{H}_{16}\text{O}_8$	359.0757	-4.08
Luteolin-3-glucuronide	$\text{C}_{21}\text{H}_{18}\text{O}_{12}$	461.0712	4.35
Feruloylnepitrin isomer II	$\text{C}_{31}\text{H}_{28}\text{O}_{14}$		-6.75
Luteolin 3'-O-(o-acetyl)- β -glucuronide isomer I	$\text{C}_{23}\text{H}_{20}\text{O}_{13}$	503.0809	4.68
Luteolin 3'-O-(o-acetyl)- β -glucuronide isomer I	$\text{C}_{23}\text{H}_{20}\text{O}_{13}$	503.0805	5.8
Luteolin 3'-O-(o-acetyl)- β -glucuronide isomer II	$\text{C}_{23}\text{H}_{20}\text{O}_{13}$	503.0808	4.92
Rosmanol	$\text{C}_{20}\text{H}_{26}\text{O}_5$	345.1694	5.53
Epirosmanol	$\text{C}_{20}\text{H}_{26}\text{O}_5$	345.1690	4.25
17-Hydroxy cryptotanshinone isomer	$\text{C}_{19}\text{H}_{20}\text{O}_4$	311.1343	-5.88
17-Hydroxy cryptotanshinone isomer	$\text{C}_{19}\text{H}_{20}\text{O}_4$	311.1267	7.16
Rosmadiol	$\text{C}_{20}\text{H}_{24}\text{O}_5$	343.1532	6.08
Diterpenic metabolite (carnosic precursor)	$\text{C}_{19}\text{H}_{22}\text{O}_3$	298.1574	-1.6

Fourier transform infrared spectroscopy (PerkinElmer Spectrum One FT-IR with ATR) was used to compare the native constituents with their resulting eutectic mixtures. Figure S1 shows FT-IR spectra of pure rosmarinic acid and DESs which are formed by pure rosmarinic acid and dried leaves of rosemary with choline chloride. The broader peaks of the DESs are due to the presence of extensive hydrogen bonding.

The salt:natural product mixture seems to have a steady state amount of water that it can absorb. If the sample is left for extended periods, it does not appear to absorb significantly more moisture. This is an area that requires further investigation, but clearly, the time taken to reach the steady

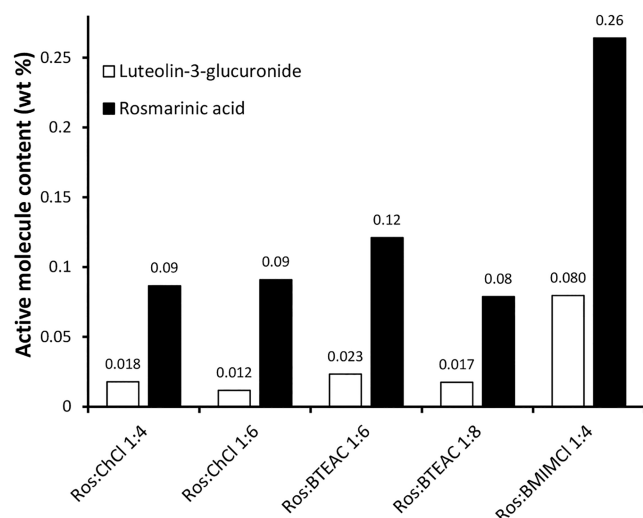


Figure 3. Content of luteolin-3-glucuronide (RMM 462.4 g/mol) and rosmarinic acid (RMM 360.3 g/mol) extracted from dried rosemary leaves (denoted as Ros) with pure QAS (choline chloride, ChCl; benzyltriethylammonium chloride, BTEAC; 1-butyl-3-methylimidazolium chloride, BMIMCl).

state water content will vary depending on the moisture content of the natural product and humidity and temperature.

At this point, it could be argued that the only effect of the QAS is to extract the fluid from the plant material. If this were the case, then simple inorganic salts should have the same effect. To test this hypothesis, the above experiments were repeated for rosemary leaves using sodium chloride, potassium chloride, and lithium chloride. No liquid phase was observed with sodium or potassium chloride, but LiCl absorbed moisture. Spectroscopic tests showed that no natural products were extracted using this salt. To ensure that it was not an effect of the cation, choline bitartrate was used to extract the rosemary leaves, but again no liquid phase was formed. This is very strong evidence to confirm that extraction only occurs when a deep eutectic mixture forms between the specific QAS and the HBD available in the natural product.

