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RESEARCH ARTICLE

Application of natural deep eutectic solvents for the "green"extraction of vanillin from vanilla pods

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Abstract

In recent years, a new type of solvents, natural deep eutectic solvents (NADES), have been developed and are gaining popularity as an alternative "green solvent" to conventional organic solvents. The traditional extraction of flavor or fragrance from plant materials such as vanillin is a tedious procedure that involves the use of organic solvents. In this case the solubility of vanillin and its extractability from vanilla pods in 14 NADES was evaluated. The solubility in some NADES such as lactic acid: propanediol (620 mg/mL) and lactic acid: fructose (320 mg/mL) was found to be almost similar to that of organic solvents e.g. methanol (632.94 mg/mL) or ethanol (375.81 mg/mL). Interestingly, the extractability of vanillin from vanilla pods did not reflect the results observed for the solubility tests. Some NADES with constituents that are suitable as food ingredients such as citric acid-fructose-glucose (CAFG), fructose-glucose (FG), malic acid-glucose (MAG), and malic acid-fructose-glucose (MAFG), showed a higher extraction power (7.6, 10.1, 16.7, and 16.3 mg/g DW, respectively) than ethanol (4.4 mg/g DW). The discrepancy between solubility and extractability might be due to differences in the interaction with the matrix depending on the NADES. The effect of water content in some NADES on the extractability of vanillin was also studied. With the high extraction capacity and the absence of toxicity of their components, NADES are very suitable for extraction of flavours and fragrances used in food and cosmetics.

KEYWORDS

extractability, green processing, NADES, solubility, vanillin

1 | INTRODUCTION

Vanilla, one of the most popular flavors worldwide, is used in a wide array of products such as confectionery, ice-cream, food, perfume and beverages. Among the many volatile aromatic compounds of vanilla extracts, vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most characteristic component for the flavor. Vanillin is produced in ripe pods of *Vanilla planifolia*, through an elaborate curing process of at least six months, during which the pods turn to a black color and develop their characteristic flavor.[1] *Vanilla planifolia* is a species of tropical climbing orchids native to Mexico, but is currently cultivated mainly in Madagascar, Indonesia, India and Tahiti.[2] The vanilla production fluctuates between 2,000 and 3,000 tons per year, Madagascar being the largest producer with 1,750 tons in 2008.[3] Vanillin can be obtained synthetically or via fermentation processes from other natural products. Currently, less than 1%, approximately 12,000 tons of the vanillin in the flavor market is derived naturally from vanilla plants.[4] However, in terms of quality, natural vanilla has a superior aroma and fragrance performance than pure vanillin. This is due to the presence of various minor compounds in the pods that give special notes to the vanilla flavor. The highest quality vanilla extract is obtained from vanilla pods using the percolation or oleoresin method.[5] The percolation method consists of circulating aqueous ethanol (35–50%, v/v) over the pods, under vacuum. This process takes between

