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Safety assessment of citrus and olive by-products using a sustainable methodology based on natural deep eutectic solvents

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ABSTRACT

In this work, the application of betaine-based hydrophilic natural deep eutectic solvents (NADESs) as green extraction solvents was proposed for the first time for the evaluation of twelve pesticides in citrus and olive by-products intended to be applied as potential sources of compounds with neuroprotective activity against Alzheimer Disease. Ultrasound-assisted extraction of selected pesticides was followed by separation and determination using gas chromatography coupled to single quadrupole mass spectrometry. Eight NADESs were tested using different hydrogen bond donors (i.e. citric and lactic acid, fructose, glucose, glycerol, propylene glycol, propionic and butanoic acid). Other factors affecting extraction efficiency were also evaluated using a step-by-step approach. Eight mL of a mixture composed of 60% betaine:propylene glycol NADES at a molar ratio 1:4 and 40% of water, as well as 30 min of ultrasound-assisted extraction were selected as the most adequate conditions. The methodology was validated prior to its application in citrus and olive by-products. Recovery values were between 73 and 115% (RSD% < 20%), while limits of quantification of the method were in the range 8.5–128.8 µg/kg, which demonstrates the suitability of the procedure to determine the selected group of pesticides, usually applied in citrus and olive crops, at the legislated levels. The greenness of the procedure was also evaluated using AGREE calculator. Finally, the whole method was applied for the safety assessment of seven olive leaf samples and seven citrus by-products produced in Spain, finding the presence of several of the evaluated compounds at concentrations higher than the established limits for similar products.

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1. Introduction

The world population growth and the global market have brought about a sharp development of food industry. However, such rise also has derived in an indiscriminate use of natural resources and, consequently, in the generation of huge amounts of waste, as well as a lack of resources and an increase in people suffering hunger [1]. Indeed, a recent study estimates that nearly 2.37 billion people did not have access to adequate food in 2020 [2]. The strategies offered by circular economy constitute suitable tools to address this issue; increasing the life cycle of products by sharing, leasing, reusing, repairing, refurbishing, and recycling existing materials.

As for the food industry, the possibility of reducing the generation of wastes and the reuse (or revalorization) of agricultural by-products can push towards the achievement of the Sustainable Development Goals (SDGs). These premises constitute remarkable tools in fruit and vegetable industries since those are the fastest-growing agricultural sectors and, consequently, some of the highest producers of agro-waste [1,3]. Besides, by-products generated in these industries, such as seeds, peels, pomace or leaves, are a valuable source of numerous bioactive compounds including phenols, peptides, terpenoids, anthocyanins, and fatty acids, among others, that can be used for the prevention and treatment of several diseases or as nutritional ingredients in functional foods, for the preparation of cosmetics, etc. [1]. Particularly remarkable in the agricultural sector in Spain are the industries of citrus and olive. In fact, Spain is the first producer of olive oil around the world and the country with the highest olive farming surface, with more than 2.5 million hectares, while almost 300000 hectares are des-

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tinated to citrus growth, being the highest exporter of these fruits worldwide [4]. These productions generate a huge amount of by-products of interest due to the presence of bioactive compounds with potential health benefits such as their neuroprotective activity against Alzheimer Disease as it has been studied by our group [5–7]. However, almost no previous works can be found related to safety evaluation of olive and citrus by-products [8].

To the best of our knowledge, there is only one publication evaluating the presence of pesticides, commonly used in both kinds of crops, in olive leaves and no work has been carried out in citrus by-product so far [9]. Therefore, it is of utmost importance to develop new methodologies allowing the reliable safety assessment of these and other types of residues that can endanger consumer health in valorized by-products. In this sense, QuEChERS method has been mostly applied to the analysis of pesticides [10], although miniaturized sorbent-based procedures using nanomaterials and miniaturized solvents-based extraction have been also largely applied [11], based on Green Chemistry and Green Analytical Chemistry principles [12]. Moreover, the use of deep eutectic solvents (DESs) and, particularly, natural deep eutectic solvents (NADESs), as alternative extraction materials to the conventional and toxic organic solvents has gained great attention in the past few years [13], as a result of their easy and cheap synthesis, great versatility and low toxicity.

NADESs are exclusively constituted by secondary metabolites or other major compounds found in cells, which provide to these novel solvents outstanding biocompatibility and make them ideal agents for the extraction of organic compounds. Among the different components, the use of quaternary ammonium salts, such as choline chloride (ChCl), or betaine, as hydrogen bonds acceptors (HBAs) in combination with different alcohols, organic acids, carbohydrates, urea, etc., as hydrogen bonds donors (HBDs), has been frequently studied with good results. Although ChCl has been the most widely HBA used so far, also for pesticides analysis; it has been demonstrated that betaine-based NADESs have lower toxicity. In addition, the particular zwitterionic amphiphilic nature of betaine enhances the effectivity of the extraction process [14,15], which makes this kind of solvents interesting for the development of sustainable methodologies for determining and monitoring pesticides residues in valorized food by-products.

In this work, different betaine-based NADESs were evaluated for the first time as extraction solvents for the ultrasound-assisted extraction (UAE) of pesticides from olive and citrus by-products prior to gas chromatography-mass spectrometry (GC-MS) determination. A group of twelve pesticides (4 organophosphate pesticides, 3 organochlorinated, 1 chlorotriazine, 1 tiadiazine, 1 strobilurin, 1 pyrazole and 1 pyrethroid), commonly used in olive and citrus crops and during storage processes [16,17], was monitored in order to evaluate the safety of valorized by-products with neuroprotection potential against neurodegenerative diseases such as Alzheimer Disease. To the best of our knowledge, this is the first work in which the safety evaluation of citrus by-products has been carried out and the first time in which NADESs have been applied as extraction solvents for safety assessment of food by-products.

2. Experimental

2.1. Chemicals and materials

Analytical standards of chlorpyrifos (CAS 2921-88-2), chlorpyrifos methyl (CAS 5598-13-0), cyhalothrin-γ (CAS 91465-08-6), endosulfan α,β (CAS 115-29-7), endosulfan sulfate (CAS 1031-07-8), fenthion (CAS 55-38-9), malathion (CAS 121-75-5), terbuthylazine (CAS 5915-41-3), pyriproxyfen (CAS 95737-68-1), buprofezin (CAS 69327-76-0), trifloxystrobin (CAS 141517-21-7), and triphenyl phosphate (TPP) (CAS 115-86-6), with purity higher than 98%, were supplied by Sigma-Aldrich Chemie (Madrid, Spain) and Cymit Chimica (Barcelona, Spain).

Stock solutions, previously prepared in acetone at 1000 mg/L, were used for the preparation of daily working mixtures of pesticides by dilution using ethyl acetate/acetone (9/1; v/v). All solutions were stored in the darkness at -18 °C.

All chemicals were of analytical reagent grade (except in those cases specifically indicated) and used as received. Ethyl acetate and ethanol LC-MS grade, acetone HPLC grade, as well as cyclohexane and heptane GPR rectapur grade (purity >99%) and citric acid (>99.9%) were from VWR Chemicals (Leuven, Belgium). L-Lactic acid (>85%), 1,2-propylene glycol (PPG) (>99%), propanoic and butanoic acid (>99%) and betaine anhydrous (>97%) were from TCI (Tokio, Japan). Glycerol, fructose, glucose of reagent grade were from Sigma-Aldrich (Madrid, Spain). Deionized water was obtained from a Milli-Q system A10 (Millipore, Massachusetts, USA).

