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Research Article

Hydrophobic Eutectic Solvent with Antioxidant Properties: Application for the Dispersive Liquid−Liquid Microextraction of Fat-Soluble Micronutrients from Fruit Juices

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ABSTRACT: Despite the great interest devoted to eutectic solvents with significant negative deviations from ideality (namely, deep eutectic solvents), many hydrophobic liquid mixtures are ideal or quasi-ideal systems. In this regard, we propose the introduction of a hydrophobic eutectic solvent based on L-menthol and butylated hydroxytoluene, blended in a molar ratio of 3:1. The physicochemical characterization by means of differential scanning calorimetry and infrared spectroscopy has identified that it is an ideal eutectic solvent. Compared to other hydrophobic mixtures, this one represents an advanced solvent system due to its intrinsic antioxidant activity, which makes it an ideal green choice for the extraction and preservation of easily oxidizable lipophilic compounds. In order to evaluate its efficiency, it was applied for the first time as an extractant for the dispersive liquid−liquid microextraction of carotenoids and fat-soluble vitamins from fruit juices, carried out at 298 K. All extracts were analyzed by high-performance liquid chromatography−tandem mass spectrometry. The developed method was then validated, providing precise (4−8%) and accurate (4−6%) results on a commercial fruit juice containing declared values of β-carotene and α-tocopherol acetate. Recoveries were ≥70%, while the detection limits were 0.05 μg L^{-1} for β-carotene and 0.28 μg L^{-1} for α-tocopherol acetate. Here, we also propose an original approach to properly determine the antioxidant activity of eutectic mixtures since the available commercial kits cannot be applied to such systems without altering their nature. Last but not least, a circular process of reuse of this ideal eutectic solvent has been developed using a low-cost activated carbon material obtained from coconut shells.

KEYWORDS: *deep eutectic solvents, dispersive liquid−liquid microextraction, vitamins, carotenoids, antioxidant activity, menthol, butylated hydroxytoluene, recycling*

INTRODUCTION

The ongoing research on deep eutectic solvents (DESs) has very recently led to the identification of a new class of solvents defined as type V DESs.[1] These are hydrophobic mixtures based on non-ionic species containing phenolic and aliphatic hydroxyl groups, such as the terpenoids thymol and menthol.[1] Thorough investigations of their physicochemical properties have unraveled that many menthol-based systems are actually ideal or quasi-ideal eutectic mixtures, exhibiting no significant depression of their melting point depending on the nature of the intermolecular forces in the mixture.[2] The rapid evolution of this research area and the confusion in the use of terms urgently require a distinction between ideal and non-ideal eutectic mixtures. In this regard, we think that "IESs" is the most appropriate acronym to identify ideal eutectic solvents and to distinguish them from non-ideal "DESs"; then, "ESs" (eutectic solvents) is the generic term suitable to include both families.

From an analytical point of view, all hydrophobic ESs (IESs and type V DESs) are of great interest when being liquid at room temperature. In fact, like ionic DESs which belong to the types I−IV, they can be prepared from natural/innocuous components, representing a green alternative to the conventional organic solvents. Nevertheless, hydrophobic ESs are more advantageous for their low viscosity and for being completely immiscible and more stable in water, especially when compared with the type III "hydrophilic"[3,4] and "quasi-hydrophobic"[5,6] choline chloride-based DESs, which currently are the most used ones for extraction purposes.[7] In particular, hydrophobic ESs are more valid replacements of halogenated solvents in the dispersive liquid−liquid microextraction (DLLME) of low-polar compounds from aqueous samples.[8] The further benefit deriving from their application is the possibility to make DLLME an even greener procedure by

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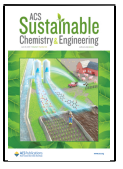

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either selecting an eco-friendly dispersing solvent, such as ethanol, or completely avoiding its use. In the latter case, the DLLME is reduced to a biphasic process.

Another great advantage of ESs over molecular solvents is their outstanding formulation flexibility, which allows one to design them through the reasoned selection of starting components, for example, to impart antioxidant properties to them. At present, the literature only describes few examples of hydrophilic ESs with an intrinsic antioxidant activity and their application for the extraction of quite polar analytes such as polyphenols.[9−11] On the other hand, no hydrophobic ESs with analogous capability have been reported so far, even if there are many lipophilic compounds sensitive to the combined action of light and oxygen. Among them, fat-soluble vitamins and carotenoids arouse deep interest due to the role they play in numerous metabolic pathways and in the antioxidant defense system against oxidative stress.[12] It is known that fruits, vegetables, and milk are rich sources of such micronutrients,[13−17] but their accurate determination is challenging also for the abovementioned reasons.[18,19] Particularly tricky is the sample preparation step that should be as fast as possible and should provide adequate protection against photooxidation. This is typically achieved by applying conventional extraction procedures based on the use of antioxidant additives such as butylated hydroxytoluene (BHT) in non-polar solvents (such as hexane, chloroform, petroleum ether, *etc.*), mostly at 0.01−0.1% w/v;[19,20] usually, such methods involve the employment of tens of milliliters of solvent. Considering the great importance assumed by green chemistry, nowadays, an analytical method should be evaluated also in terms of sustainability and not just for its cheapness, speediness, and figures of merit.[21] From this perspective, liquid microextraction techniques possess all these characteristics and appear suitable for vitamin analysis,[22] even if only few studies are present in the literature.[23−25] Among these, just two sustainable methods, combining DLLME with the use of an hydrophobic DES, were developed to isolate carotenoids from fruit juices.[24,25] In both studies, long-chain fatty compounds lacking in antioxidant activity were used as extracting solvents. In detail, Li *et al.*[24] recovered β-carotene and lycopene with a DES composed of three fatty acids (C_9, C_{10}, and C_{11} in a molar ratio of 2:1:1); the mixture was dispersed into the aqueous sample through the addition of an ammonia solution, acting as an emulsifier; the phase separation was then induced with hydrochloric acid used as a de-emulsifier. Finally, the extract was diluted with methanol and analyzed with HPLC-UV. On the other hand, Sricharoen *et al.*[25] used 1-dodecanol for the air-assisted, low-density solvent-based liquid−liquid microextraction and solidified floating organic droplets (AA-LDS-LLME-SFOD) of β-carotene, which was determined by microscale UV−vis spectrophotometry.

