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Enhanced fluorescence detection of ergosterol by hydrophobic fluorescent natural deep eutectic solvent

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ABSTRACT

Ergosterol (ERG) is a sterol found at high levels in cell membranes of fungi and is a smart option to be used as proof of fungal contamination in food samples. Therefore, the objective of this work was to develop a fast, sensitive and ecological methodology based on the use of hydrophobic fluorescent natural deep eutectic solvents (HPB-FLUODES) to determine ERG by fluorescence detection in edible mushrooms. The proposed approach involves a green sample preparation and a low cost instrumental technique leading to an efficient and simple analytical methodology suitable for high-throughput analysis (2.4 samples per minute). The experimental variables, such as the type of HPB-FLUODES, sample/solvent mass/volume ratio, time, and temperature of extraction were studied and optimized. The selected variables were HPB-FLUODES thymol:lactic acid 1:2, 2.5 mg of sample, 1 mL of HPB-FLUODES, and 45 min of extraction at 40°C. The LOD and LOQ were 0.09 μg g^{-1} and 0.29 μg g^{-1}, respectively. The results show an enhancement of ERG fluorescence signal of 1825% against the ERG signal in methanol media. The present approach shows outstanding green performance using AGREE®, AGREEprep® and WAC principles. Finally, the analysis of edible mushrooms demonstrates the feasibility of the proposed green methodology.

1. Introduction

The green chemistry revolution brings new challenges to those who practice chemistry in industry, research, and education. These challenges imply opportunities to discover and improve the economics of chemical manufacturing [1]. The difficulty and time involved in sample preparation are well-known in the industry and analytical procedure developments. Conventional solvents in sample preparation and their waste generation, imply a risk for the analyst and the environment [2].

Several analytical developments still use toxic solvents such as halogenated and petroleum derivate reagents [3]. Thus, a new generation of green solvents have been proposed. Natural deep eutectic systems (NADES) offer a range of new ecological solvents prepared from metabolites occurring naturally in all cells and organisms, such as sugars, amino acids and organic acids; among others [4]. The eutectic mixture forms supramolecular structures due principally to hydrogen bonds that melt at a much lower temperature than the melting points of the separate constituents [5]. NADES have advantages over conventional solvents due to their rapid and simple preparation, low cost and toxicity and biodegradability [4]. Recently, it has been discovered that some of these liquids present native fluorescence [6], which makes them suitable as a new alternative media in fluorescence detection. Therefore, the term FLUODES (Fluorescent NADES) is proposed here as a new class/family of eutectic solvents. On the other hand, hydrophobic NADES is a new generation of water-immiscible solvents introduced in 2015, usually attributed to various combinations of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) that result in unique interactions (such as hydrogen and π-π bonding) [7,8].

Ergosterol (ERG) is a sterol produced by fungi and can occur in free and esterified forms. This compound is found at high levels in fungi, and it is a principal structural component of cell membranes, conferring fluidity and permeability [9]. ERG quantification is a suitable and sensitive indicator for detecting fungal contamination in food samples [10–12]. Numerous instrumental techniques have been reported for the detection and quantification of ERG, mainly chromatographic methods such as gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) [9,13-15]. These instrumental techniques have several disadvantages, such as high consumption of solvents and time, the need of skilled personnel and usually require exhaustive purification steps before the analysis. Fluorescence-based techniques emerge as an alternative method with excellent features, principally high sensitivity and selectivity, speed, simplicity, low cost, low reagent consumption, and ease of operation [16]. Since ERG exhibits

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intrinsic fluorescence properties attributable to the 1,3-diene chemical structure, it is suitable for analysis by fluorescence-based techniques [17]. Nevertheless, few studies have determined ERG by fluorescence methods [16,18]. Thus, the main objective of this work was to explore the possibilities of using hydrophobic FLUODES (HPB-FLUODES) as fluorescent probes for enhancing the fluorescent detection of ERG in food samples. The selected HPB-FLUODES was applied for the easy extraction and subsequent detection of ERG by fluorescence using Portobello mushroom samples.