2.2. Apparatus and software

Synthesized NADESs were characterized by Fourier transformed infrared (FTIR). Spectra were obtained using a microscope IR Spotlight 200i (Perkin Elmer) and measuring the Attenuated Total Reflectance (ATR) with diamond crystal in the range of medium IR (4000–450 cm^{-1}) applying 20 scans, aperture of 8.94 mm and resolution of 4 cm^{-1}. Moreover, viscosity studies were carried out using a Viscosimeter VSM 3000 Stabinger (Anton Paar® GmbH) at 25 °C. The viscosity was measured at 25 °C by equilibrating the sample temperature for 5 min. Each measurement was carried out in triplicate.

Analyzes of pesticides were carried out in a GC system QP2010 Ultra equipped with an auto-injector AOC-20i and an auto-sampler AOC-20s using electron impact ion source interface and a single quadrupole (Q) as analyzer from Shimadzu Corporation (Tokio, Japan). GCMS RealTime and PostRun Analysis software from Shimadzu Corporation were used to control the GC and MS parameters, as well as the collection and process of spectrum data, respectively. Separation was carried out in an RtX-5MS column (5% diphenyl, 95% dimethylpolysiloxane, 30 m x 0.25 mm ID, 0.1 µm film thickness) from Restek Corporation (Madrid, Spain). Ultrapure Helium was used as carrier gas.

2.3. GC-Q-MS method

A volume of 2 µL of a standard or sample solution was injected in splitless mode at 300°C with a sample time of 1.5 min.

The column temperature was initially settled at 80 °C and directly increased to 190 °C at a rate of 60 °C/min, then raised to 220 °C at a rate of 6 °C/min and, afterwards increased at a rate of 15 °C/min until 300 °C. Finally, it was held for 10 min to assure the correct cleaning of the column.

The MS analysis was performed in single ion monitoring (SIM) mode using 0.3 s as event time. The electron impact energy was stablished at 70 eV and its temperature at 300 °C. The transfer line was set at 280 °C and the detector voltage 1kV. Retention time and two different fragments were used as identification points for each analyte. The quantification was carried out using the highest intensity m/z (see Supplementary data Table S1).

2.4. Synthesis of betaine-based NADESs

In this work, eight NADESs were prepared by mixing betaine as HBA and different HBDs including citric and lactic acid, fructose, glucose, glycerol, PPG, propanoic and butanoic acid in different molar ratios since the eutectic point was found at different ra-

tios for each combination. For synthesis, the NADES components were placed in a centrifuge tube and stirred at 80 °C until a homogeneous liquid was formed (30 min) in a Thermomixer comfort (Eppendorf AG, Hamburg). The addition of water during the synthesis process was necessary in some cases to get a stable homogeneous material [18,19]. The solvents were cooled to room temperature and stored in a vacuum desiccator to avoid the absorption of moisture. The characteristics and molar ratio of prepared DES are included in Supplementary data Table S2.

2.5. Samples selection

Seven olive leaves products were selected for this study, one was supplied by a local producer (Murciana de Herboristería S.A., Murcia, Spain) (OL_C_1), three were acquired in different supermarkets (OL_C_(2-4)), and the other three were obtained from non-commercial family farming producers located in Tenerife (Canary Islands) and Madrid (Spain) (OL-D-(1-3)). Samples were lyophilized in a freeze drier (Lyobeta 15 Telstar, Terrassa, Spain), ground using an IKA® M 20 grinder at 20000 r.p.m. and stored at -18 °C until their analysis. Seven orange by-products, including peel and pulp, were also selected for this study. Five of them were obtained from commercial oranges, one was supplied by J. García Carrión, S.L (Huelva, Spain) (Cit_C_1) and four were acquired in different supermarkets (Cit_C_(2-5)). The other two samples were obtained from family farming crops located in Tenerife (Canary Islands) (Cit_D_1; M_D_1). All orange samples were initially lyophilized, ground and stored under the same conditions as olive leaves. Origin and other specific characteristic of selected samples are compiled in Supplementary data Table S3.

Samples OL_C_1 and Cit_C_1 were selected to carry out the optimization and validation of the methodology after confirming the absence of pesticides residues in such matrices.

2.6. Solid–liquid microextraction procedure

Five hundred milligrams of spiked or not spiked citrus or olive powder by-product was located into a 50 mL polypropylene centrifuge tube; when necessary, samples were spiked 24 h before extraction. Then, 8 mL of NADES with 40% of water (v/v) was added to the sample and vortexed for 1 min. Afterwards, it was sonicated during 30 min in an ultrasonic bath Elmasonic S 10 system from Elma Schmidbauer GmbH (Singen, Germany) and subsequently centrifuged at 15557 g for 20 min at 22 °C in a 5810 R centrifuge from Eppendorf (Hamburg, Germany) to achieve phases separation. Then, an aliquot of 4 mL of NADES upper enriched phase containing target analytes was collected, transferred into a 15 mL centrifuge tube and 200 µL of cyclohexane was added for analytes re-extraction, applying vortex (30 s) and sonication for 5 min. Finally, 50 µL of the supernatant was transferred into an injection vial, and 2 µL was injected into the GC-MS system.

2.7. Method validation

A thorough validation of the procedure, in terms of matrix effect (ME), linearity, extraction efficiency, reproducibility, and sensitivity, was carried out. With the aim of correcting the possible errors during the analytical procedure and improving the reproducibility of the method, a surrogate (TPP) was spiked at the beginning of the whole methodology [20]. ME study was performed at two different levels of concentration (high level: 1 mg/kg, low level: 200 µg/kg), using the Matuszewski et al. method [21]. With this aim, five extractions were carried out at each level. Samples, in which the absence of pesticides was previously checked, were spiked with the target analytes at the end of the extraction procedure. ME was calculated as the percentage of the ratio of analyte areas between the spiked sample and a standard solution prepared at the same concentration level. Values of 100% indicated the absence of ME, while those higher than 120% and lower than 80% indicate the presence of important ME and those between 80 and 120%, a slight effect. For the recovery study, peak areas obtained when matrices were spiked at two levels of concentration at the beginning and at the end of the procedure (low level: 200 µg/kg, high level: 1 mg/kg) were compared by the extraction of five replicates at each level. Sensitivity was also evaluated; limits of quantification (LOQs) of the method were defined as the concentration which provides a signal to noise ratio of 10 for the m/z used for the quantification transition.

3. Results and discussion

3.1. GC-(Q)-MS method

The characteristics of the pesticides evaluated (see Supplementary Table S4), as well as the complexity of the matrices made necessary the study of GC-MS parameters' effect on the separation and determination of the analytes to achieve the highest sensitivity, increasing analytes signal and decreasing matrix influence. In this sense, not only ramp but also sampling time (0.15–2.0 min), injector temperature (250–280 °C), MS event time (0.3–1.0 s), ionization source temperature (250–300 °C), transfer line temperature (250–300 °C) and injection mode (split using different ratios or spitless) were evaluated. The best separation and determination conditions (see Supplementary data Fig. S1) were achieved as described in Section 2.3, with an analysis time lower than 12.1 min.