In the present work, for the first time, a sustainable ES with hydrophobic, antioxidant, low-viscosity, and vapor pressure characteristics was composed not only for extraction purposes but also as a medium to preserve lipophilic compounds susceptible to oxidation. To this end, L-menthol and BHT were blended in a molar ratio of 3:1 [henceforth referred to as MEN:BHT (3:1) for conciseness], and the resulting mixture was characterized by means of differential scanning calorimetry (DSC) and infrared spectroscopy (IR). An original approach was also conceived to study its antioxidant efficiency since the available commercial kits are inapplicable to such systems. MEN:BHT (3:1) was then applied as an extractant for the fast DLLME of carotenoids and fat-soluble vitamins from fruit juices, using high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) to analyze the extracts. The reliability of the validated DLLME−HPLC−MS/MS method was assessed by analyzing a commercial fruit juice containing declared values of β-carotene and α-tocopherol acetate and some not-fortified fruit juices for which the endogenous β-carotene content was unknown. Finally, an approach to clean up the ES after an extraction process and reuse it for further applications was developed employing a low-priced sorbent material.

MATERIALS AND METHODS

Chemicals. L-menthol (natural source, food grade, ≥99% purity) and BHT (food grade, ≥99% purity) and analytical standards of β-carotene and α-tocopherol acetate were purchased from Sigma Aldrich/Merck (Milan, Italy). RS-grade (elevated purity grade) solvents (absolute ethanol, 2-propanol, hexane, and methanol) were also obtained from the same supplier. Ultrapure water was produced with a Milli-Q Plus apparatus (Millipore, Bedford, MA, U.S.A.).

Standard Solutions. Stock solutions at 1 mg mL^{-1} were prepared by dissolving weighted amounts of the analytical standards (Ohaus DV215CD Discovery semi-micro and analytical balance, 81/210 g capacity, 0.01/0.1 mg readability) in methanol (α-tocopherol acetate) or chloroform (β-carotene), both solvents containing 0.1% (w/v) BHT. The mixing standard solution used for validation was prepared from stock solutions by diluting in methanol to obtain intermediate concentrations (6.64 ng μL^{-1} and 41.76 ng μL^{-1}) and final concentrations (0.133 and 0.835 ng μL^{-1}) for β-carotene and α-tocopherol acetate, respectively. All solutions were stored in amber glassware at 255 K.

Fruit Juice Samples. Industrial fruit juices of apricot (minimum fruit content 40%) and pineapple and carrot (both 100% from the concentrate) and a multivitamin ACE were purchased from a local supermarket (Rome, Italy). All juices were sold in a Tetra Pak packaging, except for the carrot juice that was contained in a non-amber glass bottle. The optimization and validation processes were conducted using the multivitamin ACE juice since it was supplied with the β-carotene and α-tocopherol acetate concentrations reported on the label.

Preparation of the Eutectic Solvent. L-menthol and BHT were weighted according to the molar ratio of 3:1 (*e.g.*, 4.0000 g of L-menthol and 1.8801 g of BHT) into the same glass test tube. The melting occurs just by mixing the solid components at room temperature, but for rapidity, the mixture was heated to 323 K in a water bath under magnetic stirring until it became liquid. Below 293 K, the ES completely solidifies; therefore, when working in cold environments, it is suggested to keep the solvent in a warm bath.

Extraction Procedure. According to the best final conditions (Figure 1), DLLME was carried out by mixing in a centrifuge tube 150 μL of the ES with 1850 μL of absolute ethanol. The 2 mL mixture was vortex-homogenized for 30 s and quickly injected into another centrifuge tube containing 5 mL of the aqueous 100-fold diluted sample. Immediately, a cloudy solution was formed; the emulsion was first vortexed for 2 min and then centrifuged at room temperature and 9000 rpm for 10 min. Thereafter, a phase separation occurred, the upper layer being the ES. The extract was withdrawn with a syringe, recovering 120 μL, 5 μL of which was directly injected into the HPLC−MS/MS system. All operations were conducted in subdued light, with centrifuge tubes enwrapped with aluminum foil.