48 and 72 h. In the case of the oleoresin method, pods are pulverized and extracted with ethanol under vacuum at about 45°C[5] during 8–9 days. The FDA defines a vanilla extract as a solution containing no less than 35% ethyl alcohol and a vanilla constituent obtained from 13.35 of of vanilla pods if containing no more than 25% moisture in one gallon of finished product.[6] However, the high ethanol content required for solubility purposes is often undesirable and can even become an obstacle for the use of vanilla extracts in certain applications, e.g. when a zero ethanol content is required for a product. However, ethanol is the only accepted relatively non-toxic option among organic solvents that can provide an acceptable product for use as a flavoring additive. It is thus useful to search for an alternative to ethanol, and natural deep eutectic solvents (NADES) might be an option, having the additional benefit of providing a green alternative.[7] In green chemistry, ionic liquids (IL) have been shown to be versatile solvents that are used for diverse purposes from media for enzymatic reactions to the extraction of natural compounds from biomass. For example, ionic liquids (ILs) in combination with different extraction technologies, such as microwave and ultrasonic treatment have been used to extract alkaloids (*N*-nornuciferine, *O*-nornuciferine and nuciferine from lotus leaf and piperine from *Piper nigrum*) and phenolics (*trans*-resveratrol from rhizome of *Polygonum cuspidatum*)[8-10] A major drawback of these ILs are their high costs and toxicity. Recently it has been found that common naturally occurring organic acids and bases can form ionic liquids.[11] Choi et al.[11] reported on natural ionic liquids and a series of other common natural products that in certain molar ratios become liquid at ambient temperatures. They hypothesized that such liquids could explain many biological phenomena, assuming that NADES occur as a third liquid phase in all living organisms and cells. These liquids dissolve a number of natural compounds that are poorly soluble in water and lipids, e.g. taxol and rutin as well as proteins. The biosynthesis of non-water soluble compounds might thus take place in such solvents. The compounds found to form NADES, like sugars, organic acids choline and proline, have been reported to be enriched in various organisms under drought and cold stress. A clear example of the occurrence in nature was the nectar of *Cleome hassleriana* flowers, showing in ^{1}H NMR analysis the presence of a combination of sucrose, fructose and glucose (1:1:1).[11] The term Natural Deep Eutectic Solvents has then been introduced as a general term to cover a wide range of compositions of primary metabolites that in different combinations form liquids at ambient temperature, and that seem to play an important role in living organisms. The NADES consist of two or more common natural products combined in a specific molar ratio that form a liquid. This is similar to the well-known phenomenon that occurs when two solids are mixed and form a liquid due to interactions that lower their individual melting points even below normal ambient temperatures (deep eutectic solvent, DES). In the case of NADES only certain molar ratios of compounds do show this transition to a liquid. NOESY experiments in the ^{1}HNMR analysis of the NADES showed that these liquids in fact are a fully organized molecular structure controlled by extensive hydrogen-bonding between the molecules. The components are common primary metabolites present in all living cells, such as sugars, sugar alcohols, small organic acids or bases, and amino acids.[11] The NADES have shown a great potential as solvents to dissolve and extract a wide range of compounds from plants. They have similar physicochemical properties to ionic liquids based on (organic) bases and acids such as negligible volatility, melting points far below 0°C and adjustable viscosity. Their polarity ranges between that of water and alcohols and they have been reported to extract a wide variety of polar to medium polar compounds.[7,12] For example, NADES have been used to extract phenolic metabolites such as carthamin from *Carthamus tinctorius* L. or anthocyanins from *Catharanthus roseus* flowers.[13,14]

Based on their components, NADES can be classified into 5 major groups: ionic liquids, made from an acid and a base; neutral, made of sugars only, or sugars and other polyalcohols; neutral with acids, made of sugars/polyalcohols and organic acids; neutral with bases, made of sugars/polyalcohols and organic bases; and amino acids-containing NADES made of amino acids with organic acids/sugars. From various NMR studies it is clear that the NADES form a network of the compounds through extensive hydrogen bonding, showing NOE effects between the participating molecules. Upon dilution with up to 50% still interaction between the molecules can be observed as can be seen from the chemical shifts.[7,13] When applied to the extraction of natural products, NADES have several advantages over the existing alternatives. These include their low cost, high biodegradability, relative non-toxicity, sustainability and simple preparation methods.[11,13]

In the case of vanilla extraction, the replacement of conventional hydroalcoholic mixtures with NADES would produce nonvolatile and relatively nontoxic extracts with the additional benefit of an increased stability of vanillin. Furthermore, as most NADES components occur in large amounts in our daily food there are no safety concerns for their use as flavoring ingredients in food and beverages.

2 | MATERIALS AND METHODS

2.1 | Plant material

The cured vanilla pods (*Vanilla planifolia*) were provided by the Ministère de L'alimentation, de L'agriculture et de la Pèche, Paris, France. The original country of the cured pods is Reunion, collected in 2015.