Additionally, and taking into account the low volatility and high viscosity of DESs, several trials were performed modifying the split ratio and the dilution of the NADESs prior their injection in the chromatographic system. Results indicated that at least ten times dilution was necessary to achieve adequate determination under the selected conditions. Apart from that, periodic manual cleaning of the injection syringe using combinations of ethanol and cyclohexane was necessary for the correct maintenance of the GC system, while more frequent liner exchange was also needed. Hence, a re-extraction step using a very small volume of a conventional organic solvent was considered at the end of the sample preparation process, avoiding the injection of NADES in the GC system. In fact, a careful evaluation of the introduction of such step in the sample preparation process showed that the consumption of organic solvent and time was even lower than previously, in accordance with the 12 Principles of Green Chemistry. Besides, it also allows increasing the lifespan of the GC-MS and consumables.

3.2. NADES selection

The criteria for selecting the natural components to synthesize the NADESs were based on biocompatibility, biodegradability, low toxicity and good extraction efficiency. In this sense, the use of sugars, alkaloids, aminoacids or ChCl as HBAs has been the main alternative. Among them, betaine, a halogen free alkaloid, has shown several advantages compared to ChCl. Betaine is industrially obtained from renewable resources and betaine-based NADESs have demonstrated a lower cytotoxicity compared to ChCl-based solvents [18]. Additionally, it has been reported that the zwitterionic amphiphilic nature of betaine can favor the extraction process [14,15,18,22,23]. Considering all these benefits, a group of betaine-based solvents was selected for this study. Citric and lactic acids, fructose, glucose, glycerol, PPG, propanoic and butanoic acids were selected as HBDs, taking into account their different potential to establish interactions with the target analytes, as well as the results obtained in previous studies in which hydrophilic NADESs have led to good efficiency for the extraction of similar group

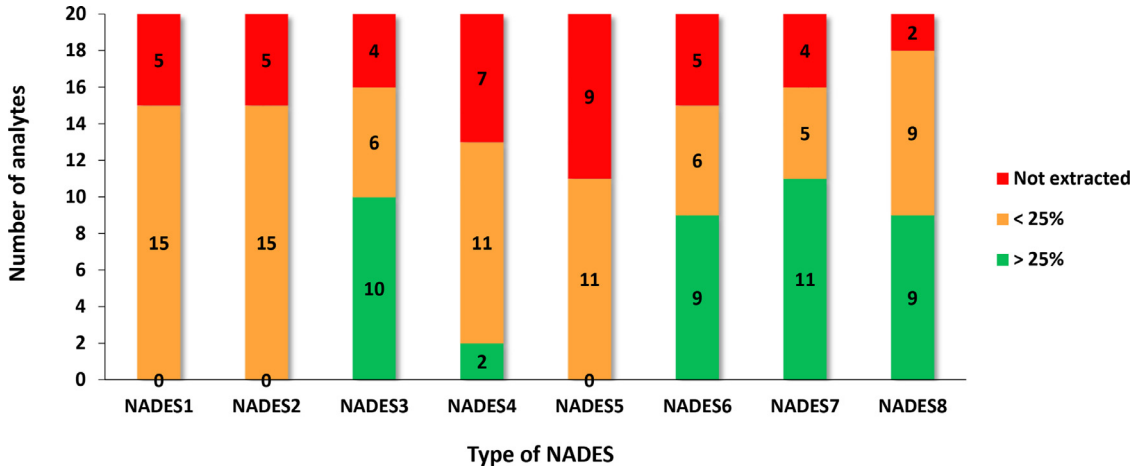

Fig. 1. Number of pesticides effectively extracted from a citrus by-product sample. NADES 1: betaine:citric acid:H_2O (1:1:4); NADES 2: betaine:glycerol (1:3); NADES 3: betaine:lactic acid (1:2); NADES 4: betaine:fructose:H_2O (1:1:4), NADES 5: betaine:glucose:H_2O (1:1:4); NADES 6: betaine:propanoic acid (1:2), NADES 7: betaine:PPG (1:4); NADES 8: betaine: butanoic acid (1:2). Extraction conditions: 1.5 ± 0.1 g of spiked citrus by-product at 1 mg/kg, 6 mL of NADES (20% water (v/v)), 50 min UAE.

of pesticides [24–26]. Synthesis was carried out as indicated in Section 2.4. and the prepared NADESs were stored in a desiccator at room temperature until their use.

For NADESs comparison, 6 mL of a mixture of each NADES at 20% (v/v) of water (except for betaine:butanoic solvent for which no water was added since it produced the disrupting of the NADES) was added to 1.5 ± 0.1 g of citrus by-product contained in a 50 mL centrifuge tube; after vortex homogenization for 1 min, the extraction was performed in an ultrasonic bath for 50 min. After centrifugation, the analytes contained in the upper phase were reextracted in 200 µL of cyclohexane and then injected in the chromatographic system. In this case, the extraction of a wide group of pesticides (20 compounds) was tested. As can be seen in Fig. 1, betaine:sugars NADESs (NADESs 4 and 5) and NADESs 1 and 2 provided the worse results, aspect that could be associated with the high viscosity of this group of solvents, which difficult the interaction solvent-analyte and, consequently the extraction performance [27]. Betaine:lactic acid (1:2) (NADES 3), betaine:propanoic acid (1:2) (NADES 6), betaine:PPG (1:4) (NADES 7) and betaine:butanoic acid (1:2) (NADES 8) were the NADESs that effectively extracted a higher number of compounds with absolute recovery >25%. However, betaine:PPG (1:4) was finally selected as the most adequate solvent since it provided the highest number of effectively extracted compounds (11), as well as the lowest number of interferences which demonstrated its higher selectivity, providing cleaner chromatograms, favoring analytes determination and the enlargement of GC-MS system lifespan.

3.3. NADESs characterization

After selecting the type of betaine-based solvent, its characterization was carried out prior to the optimization of the extraction process, to guarantee the correct formation of the solvent and with the aim of evaluating the characteristics when different molar ratios were used.

Fig. 2 shows the FTIR spectra of (a) betaine, (b) PPG and (c) betaine:PPG (1:4) NADES. The bands at 3359 and 3285 cm^{-1} in (Fig. 2a) are associated with the asymmetric and symmetric stretching of N-H bonds in the betaine structure, while the bands at 1695 and 1616 cm^{-1} are characteristic of the asymmetric and symmetric stretching of carboxylate group present in its structure [28] Fig. 2.b) shows a band at 3308 cm^{-1} related to the stretching of the hydroxylated groups of the PPG structure. The interaction of both molecules is established by hydrogen bonds between carboxylate group in betaine and the hydroxylated groups of PPG as consequence of the strong electronegativity of the O- of (COO-) group [24,28,29]. Such interaction modifies the stretching vibration of carbonyl and hydroxylated groups bringing about an increase of the band width of hydroxylated groups, as well as a shift of the wavelength as can also be seen for carboxylate bands in (Fig. 2c). However, as previously indicated by Zahrina et al. [29], this shift is lower than other polyols-betaine-based DESs because the extent of the hydrogen bonds established between PPG and betaine are lower than for other eutectic mixtures of similar nature.

3.4. NADES-UAE optimization

As mentioned, this work constitutes the first one in which betaine-based NADESs have been applied for the assessment of pesticides. For this reason, prior to the validation and application of the methodology, a thorough study of those factors with high influence on the extraction performance was carried out. In this sense, NADESs molar ratio, volume of extraction solvent, percentage of water added to the extraction solvent, and extraction time were optimized following a step-by-step optimization procedure. All experiments were performed in triplicated using 0.5 ± 0.1 g of citrus by-products spiked with the target analytes at a concentration of 1 mg/kg and using 200 µL of cyclohexane as re-extraction solvent prior to analysis by GC-MS.