Antioxidant Activity Evaluation. The antioxidant properties of the ES were tested by analyzing a 0.4 mg mL^{-1} β-carotene standard solution in MEN:BHT (3:1) before and after its exposure to UV-A LED light (light-emitting diode, 370 nm, NS370L-5RLO, 3.0 mW at 20 mA, 15°, 5 mm clear UV-resistant epoxy; from Roithner LASER Tecnik, Vienna, Austria). For comparison, β-carotene standard solutions at the same concentration were also prepared in 2-propanol and in 2-propanol containing 0.1% (w/v) BHT. The samples were

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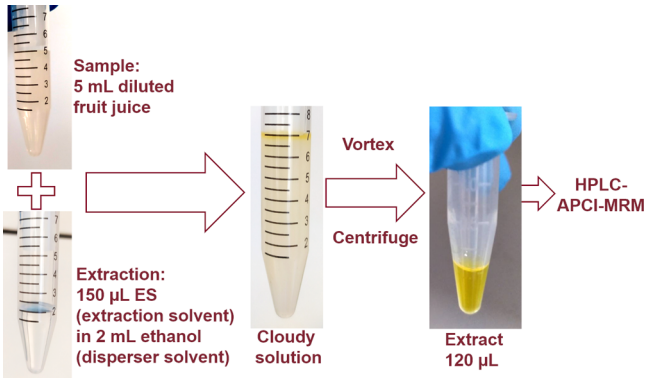

Figure 1. Scheme of the DLLME final procedure developed on a multivitamin ACE juice.

kept in a dark oven at 311 K, and the only source of radiation they received was the LED light (Figure 2). The LED was connected to a

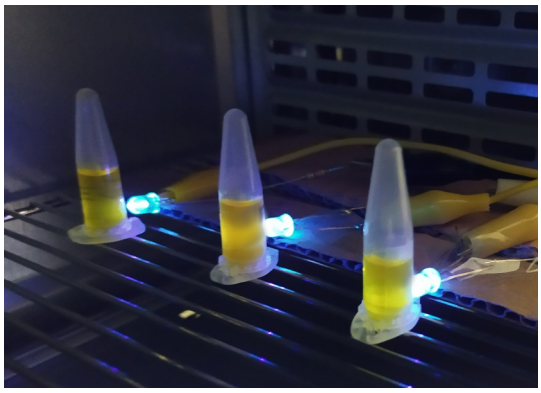

Figure 2. Experimental setup for the photodegradation of β-carotene solutions under UV-A LED light irradiation (370 nm) at 311 K.

laboratory-made electrical device (DC generator, batteries connected in series to apply a voltage of 3.3−3.6 V; electrical resistance of 14 Ω; and 20 mA). From each sample, an aliquot of 100 μL was withdrawn at time intervals of 1, 2, 4, and 15 h of exposure to 370 nm, diluted with 20 μL of solvent (2-propanol for the 2-propanol solutions and ethanol for the ES), vortexed, and directly injected (5 μL) into the liquid chromatography−mass spectrometry system.

The antioxidant properties of the different solvent systems were evaluated according to eq 1

$$\text{residual area}_{\beta\text{-carotene}}\ (\%) = \frac{\text{peak area after a certain exposure time}}{\text{peak area before exposure}} \times 100 \quad (1)$$

Differential Scanning Calorimetry. DSC curves of the eutectic mixture were acquired using a Mettler Toledo DSC 822e differential calorimeter (Mettler Toledo, Greifensee, Switzerland). A sample mass of about 4 mg was weighted in a 40 μL aluminum pan and sealed. The temperature program comprises a cooling ramp from 323 to 203 K at −10 K min^{-1}, followed by a heating ramp from 203 to 323 K at 10 K min^{-1}. Dry nitrogen was used for purging the furnace during the measurements with a flow rate of 30 mL min^{-1}.

Fourier Transform Infrared Spectroscopy. Fourier Transform Infrared (FT-IR) spectra were recorded in attenuated total reflectance (ATR) mode using a Nicolet 6700 spectrometer (Thermo Scientific, Waltham, USA) equipped with a Specac Golden Diamond ATR accessory. The spectra were acquired in the 4000−650 cm^{-1} range, by co-adding 50 scans at a resolution of 4 cm^{-1}. Pristine BHT and the eutectic mixture were analyzed at room temperature. The spectrum of liquid L-menthol was obtained by melting the compound directly on the ATR crystal at about 313 K.

High-Performance Liquid Chromatography−Tandem Mass Spectrometry. Liquid chromatography was performed as described in our previous work,[26] using a micro-HPLC series 200 (PerkinElmer, Norwalk, CT, U.S.A.) equipped with a vacuum degasser, an autosampler, and a column chiller. Briefly, the chromatographic separation of the analytes was performed on a ProntoSIL C30 column (4.6 × 250 mm, 3 μm) from Bischoff Chromatography (Leonberg, Germany), combined with a guard C30 column (4.0 × 10 mm, 5 μm), under non-aqueous reversed-phase conditions at 292 K. The mobile phases were methanol (phase A) and 2-propanol/hexane (50:50, v/v; phase B, also used as an injector-washing phase), with the following gradient profile: 0−1 min, 0% B; 1−15 min, 0−75% B; 15−15.1 min, 75−99.5% B; and 15.1−30.1 min, 99.5% B. The flow rate was 1 mL min^{-1} and was entirely introduced into a 4000 Qtrap (AB SCIEX, Foster City, CA, U.S.A.) mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) probe on a Turbo V source. The analytes were detected in the positive ion mode, setting a needle current of 3 μA and a probe temperature of 723 K. High-purity nitrogen was used as the curtain (40 psi) and collision (4 mTorr) gas, whereas air was used as the nebulizer (55 psi) and make-up (30 psi) gas. The calibration of the Q1 and Q3 mass analyzers was performed by infusing a polypropylene glycol solution at 10 μL min^{-1}. In each mass-resolving quadrupole, a full width at half maximum of 0.7 ± 0.1 unit was established, corresponding to a unit mass resolution. Data were acquired according to a targeted approach, using the multiple-reaction monitoring (MRM) mode and by selecting two MRM transitions per analyte. The quantitative analysis was conducted by considering the peak area of the most intense transition (quantifier). The qualitative analysis was based on the simultaneous application of these criteria for each analyte: the retention time matching and the presence of two characteristic transitions (qualifier and quantifier) in the expected ion ratio (see Table S1 in the Supporting Information). Data acquisition and procession were managed using Analyst 1.6.2 software (AB Sciex). A very low-sample speed injection was set for the autosampler, in order to improve the injection volume repeatability when dealing with a solvent of higher-than-usual viscosity. Before injecting the fruit juice extracts, a procedural blank was run to verify the absence of interfering signals from the solvent system.