Improvement of the Phenolic Level and Antioxidant Activity by Purification. The selective extraction process developed above produces a liquid that is overly rich in QAS, thus diluting the antioxidant compounds. One method to deplete the QAS is to contact the deep eutectic mixture with an immiscible solvent, in which the QAS can be partitioned; here oleic acid was used. Oleic acid was chosen because it is a liquid, is generally regarded as safe, and is permitted in pharmaceutical and cosmetic formulations. Oleic acid was added with the eutectic mixture prepared from the dried

rosemary leaves and BTEAC to back extract the excess QAS. Consequently, the concentration of HBDs such as rosmarinic acid and luteoline-3-glucuronide in the extract layer increased significantly. In this process, oleic acid was mixed for 30 min at 50 °C with the deep eutectic mixture at two ratios (0.75:1, v/v, and 0.5:1, v/v, respectively), then stirred, and centrifuged to separate the mixture into two phases (Figure 4). Noteworthy, the amount (in volume) of the upper phase was greater than for the lower phase after purification, which suggests that oleic acid interacted with the free BTEAC molecules. Accordingly, this would result in a concentration of the antioxidant compounds in the lower phase. Indeed, results obtained on a preliminary experiment show that the concentrations of rosmarinic acid and luteolin-3-glucuronide, two relatively hydrophilic phenolic antioxidants, are approximately twice as large in the 1 Rosemary:8 BTEAC extract after shaking with oleic acid and drying (Table S2).

Encouraged by these results, we deepened the analysis starting from the same rosemary:BTEAC (1:8) extract, although independently prepared. Figure 5A,B shows an increase of rosmarinic acid and luteoline-3-O-glucuronide. Concurrently, the BTEAC and water are partially depleted from the extracts when the purification procedure is applied, although the depletion remains modest (<10% for BTEAC; <20% for water). The BTEAC and the water removed from the extract are thus present in the upper layer of which they constitute a significant part (38–42%) almost equaling the proportion of oleic acid (43–46%) (Figure 5C). Surprisingly, despite the immiscibility of water and oleic acid, the upper layers contain around 10% of water (Figure 5C), which possibly migrated as water H-bonded to BTEAC molecules (water–BTEAC complex). In our experiments, no oleic acid migrated to the extracts during the purification step. Indeed, only low amounts of oleic acid (<1%) are found in the lower layer, whereas the nonpurified rosemary eutectic extract already contains 1.7% oleic acid (Figure 5C).

The increase in rosmarinic acid and luteoline-3-glucuronide following the application of the purification step is concomitant with an increase of the antioxidant activity of the rosemary eutectic extracts in both ORAC and CAT measurements. When 0.5 mL of pure oleic acid was used to wash 1 mL of liquid extract, the ORAC value of the resulting extract (lower layer) increased by 37% and the CAT value by 25%. Similarly, when using 0.75 mL of oleic acid (for 1 mL of extract), the ORAC value increased by 17.5% and CAT value by 19%.

Altogether, these results demonstrate that the purification step enables raising the level of active molecules through a partial migration of nonantioxidant molecules into the phase formed by the long chain fatty acid, which consequently increases the activity that is considered.

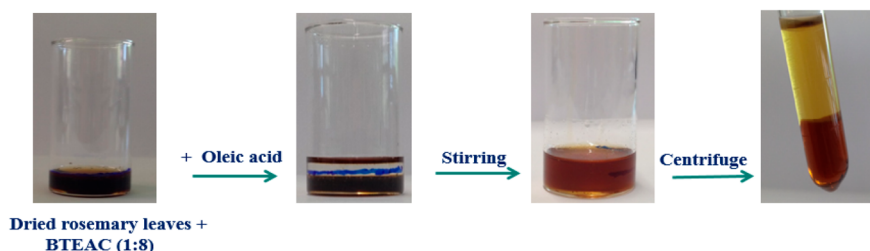


Figure 4. Purification of a rosemary eutectic extract with oleic acid used as an immiscible phase.