3.4.1. Selection of NADES molar ratio

As it has been widely described in the literature, NADES molar ratio plays an important role, not only in the synthesis process and NADES stability, but also on the characteristics of the final solvent, such as the viscosity, which have significant influence on the extraction performance. Indeed, high viscosity decreases the diffusivity of the solvents through the matrix hindering the extraction efficiency [26]. In the present work, betaine:PPG at 1:4, 1:5 and 1:6 molar ratios were tested. With this aim, 6 mL of NADES at 20% water (v/v) was used as fixed volume, applying 50 min of UAE. Previously, the viscosities of the three NADESs were measured, providing dynamic viscosities of 129.70 ± 0.18; 107.47 ± 0.34 and 94.47 ± 0.05 mPa•s, respectively, in agreement with previously reported data [30]. As can be seen in Fig. 3A, when the proportion of PPG increases, the recovery for all compounds decreases; in this sense, and even if a decrease in viscosity is observed, and

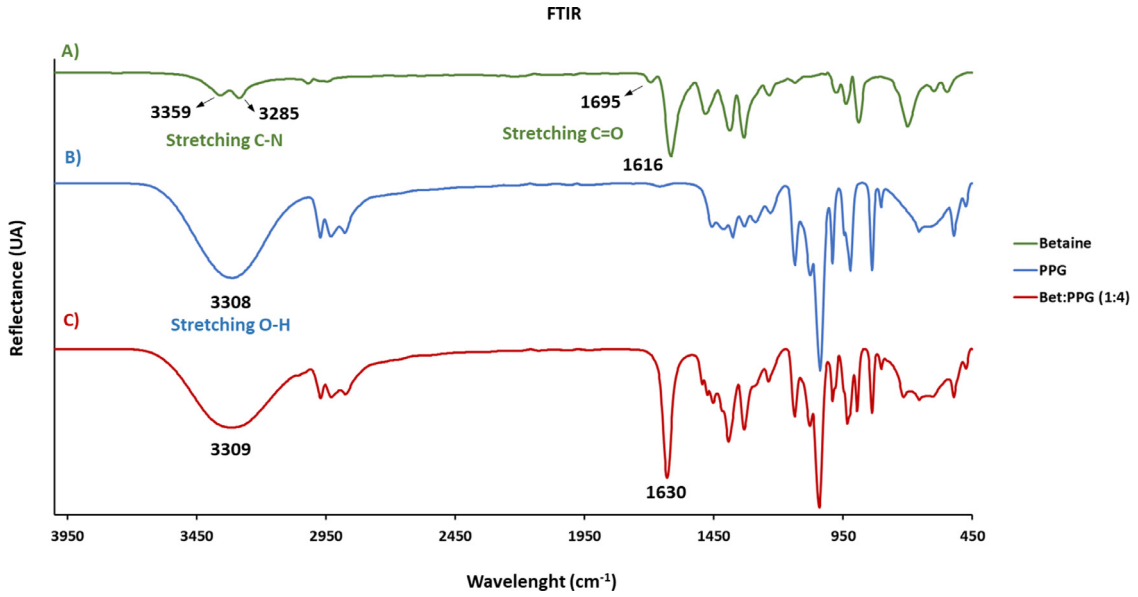

Fig. 2. **(a)** FTIR of betaine, **(b)** propylene glycol (PPG) and **(c)** Betaine:PPG (1:4) NADES.

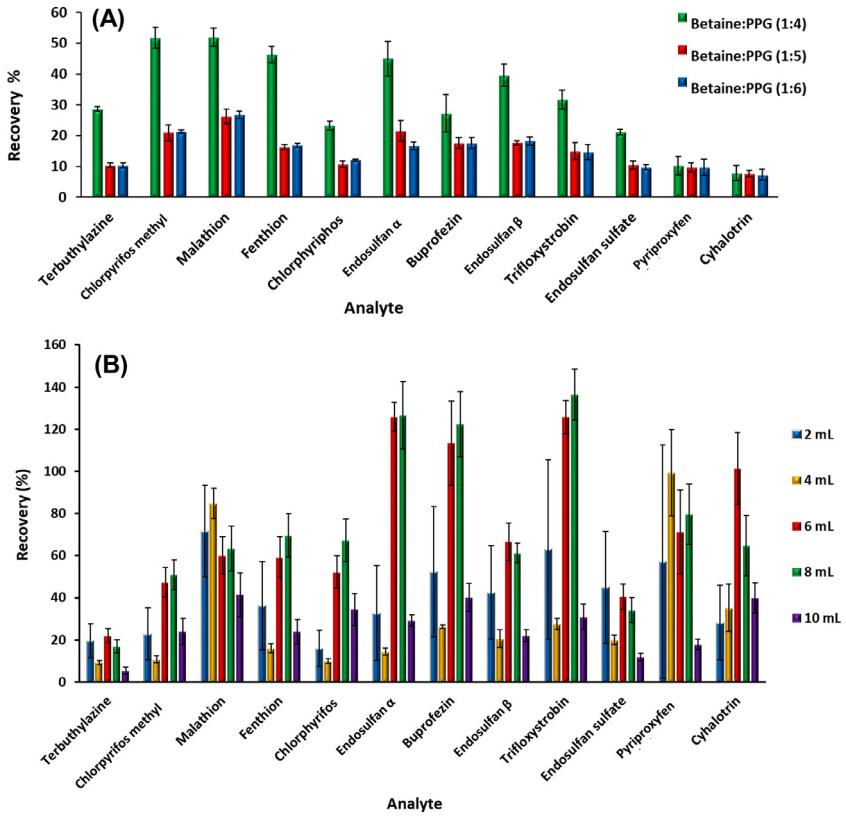

Fig. 3. (**A**) Effect of NADES molar ratio on the extraction efficiency of the target analytes after the application of the UAE procedure. Extraction conditions: 0.5 ± 0.1 g of spiked citrus by-product at 1 mg/kg, 6 mL of NADES (20% water (v/v)), 50 min UAE. **(B)** Effect of extraction solvent volume on the extraction efficiency of the target analytes after the application of the UAE procedure. Extraction conditions: 0.5 ± 0.1 g of spiked citrus by-product at 1 mg/kg, betaine:PPG (1:4) (20% water (v/v)), 50 min UAE.

therefore, a higher diffusivity is expected, other important aspects can play an important role in the extraction efficiency, such as the interactions established between each component and the target analytes. In this particular analysis, it is difficult to draw a general conclusion due to the variety of the structures of the selected pesticides (see Supplementary data Table S4). Considering the obtained experimental results, betaine:PPG (molar ratio 1:4) was selected for further studies.