Method Validation. The method validation was conducted in a matrix according to the main FDA guidelines for bioanalytical methods,[27] using a multivitamin ACE juice whose β-carotene (160 μg 100 mL^{-1}) and α-tocopherol acetate (2.4 mg 100 mL^{-1}) concentrations were declared by the manufacturer.

The quantitative analysis was conducted according to the standard addition method: seven diluted aliquots, except one, were spiked pre-extraction with increasing amounts of the standards (corresponding to the following spike levels: 0, 40, 53, 80, 133, 213, and 399 μg L^{-1} for β-carotene and 0, 250, 330, 500, 840, 1340, and 2500 μg L^{-1} for α-tocopherol acetate); thereafter, all the calibrators were adjusted to the same final volume through ethanol addition. For each analyte, the areas were plotted versus the fortification level (μg L^{-1}). The endogenous concentrations were obtained from the calibration curves by extrapolating the x value for y = 0. The calibration curves were constructed based on extractions replicated six times.

The accuracy was calculated as the mean of five replicates, according to the following equation

$$\text{accuracy } \% = \frac{\text{concentration}_{\text{measured}} - \text{concentration}_{\text{declared}}}{\text{concentration}_{\text{declared}}} \times 100 \quad (2)$$

The recoveries were calculated on five replicates, spiked before the extraction with a solution of both analytes at levels twice as high as their endogenous concentrations. Another sample was spiked post-extraction with the same amount of standards, while another one was extracted without applying any fortification. After adjusting the final volume with ethanol (V_f = 150 μL), each extract was vortex-mixed

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before being injected into the HPLC−MS/MS system. The recovery was calculated according to eq 3

$$R\ \% = \frac{\text{area}_{\text{pre-extraction spiked sample}} - \text{area}_{\text{unspiked sample}}}{\text{area}_{\text{post-extraction spiked sample}} - \text{area}_{\text{unspiked sample}}} \times 100 \quad (3)$$

The intra- and inter-day precisions were expressed as relative standard deviation (RSD) of the mean recovery obtained from five replicates performed within the same day or 2 weeks, respectively.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as those concentrations which exceed three and 10 times the noise level, respectively (five replicates).

Linear regression analysis, determination coefficients, means, and standard deviations were calculated with Microsoft Excel 2010.

RESULTS AND DISCUSSION

Preparation of the Antioxidant Eutectic Solvent. An important condition for the practical use of an ES in analytical applications is its liquid status at room temperature. Considering that menthol is a crystalline solid with different melting temperatures as a function of both the enantiomeric forms (L, D, or the racemic mixture) and the polymorphic phase (the α and β being the most stable of the four known polymorphs), several eutectic mixtures with BHT can be predicted from the solid−liquid equilibrium phase diagrams, however often only in an approximate way.[28] On this basis, in this work, different mixtures of BHT with DL-, L-, or D-menthol were prepared at different molar ratios (1:1, 2:1, 3:1, 4:1, and 5:1). Finally, the L-MEN:BHT (3:1) mixture was selected: (i) for the low eutectic temperature, which results in its use as a solvent possible at room temperature; (ii) for the greatest BHT molar fraction, which guarantees maximum antioxidant protection; and (iii) for being composed of the natural enantiomer L-menthol. Finally, it has been verified that the prepared solvent can be used for months without losing efficiency if, when unused, it is protected from light and oxygen in a closed screw-cap tube enwrapped with aluminum foil.

Characterization of a Pure ES: DSC and FT-IR Spectroscopy. The DSC traces of MEN:BHT (3:1) are shown in Figure 3a. During the cooling ramp at 10 K min^{-1} from 323 to 203 K, the crystallization of the ES occurs at about 273 K. During the heating stage at 10 K min^{-1}, the glass transition temperature of the amorphous fraction is observed at T_g = 220 K. Subsequently, a first partial melting occurs in a wide temperature range, starting from about 258 K up to T_{m1} = 285 K. The appearance of an exothermic peak at T_c = 292 K is likely ascribed to a crystalline reorganization (possibly related to the L-menthol polymorphic interconversion), followed by the final melting at T_{m2} = 298 K. This temperature approaches the eutectic one (T_e ideal = 295 K, considering the L-menthol α polymorph),[1] and it is obviously lower than the individual melting temperatures of the pure compounds (343.2 K for BHT and 316.1 K for α L-menthol; Figure S1). The calorimetric results suggest that the liquid state of MEN:BHT (3:1) is thermodynamically stable at room temperature and above that (≥298 K). However, this eutectic mixture persists in the liquid phase even in the range 293−298 K. This phenomenon can be due to the kinetic hindrance during crystallization of the eutectic systems containing L-menthol,[29] even if the role of the sterically hindered BHT in the delay of L-menthol crystalline reorganization cannot be excluded.