2.2 | Chemicals and reagents

Malic acid, citric acid, lactic acid, choline chloride, L-serine, β-alanine, betaine, lactic acid, 1,2-propanediol and vanillin were purchased from Sigma (St. Louis, MO, USA); D-fructose, D-glucose monohydrate and sucrose from Boom B.V. (The Netherlands) and glycerol from Merck (Darmstadt, Germany). *n*-Butanol and acetic acid of analytical grade were purchased from Biosolve B.V. (Valkenswaard, The Netherlands).

2.3 | Natural deep eutectic solvents preparation

Natural deep eutectic solvents were prepared by combining the components in certain molar ratios and stirring the mixture at 50°C.[7] The NADES used in this experiment were choline chloride-malic acid (CCMA), choline chloride-citric acid (CCCA), choline chloride-glycerol (CCGo), malic acid-fructose-glucose (MAFG), citric acid-fructose-glucose (CAFG), fructose-glucose (FG), malic acid-glucose (MAG), betaine-sucrose (BeS), betaine-malic acid-glucose (BeMAG), betaine-citric

TABLE 1 Natural deep eutectic solvents (NADES) used for the extraction of vanillin from vanilla pods and solubility testing of pure vanillin at 50°C

Name	Combination	Molar ratio	Solubility of vanillin mg/mL
CCCA	Choline chloride: citric acid: water	1:1:6	90
CCMA	Choline chloride: malic acid: water	1:1:6	90
CCGo	Choline chloride: glycerol	1:1	270
FG	Fructose: glucose: water	1:1:6	30
MAG	Malic acid: glucose: water	1:1:6	30
BeS	Betaine: sucrose: water	2:1:6	30
BeCA	Betaine: citric acid: water	1:1:6	60
BeMAG	Betaine: malic acid: glucose: water	1:1:1:9	30
CAFG	Citric acid: fructose: glucose: water	1:1:1:9	30
MAGF	Malic acid: glucose: fructose: water	1:1:1:9	30
SrMA	L-Serine: malic acid: water	1:1:6	30
bACA	β-alanine: citric acid: water	1:1:6	30
LAPo	Lactic acid: 1,2-propanediol	1:1	620
LAF	Lactic acid: fructose	5:1	320

Solubility of vanillin in ethanol (375.81 mg/mL), methanol (632.94 mg/mL), and water (30 mg/mL).

acid (BeCA), β-alanine-citric acid (bACA), L-serine-malic acid (SrMA), lactic acid-1,2-propanediol (LAPo) and lactic acid-fructose (LAF). Table 1 shows the molar ratios of the NADES components.

2.4 | Solubility test

Solubility tests were carried out by adding vanillin to each of the NADES listed above with a concentration range of 30 mg/mL to 620 mg/mL and leaving them at 50°C for 1 h. Water, methanol and ethanol were used as control experiments. In the case of methanol and ethanol, the solubility tests were performed at room temperature. Experiment were done in triplicate.

2.5 | Extraction of vanillin from vanilla pods with different NADES

Extraction was performed by mixing 1 mL of NADES with 50 mg of ground cured vanilla pods in a 2 mL-Eppendorf tube. The mixture was vortexed, and kept at 50°C for 1 h and then centrifuged for 30 min at 1300 rpm. An aliquot of 400 μL of the supernatant was transferred to a vial, diluted with 600 μL of the HPLC mobile phase and homogenized. The sample was filtered with a 0.20 μm filter prior to HPLC analysis. As a control, 50 mg of ground vanilla pods were extracted separately with 1 mL of methanol or ethanol at room temperature and analyzed by HPLC in similar conditions to those used for the NADES tests.