3.4.2. Selection of NADES volume

Ratio sample-solvent is a relevant aspect in any extraction, and particularly critical for UAE using high viscose and low volatile

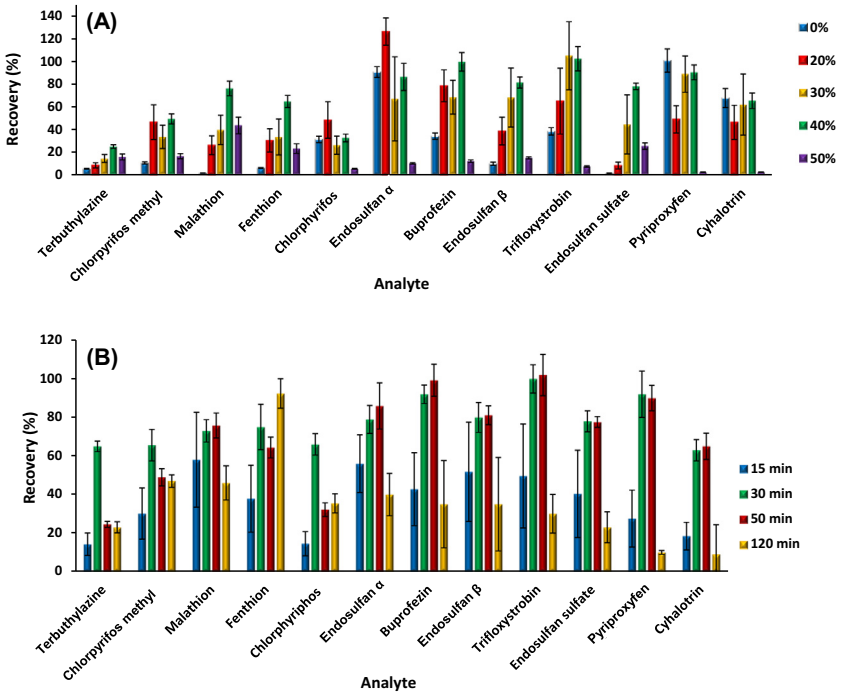

Fig. 4. (**A**) Effect of water addition on the extraction efficiency of the target analytes after the application of the UAE procedure. Extraction conditions: 0.5 ± 0.1 g of spiked citrus by-product at 1 mg/kg, 8 mL betaine:PPG (1:4) with different percentages of water, 50 min UAE. **(B)** Effect of UAE time on the extraction efficiency of the target analytes after the application of the UAE procedure. Extraction conditions: 0.5 ± 0.1 g of spiked citrus by-product at 1 mg/kg, 8 mL betaine:PPG (1:4) (40% water (v/v)).

NADESs [30,31]. It is therefore important to reach a compromise between adding enough solvent to reach efficient interaction NADES-analytes while minimizing NADES volume to get good sensitivity, since preconcentration of the extract is not possible. Considering all these aspects, as well as the principles of Green Chemistry, in which the reduction of materials and solvent is encouraged, volumes of solvent between 2 and 10 mL were evaluated maintaining the rest of parameters fixed: 0.5 g of sample, 20% of water and 50 min of extraction using betaine:PPG in a ratio 1:4.

Fig. 3B shows the recovery obtained for each volume tested. The extraction efficiency increases with the volume of solvent up to 8 mL and then slightly decreases. These results suggest that a volume smaller than 8 mL is not enough to interact with 0.5 g of sample, whereas higher values do not provide higher efficiency and, in addition, increase materials consumption. Based on that, 8 mL was applied in subsequent studies.

3.4.3. Selection of percentage of water in NADES

The addition of water to hydrophilic NADESs is a common practice, not only during the synthesis, favoring the process and NADESs stability, but also after formation, to decrease the viscosity and favor extraction performance [32,33]. In this case, percentages of water in the range 0–50% (v/v) were added to betaine:PPG (1:4) NADES to reach a total volume of 8 mL. The rest of parameters were not modified. Results (see Fig. 4A) showed a clear trend for most analytes in which recovery improved when water % was increased up to 40%, drastically decreasing at higher %. A possible explanation is related to the behavior of viscous liquids in UAE; in this sense, the activation energy needed to create acoustic bubbles and produce collapse events in viscous solvents is high due to the stronger cohesive forces. When water is added, viscosity decreases and, consequently, the extraction performance is favored. However, if the percentage of water is too high, its interaction with each component competes with the interactions HBA-HBD, breaking the NADES by solvation of each hydrophobic component separately, and hindering the extraction process [32,33]. Based on that, 40% of water was selected.

3.4.4. Evaluation of extraction time

The effect of the extraction time was studied considering periods between 15 and 120 min Fig. 4.B shows the recovery obtained for all pesticides evaluated. As can be seen, 15 min was not enough time to achieve the effective extraction of target analytes, while using long periods (120 min), the extraction decreased, probably due to the equilibrium distribution of the analytes between the sample and the NADES phase. Extraction time in the range 30–50 min provided the best results without significant differences between them. Considering this and the principles of Green Chemistry that urge the necessity of reducing energy consumption and simplifying procedures, 30 min was selected as the most suitable extraction time.

3.4.5. Evaluation of re-extraction conditions

Finally, in order to assure the correct recovery of the whole group of analytes from the NADES, variation of the polarity of the re-extraction solvent was tested. In this sense, cyclohexane was compared with a mixture cyclohexane:ethanol (1:1; v/v). Not significant differences were observed in the extraction rate; thus, cyclohexane was selected as the most adequate re-extraction solvent.

3.5. UAE-GC-MS validation

Safety assessment of by-products is of great importance to guarantee consumers health. However, although valorization studies of different by-products have been widely reported in the literature, the evaluation of possible contaminants that can endanger de quality and safety of the final products is still scarce [8]. Pesticide occurrence on two different by-products (orange and olive leaves), whose valorization has been previously studied in our laboratory, [5–7] was evaluated.

Table 1
Results of the matrix effect study (n=5) of the UAE-GC-(Q)-MS method for the target compounds in the selected by-products at two levels of concentrations.

Analyte	Type of by-product	Matrix effect % (RSD, %)
Terbuthylazine	Orange	46 (17)
	Olive leaves	43 (12)
Chlorpyrifos-methyl	Orange	139 (19)
	Olive leaves	76 (16)
Malathion	Orange	58 (12)
	Olive leaves	37 (18)
Fenthion	Orange	123 (9)
	Olive leaves	111 (10)
Chlorpyrifos	Orange	45 (8)
	Olive leaves	21 (3)
Endosulfan α	Orange	158 (14)
	Olive leaves	140 (17)
Buprofezin	Orange	114 (20)
	Olive leaves	99 (20)
Endosulfan β	Orange	131 (1)
	Olive leaves	115 (18)
Trifloxystrobin	Orange	141 (15)
	Olive leaves	126 (16)
Endosulfan sulfate	Orange	166 (12)
	Olive leaves	116 (6)
Pyriproxyfen	Orange	142 (20)
	Olive leaves	153 (6)
Cyhalothrin	Orange	56 (14)
	Olive leaves	42 (9)

Concentrations of the analytes in the samples: 1 mg/kg and 200 μg/kg.

The complexity of food matrices such as fruit and vegetables can bring about suppression or enhancement of MS signal due to a phenomenon known as ME [34] Table 1. compiles the results obtained from ME study. As can be seen, most of compounds presented moderate or slight increase of the signal in both matrices whereas terbuthylazine, chlorpyrifos methyl, malathion, chlorpyrifos and cyhalothrin showed a strong suppression effect in olive leaves and orange (except chlorpyrifos-methyl that show moderate enhancement in orange). Based on that, matrix-matched calibration curves for each matrix were obtained by injecting seven different concentration levels ($n = 7$) in duplicate to check the linearity of the methodology in the range of concentration of interest. Matrices used for the study were previously evaluated to verify the absence of the target pesticides that could compromise the reliability of the study. As it is shown in Table 2, good linearity was obtained for all compounds in orange and olive by-products with R^2 values higher than 0.9902 in all cases.