In order to study the intermolecular interactions among −OH groups in the eutectic mixture, a comparison of the ATR FT-IR spectra of the MEN:BHT (3:1) mixture, liquid L-menthol, and crystalline BHT is shown in Figure 3b. The phenolic stretching of crystalline BHT is observed at high wavenumbers (3618 cm^{-1}) as a sharp band, and this feature is a characteristic of weak or negligible H-bonds in the crystal structure. On the other hand, the −OH stretching of liquid L-menthol appears as a broad band centered at 3347 cm^{-1}, suggesting the formation of H-bonds among the L-menthol −OH groups. In the vibrational spectrum of the eutectic mixture, the L-menthol OH stretching band remains unchanged at 3347 cm^{-1}, whereas the BHT phenolic stretching is blue-shifted at 3654 cm^{-1}, indicating the loss of the weak interactions observed in the crystalline structure. In the lower wavenumber region (1600−650 cm^{-1}), the IR spectrum of the eutectic mixture is characterized by the superimposition of the spectral features of both pure L-menthol and BHT.

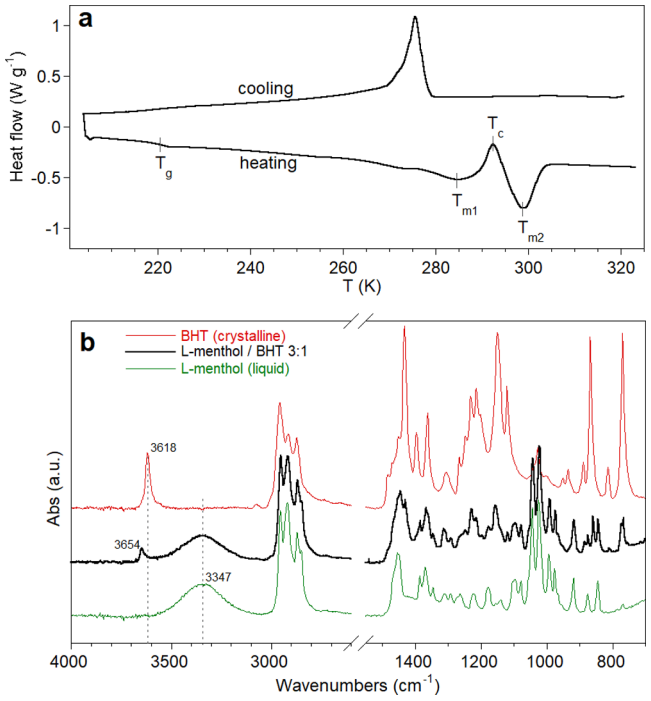

Figure 3. (a) DSC curves of the L-menthol/BHT 3:1 mixture recorded at 10 K min^{-1} heating/cooling rate. (b) ATR−FTIR spectra of the eutectic mixture (L-menthol/BHT 3:1, black line) and its precursors (BHT crystals, red line; L-menthol liquid, green line).

The overall results indicate that the mixture of L-menthol and BHT can be considered as an IES because of the negligible formation of H-bonds between the two components. This is imputable to the high steric hindrance around the BHT phenolic group that prevents the formation of a type V DES.[1]

Antioxidant Activity. Most of the developed assays to measure antioxidant activity are spectrophotometer-based. In these cases, the changes in absorbance of a suitable chromophore, such as the radicals 2,2′-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$)[30] or 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS$^{\bullet+}$),[31] are evaluated in the presence of an antioxidant acting as a scavenger. These methods obey the Beer−Lambert law; therefore, the maximum concentration of the chromophore solution is limited by the related absorbance, whose value should be lower than 1.0. Moreover, the molar ratio between the analyzed antioxidant and the chromophore should be chosen, so that the kinetics of the single-electron transfer or hydrogen atom transfer reactions are neither too

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fast nor too slow. As a consequence, samples with too high antioxidant activities should be diluted in a suitable organic solvent. However, when dealing with ESs, too high dilution factors would alter the nature of these systems, giving rise to distorted results. In the specific case, the analyzed system would become a solution of BHT in a traditional solvent. In order to overcome this issue, the antioxidant activity of MEN:BHT (3:1) was evaluated by means of the direct HPLC−MS/MS determination of β-carotene, as described in the paragraph "Antioxidant Activity Evaluation". It is worth highlighting that the same approach can be applied to test the antioxidant activity of hydrophilic ESs, by dosing a photosensitive hydrophilic compound such as B_2 vitamin instead of β-carotene.

The superior antioxidant properties of MEN:BHT (3:1) are evident from the comparison of the residual β-carotene areas in the different solvent systems as a function of the UV-A light exposure period (see Figure S2). In 2-propanol without BHT, the full degradation of β-carotene occurs within 4 h of exposure. On the other hand, considering the same exposure time, the protection against the photo-oxidation of β-carotene is ensured by both MEN:BHT (3:1) and 2-propanol containing 0.1% (w/v) BHT since its residual chromatographic areas are 100% for both the systems. However, after 15 h of exposure, the β-carotene residual area drops to 1% in 2-propanol with 0.1% (w/v) BHT, but it is 85% in MEN:BHT (3:1) due to its higher content of BHT. Such a decrease can be considered negligible, falling within a permissible error of 20%. This outcome has another important practical implication: besides preserving analytes susceptible to photo-oxidation during extraction, such a solvent medium can protect extracts more efficiently and for a longer time than other conventional solvents containing BHT in the following steps of an analysis. This is particularly important when a large number of samples have to be analyzed and their extracts are kept in an autosampler waiting for the injection.