2.6 | The effect of water in NADES on the extractability of vanillin from vanilla pods

Four types of NADES (FG, BeCA, LAF and LAPo) were diluted with de-ionized water, in diverse ratios (90:10, 75:25, 60:40, 40:60 *v*/v). Fifty mg of ground vanilla pods were extracted with 1 mL of each diluted NADES and with 1 ml of the non-diluted NADES. The resulting extracts were analyzed by HPLC in similar conditions to those described previously.

2.7 | HPLC-DAD analysis

Vanillin, the major volatile compound in vanilla beans was quantified by reversed-phase HPLC with an Agilent 1200 chromatographic system equipped with a photodiode array detector (DAD). The HPLC column was a Phenomenex C18 column (150 x 4.60 mm, 5 μm). The mobile phase consisted of 2.5% butanol in water with 0.2% acetic acid with a flow rate of 1.0 mL/min.[15] The injection volume was 20 μL and the chromatograms were recorded at 310 nm. A calibration curve with a range of 50–625 ug/mL was prepared by triplicate.

3 | RESULTS AND DISCUSSION

3.1 | Solubility of vanillin in different NADES

Among the many existing NADES, 14 were selected, representing different classes of NADES (Table 1). Four ionic liquid-type (BeCA, BeMAG, CCMA, CCCA), one neutral type (FG), two mixtures of amino acid – acid combination (SrMA, bACA), two neutral-base type (BeS, CCGo) and five neutral-acid (CAFG, MAG, MAGF, LAF; LAPo). One of the difficulties in handling NADES is their higher viscosity. Previous studies showed that temperature as well as water content, greatly influences the viscosity. In general, the viscosity of NADES rapidly drops above 40°C. Therefore, the solubility tests of vanillin in diverse NADES for comparative purposes was done at 50°C (for 1 h).

The solubility of vanillin in methanol was 632.94 mg/mL, 375.81 mg/mL in ethanol and only 30 mg/mL in water.[16,17] In the case of the NADES, the solubility of vanillin was highest in LAPo (620 mg/mL) followed by LAF (320 mg/mL) and CCGo (270 mg/mL), very likely due to the better ability of vanillin to form hydrogen bonds with these NADES. It was also soluble, though in a lesser degree, in CCCA (90 mg/mL), CCMA (90 mg/mL) and BeCA (60 mg/mL), whereas BeMAG, CAFG, MAFG, MAG, bACA, SrMA, FG and BeS showed a much lower capacity to dissolve vanillin, similar to that of water (Table 1).

3.2 | Extractability of vanillin from vanilla pods with diverse NADES

The extraction efficiency of NADES was compared to that of ethanol and methanol since vanillin is very soluble in these organic solvents. In the case of ethanol, it was particularly important to compare yields since it is used to produce the extracts for food flavoring purposes. The determination of vanillin in the NADES extracts was done by HPLC/DAD, vanillin was identified by comparison of its retention time and UV-spectra with a pure reference compound (Figure 1). The results showed that methanol is 3–4 times more efficient than ethanol as an extraction solvent (18.0 and 4.4 mg/g DW, respectively). Among the NADES tested, the highest amounts of vanillin extracted from vanilla pods were obtained with CCCA, BeCA, LAPo and LAF (18.6 mg, 19.8 mg, 19.9 mg and 21.8 mg/g DW, respectively), which were higher than that those obtained with methanol. The combination of choline chloride with malic acid (CCMA) showed a similar yield of vanillin (17.9 mg/g DW) to that of methanol, whereas some other NADES such as BeMAG, bACA, MAG, MAFG, CCGo and SrMA extracted a slightly lower amount (17.1 mg, 17.6 mg, 16.7 mg, 16.3 mg, 15.7 mg and 14.5 mg/g DW vanilla pods, respectively). Others, such as CAFG (7.6 mg/g DW), FG (10.1 mg/g DW) and BeS (6.2 mg/g DW) were clearly less efficient (Figure 2). Thus, as can be observed, all the tested NADES had a higher extraction capacity for vanillin than ethanol, and many had approximately the same or even a slightly higher yield than methanol (Figure 2). However, as well known, methanol is toxic for humans and cannot be used in any step of food processing. Interestingly there was no correlation between the solubility of vanillin in the NADES and the extractability of vanillin from the pods with the NADES, implying that the removal of vanillin from its matrix is an important factor in obtaining an exhaustive extraction of this compound from the pods.