Recovery study results are shown in Table 3, as can be observed good efficiency and reproducibility of the extraction process were obtained, with relative recovery values in the range 73–110% for orange by-product and 73–115% for olive leaves, and relative standard deviation (RSD) values lower than 20% for all analytes.

LOQs of the method, were in the ranges 8.5–128.8 µg/kg for orange and 8.5–67.5 µg/kg for olive by-products (see Table 3). There are no maximum residues limits (MRLs) for pesticides in those by-products. However, taking into account the data available for table olives and citrus, it should be highlighted that the developed method allows the determination of selected pesticides below the legislated levels, except for buprofezin and endosulfan β. Comparison in terms of LOQs with previous works in which pesticides have been extracted from solid foods using DESs is quite difficult since in most cases the matrix is previously extracted using organic solvents and the values are referred to these extracts [24,25,35,36]. The revision of those articles in which LOQs can be compared come up with values higher [37], similar or lower [38–40] than those obtained in the present study. However, in those cases, a lower number of pesticides was simultaneously evaluated [37–40]. Besides, it should be highlighted that, although the sensitivity reached in this work is not the best reported so far, the present study is the first one in which a DES has been directly used for the extraction of pesticides from the solid matrix and, additionally, not further clean-up steps have been employed. From a sustainable point of view, such aspect is of great importance because not only green solvents have been applied, but also the use of some mL (10-1.5 mL) of conventional organic solvents (i.e., methanol or acetonitrile) has been avoided. Apart from that, additional steps that increase time consumption, as well as the use of additional sorbents were also removed from the process, obtaining good extraction efficiency and reproducibility and acceptable LOQs, which can be further improved using analyzers with higher sensitivity, such as triple quadrupole working in tandem mode. Thus, this work constitutes a promising initial step in the searching of sustainable procedures that minimize the use of organic solvents in solid food samples analysis by direct application of the DES to the raw matrix.

3.6. Safety assessment of citrus and olive by-products

The validated methodology was applied for safety assessment of fourteen samples, including seven olive leaves and seven citrus by-products. With the aim of evaluating a varied spectrum of samples, some of them were acquired in different supermarkets while others were obtained from family farms in different points of Spain, as it was indicated in Table S3. Table 4 compiles the results of these analyses and Fig. 5 shows the chromatogram of the analytes found in OL_C_3. Eleven of the evaluated samples contained at least one residue of the pesticides, whereas, only OL_C_1, Cit_C_1 and Cit_C_5 were free of residues. Terbuthylazine, chlorpyrifos-methyl, malathion and endosulfan β, which have been reported to be commonly used in olive production [16], and pyriproxyfen, whose use has been confirmed by citrus producers from Spain, were the analytes identified in these samples, although in most cases they were below the LOQ of the developed methodology. Malathion has been the pesticide most frequently found in the evaluated samples, particularly in olive leaves. Since there are no established MRLs for olive or citrus by-products, these results have been analyzed considering the values legislated by the European Union for olives and citrus fruit. In this sense, it should be highlighted that, except pyriproxyfen, the rest of residues quantified in the evaluated samples were found above the MRLs, as can be observed in Table 4 [41]. Especially remarkable is the presence of Endosulfan β, whose use as phytosanitary product in the EU was banned in 2005, and its use is forbidden in Spain for olives or oranges production [42–44].

As previously indicated, safety evaluation of valorized by-products is scarce. However, these results could be compared to other previous works, in which olives, olive oils, olive leaves or orange have been assessed. In this sense, malathion had been previously found in Valencian oranges in the range 50–100 µg/kg [45], similar values as the ones obtained in this study for two citrus by-products produced also in Valencia (Cit_C_3 and Cit_C_4), which were in the range 45.18-45.33 µg/kg. Regarding pyriproxyfen, its evaluation has been previously reported in citrus fruit from Valencia by Juraske and Sanjuán [17]. The presence of this pesticide was found in the present work in two Valencian orange by-products and one from Canary Islands, supporting the persistence of such substance in the final products after their use during fruit growth.

Regarding olive derived matrices, Žuntar [9] found concentrations lower than 2 µg/kg for the sum of endosulfan α/β and endosulfan sulfate in olives leaves from Croatia; while Likudis et al. [46] evaluated 77 olive oil samples from Greece detecting those analytes in a large number of samples. However, concentrations were in the range 10.2-29 µg/kg for endosulfan β, lower than the one found in this work (120.6 µg/kg) for OL_C_3. These results confirm

Table 2
Matrix-matched calibration data of the target compounds in the selected by-products.

Analyte	Type of by-product	Calibration data (n = 7)			
		Range of concentration studied (μg/L)	Slope	Intercept	R^2
Terbuthylazine	Orange	30-2000	$5.77 \cdot 10^{-2} \pm 1.84 \cdot 10^{-3}$	$-2.58 \cdot 10^{-0} \pm 1.02 \cdot 10^{-0}$	0.9947
	Olive leaves	30-2000	$4.38 \cdot 10^{-2} \pm 1.12 \cdot 10^{-3}$	$-2.19 \cdot 10^{-0} \pm -5.64 \cdot 10^{-0}$	0.9909
Chlorpyrifos-methyl	Orange	30-2000	$6.48 \cdot 10^{-2} \pm 1.67 \cdot 10^{-3}$	$-1.34 \cdot 10^{-0} \pm 1.45 \cdot 10^{-0}$	0.9916
	Olive leaves	30-2000	$6.75 \cdot 10^{-2} \pm 1.73 \cdot 10^{-3}$	$-4.57 \cdot 10^{-0} \pm 3.69 \cdot 10^{-0}$	0.9921
Malathion	Orange	90-2000	$1.92 \cdot 10^{-2} \pm 5.32 \cdot 10^{-3}$	$-7.61 \cdot 10^{-1} \pm 2.11 \cdot 10^{-1}$	0.9949
	Olive leaves	40-2000	$2.18 \cdot 10^{-2} \pm 5.59 \cdot 10^{-3}$	$-1.11 \cdot 10^{-1} \pm 2.85 \cdot 10^{-1}$	0.9930
Fenthion	Orange	30-2000	$3.14 \cdot 10^{-2} \pm 8.08 \cdot 10^{-3}$	$-1.78 \cdot 10^{-0} \pm 4.58 \cdot 10^{-1}$	0.9923
	Olive leaves	30-2000	$3.48 \cdot 10^{-2} \pm 8.43 \cdot 10^{-3}$	$-2.56 \cdot 10^{-0} \pm 1.65 \cdot 10^{-0}$	0.9939
Chlorpyrifos	Orange	30-2000	$1.40 \cdot 10^{-2} \pm 3.59 \cdot 10^{-3}$	$-7.00 \cdot 10^{-1} \pm 8.79 \cdot 10^{-2}$	0.9906
	Olive leaves	30-2000	$1.44 \cdot 10^{-2} \pm 3.51 \cdot 10^{-3}$	$-1.38 \cdot 10^{-0} \pm 3.24 \cdot 10^{-1}$	0.9902
Endosulfan α	Orange	90-2000	$6.14 \cdot 10^{-3} \pm 1.57 \cdot 10^{-4}$	$8.69 \cdot 10^{-2} \pm 2.23 \cdot 10^{-2}$	0.9950
	Olive leaves	30-2000	$2.11 \cdot 10^{-3} \pm 5.17 \cdot 10^{-4}$	$1.04 \cdot 10^{-1} \pm 2.55 \cdot 10^{-1}$	0.9910
Buprofezin	Orange	30-2000	$3.04 \cdot 10^{-2} \pm 7.81 \cdot 10^{-3}$	$-2.31 \cdot 10^{-2} \pm 9.56 \cdot 10^{-3}$	0.9913
	Olive leaves	50-2000	$1.88 \cdot 10^{-2} \pm 4.82 \cdot 10^{-3}$	$-9.14 \cdot 10^{-2} \pm -2.35 \cdot 10^{-1}$	0.9918
Endosulfan β	Orange	200-2000	$6.92 \cdot 10^{-4} \pm 1.92 \cdot 10^{-5}$	$-5.11 \cdot 10^{-2} \pm 1.42 \cdot 10^{-2}$	0.9930
	Olive leaves	200-2000	$3.44 \cdot 10^{-4} \pm 8.83 \cdot 10^{-5}$	$2.60 \cdot 10^{-2} \pm 6.66 \cdot 10^{-2}$	0.9913
Trifloxystrobin	Orange	30-2000	$5.60 \cdot 10^{-3} \pm 1.43 \cdot 10^{-4}$	$-2.74 \cdot 10^{-2} \pm 7.05 \cdot 10^{-3}$	0.9940
	Olive leaves	30-2000	$4.66 \cdot 10^{-3} \pm 1.20 \cdot 10^{-4}$	$1.88 \cdot 10^{-2} \pm 4.83 \cdot 10^{-2}$	0.9980
Endosulfan sulfate	Orange	75-2000	$1.44 \cdot 10^{-3} \pm 1.91 \cdot 10^{-4}$	$-7.46 \cdot 10^{-2} \pm 1.917 \cdot 10^{-2}$	0.9955
	Olive leaves	40-2000	$9.91 \cdot 10^{-4} \pm 2.42 \cdot 10^{-5}$	$1.92 \cdot 10^{-2} \pm 4.69 \cdot 10^{-3}$	0.9956
Pyriproxyfen	Orange	75-2000	$1.67 \cdot 10^{-3} \pm 4.29 \cdot 10^{-4}$	$-2.62 \cdot 10^{-2} \pm 6.72 \cdot 10^{-3}$	0.9951
	Olive leaves	50-2000	$6.68 \cdot 10^{-3} \pm 1.63 \cdot 10^{-4}$	$-2.81 \cdot 10^{-1} \pm 6.87 \cdot 10^{-2}$	0.9930
Cyhalothrin	Orange	90-2000	$5.25 \cdot 10^{-3} \pm 1.45 \cdot 10^{-4}$	$1.24 \cdot 10^{-1} \pm 3.44 \cdot 10^{-2}$	0.9933
	Olive leaves	90-2000	$3.20 \cdot 10^{-3} \pm 7.84 \cdot 10^{-4}$	$-4.03 \cdot 10^{-1} \pm 9.86 \cdot 10^{-2}$	0.9914