This experiment also proves that this IES retains unaltered the BHT antioxidant activity, just because of the marginal involvement of its OH group in the formation of the ES. In fact, it is known that the antioxidant power of a phenolic compound depends on the H lability of its OH group (which can be easily abstracted by a free radical) but, above all, on the stability and reactivity of the generated phenoxyl radical. In BHT, the phenoxyl radical is stabilized by inductive, resonance, and steric factors especially thanks to the electron-donating *tert*-butyl substituents on the 2- and 4-positions of the phenolic ring.[32] Thus, the greater the steric hindrance of a phenol, the better its antioxidant activity but the less its ability to form a hydrogen bond and, consequently, a type V DES, as experimentally verified in this work. An opposite example is that of the hydrophobic mixture composed by menthol and thymol: the latter is much less hindered than BHT, so it can better interact with menthol via H-bonds, giving rise to a type V DES.[1] Therefore, the concept entertained by Abranches *et al.* of a type V DES retaining the antioxidant properties of BHT is obviously unsound.[1] From an applicative point of view, MEN:BHT (3:1) represents an efficient medium to dissolve and extract low-polar oxidizable compounds but also a workable solution to preserve such compounds longer than other conventional solvents and type V DESs.

DLLME Optimization. The DLLME extraction procedure was optimized to maximize the analyte chromatographic peak areas of β-carotene and α-tocopherol acetate, using a multivitamin ACE juice. Due to the high concentrations of the studied micronutrients, 5 mL of diluted samples and a 2 mL solvent system, composed of 150 μL of extractant and 1850 μL of dispersant, were employed for the several experiments.

The first tests were aimed at establishing the correct sample dilution factor. To this end, three samples were prepared at the dilution factors 20, 50, and 100, obtained by diluting 500 μL of multivitamin ACE juice to 10, 25, and 50 mL with ultrapure water, respectively. Figure 4 illustrates the obtained results: at

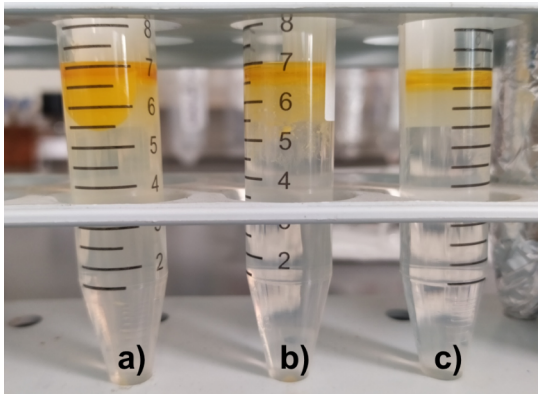

Figure 4. Effect of different sample dilutions on phase separation: (a) 20-fold dilution; (b) 50-fold dilution; and (c) 100-fold dilution.

low dilution factors (a and b in Figure 4), the presence of the floating material from the matrix prevented a sufficient volume of clean extract from being taken, while a clear phase separation was obtained when a 100-dilution factor was applied (c in Figure 4). In the latter case, the dilution factor allows one to make the upper phase withdrawal easier and to obtain high signals of the analytes.

Thereafter, the effect of the dispersing solvent was evaluated by carrying out DLLME with 150 μL of MEN:BHT (3:1) with the addition of different ethanol volumes (0, 350, 850, and 1850 μL to have a total volume of 150, 500, 1000, and 2000 μL, respectively). The use of ethanol as the dispersing solvent has significant influence neither on the obtained areas (Figure 5a) nor on the recovered extract volume (120 μL), but it is a convenient way of avoiding the extract solidification when the temperature falls below 293 K. Therefore, an ethanol volume of 1850 μL has been selected for the DLLME procedure. The choice of ethanol has been dictated by the fact that it is an eco-friendly solvent and is mixable with both MEN:BHT (3:1) and water.

Finally, higher (200 μL) and lower (100 μL) volumes of extracting solvent were evaluated (Figure 5b), keeping the dispersing solvent volume unchanged. A higher volume led to decreased chromatographic areas because of the lower enrichment factor achieved in the final extract. A lower volume was not able to improve the areas, probably because of a slight matrix effect induced by a greater concentration of the extract. Therefore, the volume of extracting solvent was kept at 150 μL.

Analytical Method Validation and Quantitative Analysis Results. The method was validated according to the main FDA guidelines of bioanalytical methods[27] using a commercial multivitamin ACE juice, whose concentrations of β-carotene and α-tocopherol acetate were declared on the label. The estimated figures of merit are listed in Tables 1−4.