3.3 | Effect of water on NADES for the extractability of vanillin from vanilla pods

The viscosity of a NADES differs according to its composition and the addition of small amounts of water decrease the viscosity considerably.[18] The lower the viscosity of the NADES, the easier it is to handle and to work with for practical reasons. This issue is also important to be considered when developing formulations of vanilla extracts and vanillin as food additives. However, the presence of water can affect the solubility of the target compounds. Therefore, the effect of the amount of water in NADES on the solubility of vanillin and its extractability from vanilla pods was also studied. Depending on the method of preparation in some combinations water is part of interaction of the molecules and cannot be evaporated. A ca. 5% water content in the sugar containing NADES correspond with about 1 molar in the mixture. All NADES are easy to dilute with water, and from the NMR shifts of the protons one can see that with increasing dilution the interaction between the molecules gradually disappear, and at higher than about 50% water the molecules behave like as in an aqueous solution.[11] In the present work, water was added up to 60% *w*/w in some NADES to learn more about the behavior of vanillin in the NADES extracts upon dilution in

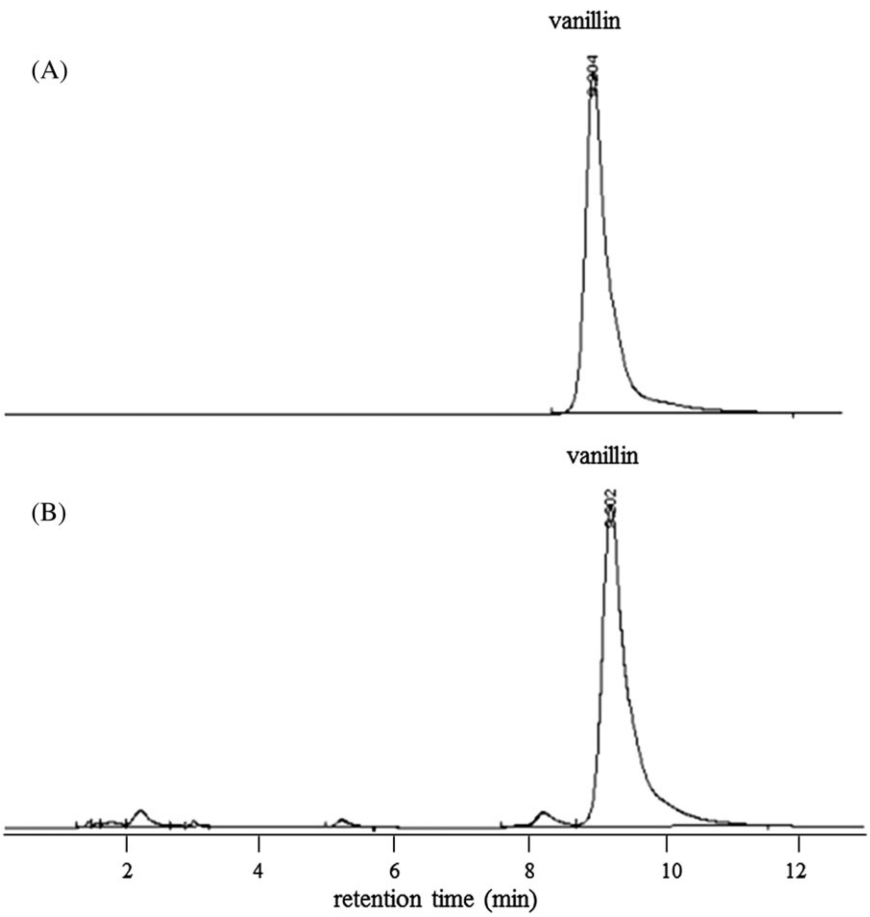

FIGURE 1 HPLC chromatograms of vanillin standard in NADES (A) and vanilla pods extract in NADES (B). The samples were diluted with the mobile phase before injection