R^2: Determination coefficient. Triphenyl phosphate (TPP) was used as IS in all cases.

Table 3
Results of the recovery study (n=5) of the UAE-GC-(Q)-MS method for the target compounds in the selected by-products at two levels of concentration.

Analyte	Type of by-product	Level 1[a] (n=5) Relative Recovery %(RSD, %)	Level 2[b] (n=5) Relative Recovery % (RSD, %)	LOQ_{method}[c] (µg/kg)	LMR[d](µg/kg)
Terbuthylazine	Orange	83 (20)	84 (20)	21.4	100
	Olive leaves	92 (10)	96 (11)	18.7	50
Chlorpyrifos-methyl	Orange	77 (11)	80 (11)	22.5	2000
	Olive leaves	86 (6)	90 (18)	10.0	10
Malathion	Orange	100 (14)	82 (18)	55.7	2000
	Olive leaves	97 (10)	96 (15)	9.6	20
Fenthion	Orange	93 (15)	89 (19)	8.5	10
	Olive leaves	107 (12)	80 (19)	8.5	10
Chlorpyrifos	Orange	89 (10)	80 (9)	22.5	1500
	Olive leaves	106 (11)	115 (3)	9.6	10
Endosulfan α	Orange	90 (10)	110 (16)	49.0	50[e]
	Olive leaves	107 (10)	75 (14)	24.0	50 [e]
Buprofezin	Orange	87 (8)	80 (9)	22.5	10
	Olive leaves	93 (7)	83 (15)	36.1	10
Endosulfan β	Orange	77 (12)	93 (14)	128.8	50 [e]
	Olive leaves	82 (14)	79 (10)	67.5	50 [e]
Trifloxystrobin	Orange	96 (14)	101 (20)	17.8	500
	Olive leaves	89 (2)	73 (10)	24.6	300
Endosulfan sulfate	Orange	77 (13)	100 (18)	44.9	50 [e]
	Olive leaves	76 (7)	89 (16)	26.9	50 [e]
Pyriproxyfen	Orange	73 (7)	79 (9)	56.9	600
	Olive leaves	103 (10)	90 (7)	33.3	50
Cyhalothrin	Orange	78 (11)	84 (9)	64.2	200
	Olive leaves	76 (12)	87 (14)	61.9	1000

a) Concentration of the analytes in the samples: 1 mg/kg. b) Concentration of the analytes in the samples: 200 μg/kg. c) Defined as the concentration which provides a signal to noise ratio of 10 for the m/z used for the quantification. d) based on Regulation (EC) No 396/2005 of the European Parliament and subsequent amends. e) Sum of endosulfan α and β as well as endosulfan-sulfate

the presence of these pesticides in different areas of the European Union, even when their use has been banned since 2005 [44].

3.7. Greenness evaluation of the developed methodology

The greenness of the proposed method was evaluated using an innovative tool called AGREE, recently developed by Pena-Pereira et al. [47]. The calculator tool is based on an eco-scale in the range 0–1. The greenest method reaches 1 and the score decreases by assigning penalties points based on the 12 Principles of Green Analytical Chemistry. Table 5 shows the main aspects considered by AGREE calculator for some recently developed methodologies based on green procedures applied for the analysis of pesticides in orange or olive matrices, as well as the score obtained for each procedure when AGREE calculator was applied, while the AGREE diagrams obtained in each case are shown in Supplementary data (Fig. S2) [48–51]. As can be seen, the betaine-based NADES-UAE-GC-MS methodology reached the third ranged

Table 4
Results of the analysis of different citrus and olive by-products using the developed methodology.

Sample	Concantration (µg/kg)[a),b)]				
	Terbuthylazine	Chlorpyrifos-methyl	Malathion	Endosulfan β	Pyriproxyfen
Olive by-products					
OL_C_1	n.d.	n.d.	n.d.	n.d.	n.d.
OL_C_2	n.d.	n.d.	64.69 ± 33.29	n.d.	n.d.
OL_C_3	57.33 ± 29.50	n.d.	64.16 ±33.02	120.6 ± 62.1	n.d.
OL_C_4	n.d.	n.d.	63.69 ± 32.77	< LOQ	n.d.
OL_D_1	< LOQ	< LOQ	64.78 ± 33.34	n.d.	n.d.
OL_D_2	< LOQ	n.d.	64.68 ± 33.28	n.d.	n.d.
OL_D_3	n.d.	n.d.	85.53 ± 44.02	n.d.	n.d.
Citrus by-products					
Cit_C_1	n.d.	n.d.	n.d.	n.d.	n.d.
Cit _C_2	< LOQ	< LOQ	n.d.	< LOQ	49.50 ± 25.47
Cit _C_3	< LOQ	< LOQ	45.18 ± 23.25	n.d.	n.d.
Cit _C_4	n.d.	n.d.	45.43 ± 23.38	n.d.	< LOQ
Cit _C_5	n.d.	n.d.	n.d.	n.d.	n.d.
Cit _D_1	n.d.	< LOQ	n.d.	< LOQ	< LOQ
MA_D_1	< LOQ	n.d.	n.d.	< LOQ	n.d.
LMR (µg/kg)[c)]					
Olive	50	10	20	50[d)]	50
Citrus	100	2000	2000	50[d)]	600

a) Results obtained as an average of two analyses for each product. b) n.d.: Not detected. c) based on Regulation (EC) No 396/2005 of the European Parliament and subsequent amends. d) Sum of endosulfan α and β as well as endosulfan-sulfate. Further description and characteristics of the selected samples can be found in Table S3.