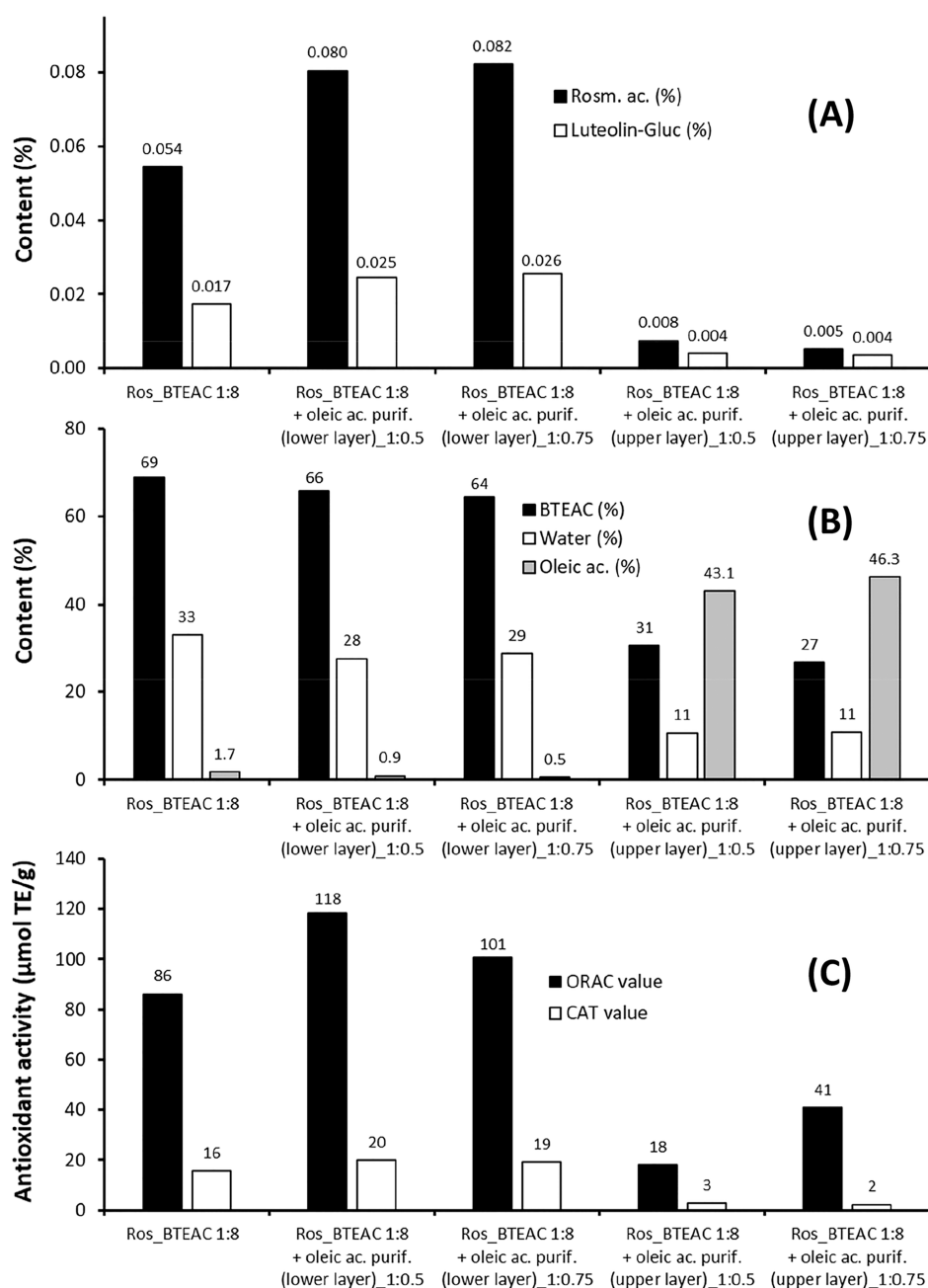


Figure 5. Effect of the purification process of a rosemary eutectic extract on its content in rosmarinic acid and luteoline-3-glucuronide (A), its content in BTEAC, water, and oleic acid (B), and its antioxidant capacity expressed as ORAC and CAT values (C).

CONCLUSION

In this study, a number of HBDs, namely, ascorbic acid, rosmarinic acid, resveratrol, quercetin, DL- α -tocopherol, tocotrienols, and caffeic acid, were used to form deep eutectic mixtures in different molar ratios with various quaternary ammonium salts such as ChCl, benzyltrimethylammonium chloride, benzyltriethylammonium chloride, and L-carnitine hydrochloride. The HBDs which are used in this work have a variety of uses in the pharmaceutical, cosmetic, and dietetic areas such as antiviral, antibacterial, anti-inflammatory, analgesic, and antioxidant. In addition, some of the selected QASs are useful and already used in the industry. For example, ChCl is nontoxic, inexpensive, and biodegradable and is used as an additive in chicken feed and as a provitamin in Europe.

Furthermore, L-carnitine hydrochloride is a natural product and is useful in terms of nutritional and pharmacological properties. The glass transition temperatures were measured by DSC for the eutectic mixtures produced. The second part of the study aimed to see if these natural product mixtures could be formed *in situ* by mixing QASs with natural products. While they did not form spontaneously in the solid state, the adsorption of atmospheric moisture was sufficient to form these natural product deep eutectic mixtures. This methodology was demonstrated using rosemary (*Rosmarinus officinalis*), but the technique was also successful for a variety of leaves, fruits, seed, barks, and roots. In all cases, natural product extracts were obtained, which predominantly contained molecules which were strong hydrogen bond donors, in

particular phenolic compounds. All of the eutectic mixtures formed showed high antioxidant activity as measured by the CAT and ORAC assays.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssuschemeng.2c06894>.

Table of natural product hydrogen bond donors that do not form DESs with QASs and the analysis of natural products extracted from rosemary using different QASs (PDF)

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Notes

The authors declare no competing financial interest.

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