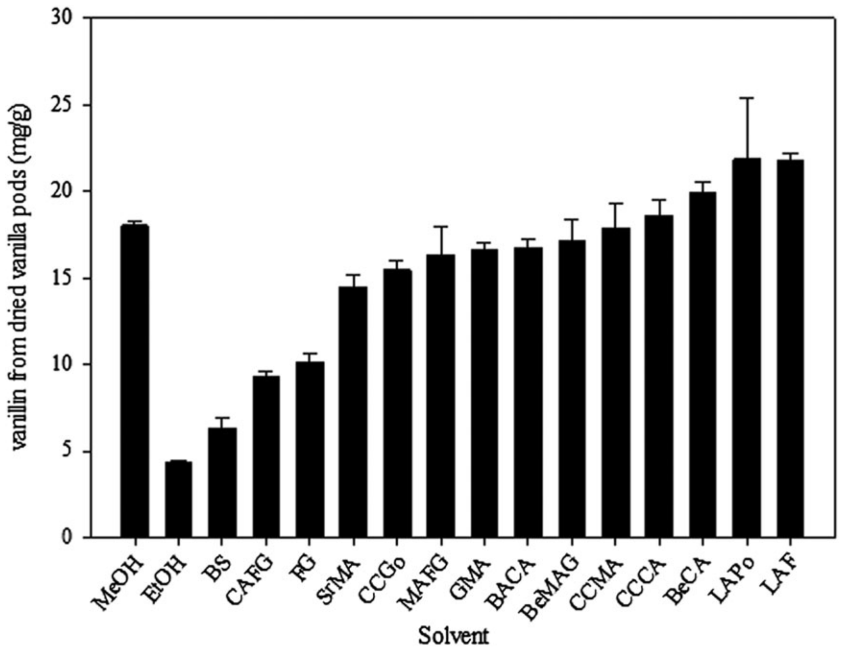

FIGURE 2 Amount of vanillin extracted from vanilla pods using methanol, ethanol and NADES. The data is expressed in mean ± SD (n = 3)