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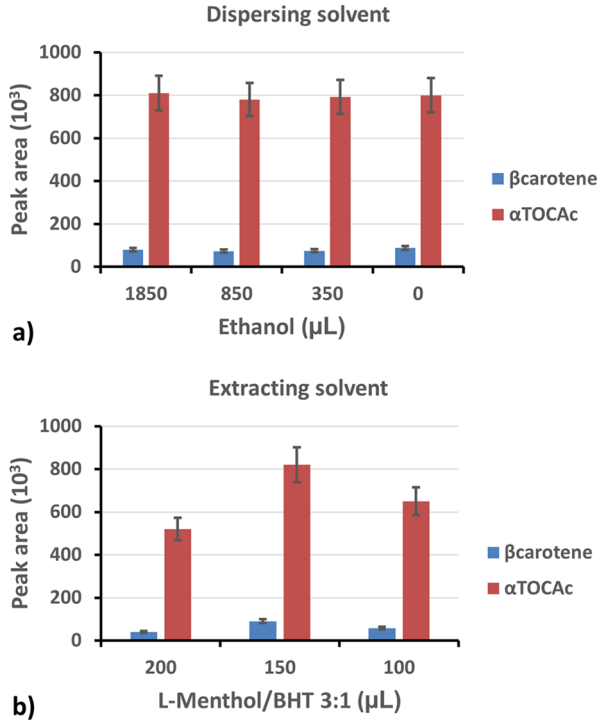

Figure 5. (a) Effect of ethanol as a dispersing solvent on mean areas (n = 5) using L-menthol/BHT 3:1 as an extracting solvent (150 μL). (b) Effect of different volumes of extracting solvent on mean areas (n = 5) using ethanol as a dispersing solvent (1850 μL).

Looking at Table 1, it can be seen that mean absolute recoveries are greater than 70% with RSD values below 5%, representative of an excellent intra-day precision. For both analytes, the inter-day precision was high as well, RSD being below 8%. Concerning LODs and LOQs, they were in the low μg μL^{-1} range.

Table 2 shows the calibration data in the studied dynamic range, the error associated to the slope (b) and intercept (a), which were estimated by means of the least-square method, and the determination coefficients, which were greater than 0.996.

In Table 3, the analysis results of a real sample and the estimated accuracy are reported. The analyte concentrations, extrapolated by the standard addition method, are in compliance with those declared by the manufacturer with considerable accuracy, its figure of merit being lower than 6%; actually, as guaranteed by the Tetra Pak packaging, the juice had efficiently been protected from the light since its production.

The developed method was also applied for the analysis of the endogenous β-carotene content in other fruit juices: 100% pineapple, 100% carrot, and 40% apricot. The measured concentrations in the respective samples (≤LOD, 24.80, 1.84 mg L^{-1}) reflect the β-carotene levels occurring in the different natural sources in accordance with the literature:[33] carrots are the richest source of β-carotene; compared with carrots, pineapple juice contains negligible β-carotene levels (nearly 0.3%), while those in apricot juice reach nearly 21%.

The qualitative analysis of the extracts also highlighted the presence, especially in the carrot juice, of other carotenoids and fat-soluble vitamins, such as phytoene, phytofluene, and α-tocopherol; lutein, phylloquinone, γ-tocopherol, and α-tocotrienol were detected as well even if to a lesser extent. Figure S3 represents a typical LC−MS/MS chromatogram of a carrot juice sample, extracted with the proposed method.

Comparison with Other DLLME Methods from the Literature. The main qualitative and quantitative parameters (recovery, precision, LOD, type and volume of organic solvents, and analysis time) of the extraction procedure, described here, were compared with those of the previous DLLME methods developed to extract β-carotene from fruit juice samples (see Table 4).[23−25] Actually, all selected methods show similar figures of merit with slight differences. Obviously, those based on MS detection (ref 23 and this work) exhibit lower and very similar LODs. As far as the recovery is concerned, our method has a yield not as high as that of the ABLLME[24] and AA-LDS-LLME-SFOD[25] methods; however, the comparison is difficult to draw since the authors did not provide the approach applied for the calculation. All methods are very fast, lasting 10−20 min per sample, and also have comparable precision values. Depending on the vortex and centrifuge capacity, our method makes feasible the simultaneous extraction of multiple samples in 15 min, the vortex and centrifuge being the time-limiting steps. The real difference among these methods concerns their sustainability and cheapness on a large scale. Finally, our method stands out for the antioxidant properties of the extractant, which make it an ideal system to preserve photo-oxidizable vitamins from the extractive step to the instrumental analysis.

Sustainability and Life Cycle Considerations. The preparation and application of MEN:BHT (3:1) is advantageous from both an economic and sustainable perspective. Indeed, both starting components are cheap and the IES production can also be made at room temperature without involving a heating source; moreover, if the preparation is on an analytical scale, manual agitation is sufficient. In addition, this eutectic mixture is safe to handle and has a low environmental impact due to the characteristics of the individual components. In fact, BHT is extensively used as a non-staining antioxidant additive by food, cosmetic, and pharmaceutical industry thanks to its low acute toxicity (oral LD_{50} values of 1700−1970 mg BHT/kg bw in rats) and slow passage through the skin (when there is a dermal contact, the majority of BHT remains on the cutaneous surface).[34] It is produced synthetically through an acid-catalyzed two-step process and is commercially available at low cost (3−12 € per kg); however, potential alternatives for the BHT production are some freshwater algae[35] and fungal strains[36] that have

Table 1. Validation Parameters on the Multivitamin ACE Juice

analyte	recovery[a] (%)	intra-day precision (RSD %)[b]	inter-day precision (RSD %)[b]	LOD[c] (μg L^{-1})	LOQ[c] (μg L^{-1})
β-carotene	72	5	8	0.05 ± 0.01	0.16 ± 0.02
α-tocopherol acetate	70	4	6	0.28 ± 0.02	0.93 ± 0.03

[a]Mean of five independent analyses. [b]RSD % of five independent analyses performed within the same day or within two weeks (intra-day precision; inter-day precision). [c]Mean and SD of five independent analyses.