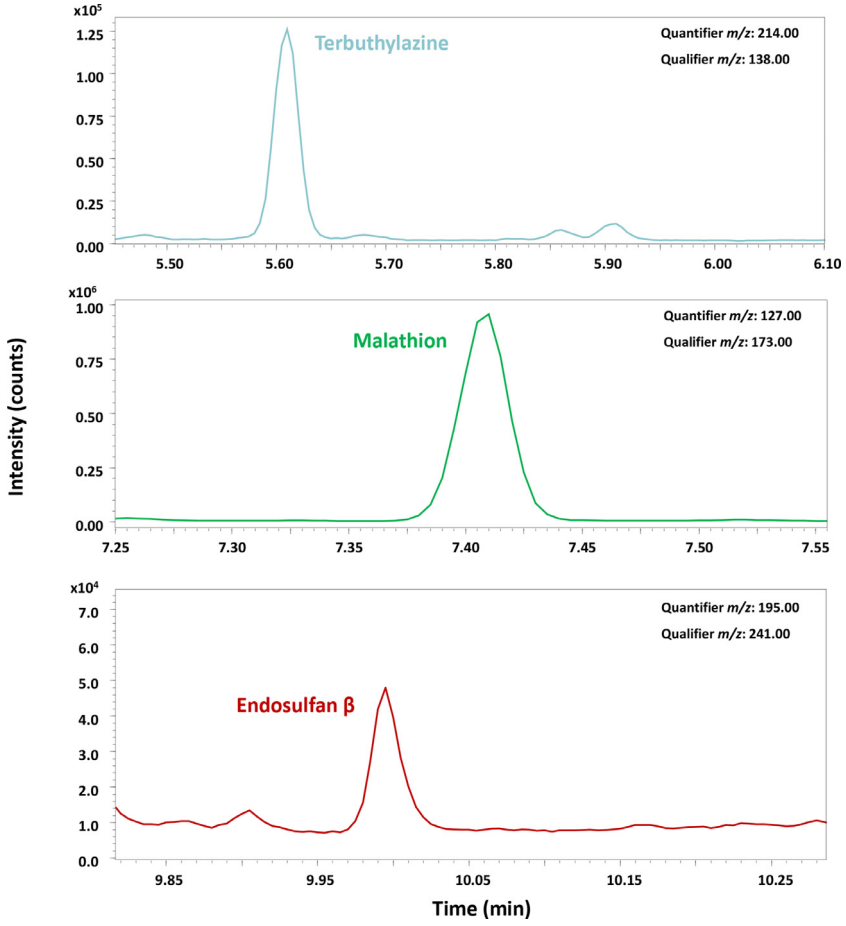

Fig. 5. GC-MS ion extracted chromatogram of the three compounds found at the sample OL_C_3 analyzed by application of the UAE-GC-MS method.

Table 5
Main aspects that are considered for greenness assessment using AGREE calculator.

Greeness comparison							
Sample (amount)	Analytical device	Sample preparation	Pretreatment steps	Pesticides simultaneously evaluated	Type of reagent/solvent	AGREEscore	Refs.
Olive oil (0.5 g)	GC-μECD	LLE+DLLME	6	16	4 mL n-hexane +2 mL ACN+ 2.5 mL NADES	0.	[48]
Orange juice (2.5 mL)	GC-FID	DLLME	7	6	ACN (1 mL) + DES (132 μL)	0.53	[49]
Olive leaves, orange by-products (0.5 g)	GC-MS	UAE	4	12	NADES (8 mL) + 200 μl cyclohexane	0.54	This work
Orange fruit juice (5 mL)	GC-FID	HS-SDME	7	7	DES (2 μL)	0.56	[50]
Olive oil (5 mL)	HPLC-DAD	HLLME+DLLME	5	6	DES (150 μL) + carbonate water solution (5 mL) + ACN (44 μl)	0.62	[51]

ACN: acetonitrile; DAD: diode array detection; DLLME: dispersive liquid-liquid microextraction; FID: flame ionization detector; HLLME: homogeneous liquid-liquid microextraction; HS-SDME: head-space single drop microextraction; μECD: micro-electrochemical detector.

score from all methods evaluated. This result demonstrates the green character of this procedure, even when it is the only work in which a solid matrix was directly evaluated, and not miniaturized procedure was applied. Another important aspect that should be remarked is that the present work is the only one in which MS was used for determination of the analytes. This aspect decreases the sustainable character of the method due to the higher energy consumed by this type of systems respect to conventional detectors. However such aspect also provides a higher reliability of the obtained results that is especially important when food safety is evaluated, and consumers health should be guaranteed.

4. Conclusions

In this work, a sustainable analytical method, based on NADES extraction procedure combined with GC-Q-MS determination, has been developed for the first time for the evaluation of 12 pesticides in citrus and olive by-products with neuroprotection potential against neurodegenerative diseases such as Alzheimer Disease. NADESs constituted of betaine as HBA and different HBDs were tested. The combination of betained:PPG in a molar ratio 1:4 provided the highest extraction efficiencies for the largest number of screened compounds and extraction conditions were further optimized. Validation of the methodology provided adequate results for both type of by-products. Good linearity was obtained in all cases with R^2 higher than 0.9902 for matrix matched calibrations. Trueness was evaluated by a recovery study obtaining values of 73-115% with RSDs lower than 20%, which demonstrated the reproducibility of the procedure. LOQs were in the range 8.5–128.8 μg/kg showing an acceptable sensitivity of the method. Besides, the greenness of the developed methodology was assessed indicating the sustainable nature of the proposed methodology even when it was compared with recently developed miniaturized procedures applied for the analysis of pesticide in related samples.

Analysis of real samples (seven olive leaves samples and seven orange by-products, including different varieties and origins) indicated the presence of terbuthylazine, chlorpyrifos-methyl, malathion, endosulfan β and pyriproxyfen in several of the evaluated samples, finding violations of the MRLs established by the European Commission for all compounds detected except for pyriproxyfen. Results highlight the relevance of safety evaluation in valorized agricultural by-products to guarantee the security of consumer health and, in addition, the suitability of sustainable procedures based on green solvents to address this issue. The proposed method is a simple, inexpensive, effective and safe alternative methodology for the evaluation of pesticides using, for the first time, betaine-based NADESs as green solvents. In addition, it is the first procedure in which NADES-based extraction has been proposed for the evaluation of pesticides in olive leaves or orange by-products.

Declaration of Competing Interest

Authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.462922.

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