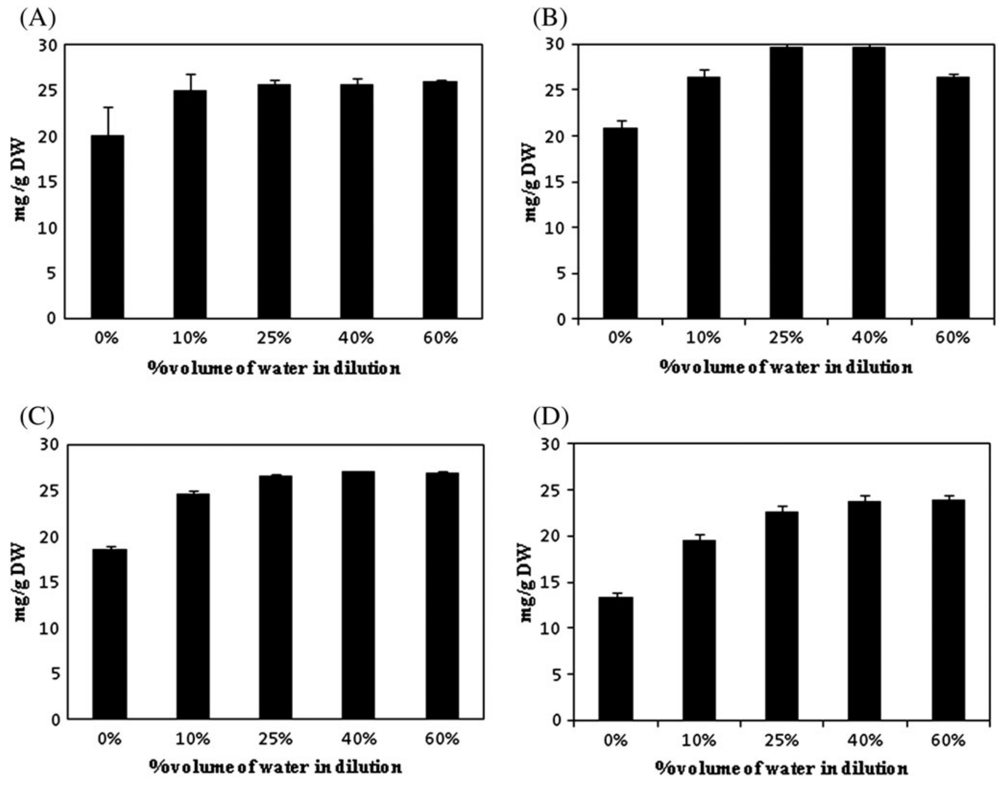

FIGURE 3 Effect of water content on the extractability of vanillin from vanilla pods with diverse NADES. The graphs show the amount of vanillin extracted from vanilla pods (mg/g DW) in four different NADES diluted with 0–60% (v/v) of water. A: BeCA (Betaine-citric acid), B: LAPo (Lactic acid-1,2-Propanediol), C: LAF (Lactic acid-Fructose), D: FG (Fructose-Glucose). The data is expressed in mean ± SD (n = 3)

connection with the potential applications of the NADES extracts. Some NADES were used as models (FG, BeCA, LAF and LAPo). Results showed that, as expected, the water content of NADES influenced the extractability of vanillin. With dilutions ranging between 10 and 60% of water, v/v, the amount of vanillin extracted increased with the increasing content of water up to 25% water in all tested NADES (Figure 3). Dilutions above 25% did not notably increase the extractability, and even decreased it in the case of LAPo. These results are in agreement with the previous study performed with quercetin, rutin and carthamin,[13] which showed that the water content can improve the solubility of medium polar compounds in NADES while more nonpolar compounds are more soluble in NADES that contain relatively lower amounts of water or are even anhydrous. However, when determining the optimal water content, other factors such as stability of the compounds and possible microbial contamination should also be considered.

In this study, we have reported our results regarding the solubility of pure vanillin in NADES on one hand, and the extraction of vanillin from vanilla pods using NADES. There are thus two distinct products that can be obtained using NADES for vanilla-like flavoring: a vanillin solution and a vanilla extract. An ideal solvent for a vanillin formulation should be non-toxic and give high yields of vanillin apart from providing a stabilizing medium. Currently, ethanol is the solvent of choice for industrial production, there are some NADES that have a similar (LAF, CCGo) or even higher (LAPo) solubility. The advantage of the NADES is that they are in general nontoxic, non-volatile, and as for all phenolics, increase their stability.[14,18] It is possible to add water to NADES since they are fully water soluble. Some NADES contain ingredients with some restrictions (LD50) that limit their use as food ingredients or drug excipients to certain levels, but in many cases, they are safe in high doses and have received GRAS certification (e.g. FG and FGS). These NADES can be included directly in food products such as ice-cream, beverages, cookies, candies, cooking in general and other kinds of desserts in restaurants. Thus, NADES can be a potential alternative solvent for the extraction of flavors from plants or can be used to dissolve flavor compounds for food additives.

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AUTHOR CONTRIBUTIONS

C. Guízar executed the experimental work, drafted the manuscript, N. R. Mustafa supervised the daily lab work and data analysis, E. G. Wilson provided general supervision, R. Verpoorte and Y. H. Choi designed the study and interpreted the results.

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