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Table 2. Method Calibration Data

analyte	linear dynamic range ($\mu g\ L^{-1}$)	calibration curves[a]		
		$b \pm S_b t_{(0.05;6)}$	$a \pm S_a t_{(0.05;6)}$	R^2
β-carotene	40−400	$48.5 \times 10^{-3} \pm 1.1 \times 10^{-3}$	83.6 ± 2.2	0.9971
α-tocopherol acetate	250−2500	$44.7 \times 10^{-3} \pm 0.9 \times 10^{-3}$	1007 ± 10	0.9965

[a]Mean of six independent analyses.

Table 3. Analysis Results of the Multivitamin ACE Juice and the Accuracy Evaluation

analyte	declared value (for 100 mL)	extrapolated value[a] (for 100 mL)	accuracy[a] (%)
β-carotene	160 μg	170 ± 8 μg	6
α-tocopherol acetate	2.4 mg	2.3 ± 0.1 mg	4

[a]Mean of six independent analyses.

recently been identified as natural sources of this molecule. L-menthol is extracted from corn mint (*Mentha arvensis*) and is also synthesized artificially to meet the high demand (20,000 metric tons/year) of food, cosmetic, and pharmaceutical industry; in fact, it is used as a flavor ingredient and for its counterirritant effect on skin and mucous membranes. It is considered very safe for human beings and eco-friendly because it is readily degradable in aquatic environments.[37] It is low priced with a cost of around 12 € per kg.

The recycling potential of this IES was evaluated by cleaning the DLLME extracts *via* dispersive solid phase extraction (d-SPE), followed by centrifugation or filtration. As a sorbent, a low-cost carbonaceous material derived from coconut shells was used. Comparing the chromatographic areas of β-carotene and α-tocopherol acetate before and after the d-SPE treatment, the removal percentage of the analytes was 98.8%; moreover, the ethanol in the cleaned extract could be easily removed by evaporation under nitrogen flow. In conclusion, this approach is very effective in cleaning up the IES and allows one to reuse it for more extraction cycles. It is also suitable for other employments such as the removal of organic contaminants from water, as emerged from preliminary experiments carried out on an analytical scale.

CONCLUSIONS

A new sustainable IES based on L-menthol and BHT has been experimentally described for the first time. Its peculiar characteristics, such as the combination of high hydrophobicity with intrinsic antioxidant activity, represent a novelty in the literature and an advanced solvent with respect to type V DESs. Moreover, the potential of this IES has successfully been exploited for the development of a sustainable analytical method for the fat-soluble micronutrient determination in fruit juices. The results have shown that this solvent system, liquid at room temperature, is a perfect candidate for the substitution of hazardous organic solvents, such as hexane added with 0.1% (w/v) BHT, traditionally used for the liquid−liquid extraction of fat-soluble easily oxidizable compounds. The application of this neoteric solvent to the microextractive technique DLLME makes this procedure even more eco-friendly. Compared to other green methods, this one is advantageous for the high antioxidant protection, which allows the extracts to be conserved for a long time before being analyzed. The MEN:BHT (3:1) mixture also has a low vapor tension that avoids problems of concentration variation due to solvent evaporation, a serious drawback when hexane is used. Finally, a strategy to reuse the IES was successfully applied, thanks to its clean-up with an inexpensive carbonaceous sorbent derived from vegetable wastes. Last but not least, for the first time, an antioxidant assay for the proper determination of the intrinsic antioxidant activity of ESs has been proposed.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c01473.

LC−MRM parameters used for the compound identification; DSC traces of pure L-menthol and BHT; photodegradation of β-carotene in different solvents as a function of exposure time to UV-A light (370 nm); and LC−MRM profiles of the main fat-soluble micronutrients in commercial carrot juice (PDF)

Table 4. Comparison of the Main Merit Figures of Some Recent Methods Aimed at the Extraction of β-carotene from Fruit Juices

method (common analytes)	recovery %	repeatability %	LOD ($\mu g\ L^{-1}$)	type and volume of solvents	extraction time (min)	reference
DLLME−LC−MS/MS	not provided	8.6	0.03	Extr[a]: CCl_4; 150 μL, Disp[b]: methanol; 1850 μL	∼20, including the evaporation and reconstitution steps of the final extract	23
ABLLME[c]−LC−UV/Vis	96−101.5	2.0−3.7	2	Extr[a]: three fatty acids C9:C10:C11 (2:1:1); 600 μL, emulsifier: $NH_3 \cdot H_2O$ 2.2 M; 400 μL, demulsifier: HCl 2.5 M	∼10	24
AA-LDS-LLME-SFOD-UV/Vis spectrophotometry[d]	93.6−101.5	4.85	40	Extr[a]: 1-dodecanol; diluting solvent: THF; 1 mL	∼15	25
DLLME−HPLC−APCI(+)-MS/MS	72.0	5	0.05	Extr[a]: MEN:BHT (3:1); 150 μL, Disp[b]: ethanol; 1850 μL	∼15[e]	this work

[a]Extr: extraction solvent. [b]Disp: dispersing solvent. [c]ABLLME: acid−base-induced DES liquid−liquid microextraction. [d]AA-LDS-LLME-SFOD: air-assisted, low-density, solvent-based liquid−liquid microextraction and solidified floating organic droplets. [e]For the simultaneous extraction of 6 samples.

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