2. Materials and methods

2.1. Reagents and solutions

ERG standard (purity >98%), levulinic acid, and dodecanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thymol was purchased from Alkemit (Argentina). L (+) lactic acid and menthol were obtained from Biopack (Buenos Aires, Argentina). For HPLC analysis, methanol (MeOH) and acetonitrile (ACN) were purchased from J.T. Baker (PA, USA) and Sintorgan (Argentina), respectively. Stock solutions of ERG were prepared in HPB-FLUODES or MeOH and stored at 20°C in darkness to prevent degradation.

2.2. Preparation of hydrophobic FLUODES

HPB-FLUODES were prepared using thymol (T), menthol (M), camphor (C), L (+) lactic acid (La), and levulinic acid (Le) at different molar ratios. Components, abbreviations, and final molar ratios are shown in **Table 1**. The components were added to a caramel vial and heated at 70°C under magnetic agitation at 450 rpm. Temperature was maintained for about 15 minutes until a clear and homogeneous liquid was obtained [19]. The stability was evaluated every 24 h; solvents were considered stable when the mixture remained in the liquid phase for a 7-day period.

2.3. Sample preparation

Portobello mushroom (*Agaricus brunnescens*) samples were purchased from a local market. Fresh mushrooms were cut into small pieces and freeze-dried for 3 days in a lyophilizer (BIOBASE Dauerhaft). Freeze-dried samples were homogenized in a grinder, sieved, and kept in glass bottles at 20°C until analysis. 25 mg of the lyophilized mushroom powder was added into a 2000 μL microtube with 1 mL of TLa 1:2. To achieve maximum extraction, the mixture was then shaken for 30 s in a vortex and sonicated for 45 min at 40°C, to achieve maximum extraction. After extraction, the tube was centrifuged at 10000 rpm for 10 min at 25°C, to accelerate the phase's separation. The supernatant was transferred to a polypropylene microtube for fluorescence analysis.

2.4. Optimization of extraction procedure

The experimental variables that could modify the analytical response of ERG were sample mass/HPB-FLUODES ratio (2.5-10 mg mL^{-1}), extraction temperature (25-55°C) and sonication time (15-60 min). The variables were evaluated by modifying one variable at the time, while keeping the remaining constant. Analyses were performed in triplicate and results were expressed as mean values of relative fluorescence units (RFU). RFU is a unit of measurement used in analysis which employs fluorescence detection. This value is the amount of emission light collected by the instrument. The term "relative" indicates that the fluorescence intensity signal measured depends on the instrument itself. The relative response (RR, %) for ERG was considered to optimize each variable.

Table 1
Composition and molar ratios of the HPB-FLUODES prepared.

Abbreviation	Component 1	Component 2	Molar Ratio
TLa	Thymol	L (+) lactic acid	1:2
TLe	Thymol	Levulinic Acid	1:1
TC	Thymol	Camphor	1:1
MT	Menthol	Thymol	1:1
MD	Menthol	Dodecanol	1:1

2.5. ERG Fluorescence analysis

Fluorescence measurements were carried out in a low cost all-in-one DeNovix QFX Fluorometer (Wilmington, USA) equipped with four LEDs as excitation sources, a photodiode detector (range 300-1000 nm), and a 7" HD touchscreen. The pre-installed app "Basic fluorometer" was used to perform all measurements. **Table S1** (supplemental material) shows the multiple wavelengths of excitation and emission channels. In routine analysis, the channels of excitation and emission chosen were those showing the maximum fluorescence intensity. Fluorescence of ERG extracted with HPB-FLUODES was measured in a 500 μL thin-wall PCR tube (polypropylene).

2.6. Liquid chromatography-UV analysis

HPLC analyses were carried out on a Shimadzu Prominence LC-20 coupled to a UV/Vis detector (Shimadzu Corporation, Japan) and operated by LabSolutions Lite (5.93 software version). The LC column used was a Zorbax C18 (100 × 4.6 mm, 3.5 μm particle size; Agilent Technologies, USA). The mobile phase was 100% ACN, for 10 min at a flow rate of 1.2 mL min^{-1}. The oven was operated at 30°C and the working wavelength was 280 nm. Peak identification in samples was accomplished by comparing retention times with ERG standard.

2.7. Analytical performance

The fluorometer and LC-UV methods were evaluated in terms of accuracy, precision, selectivity, and recovery, using two sets of samples. Set 1 was constructed using ERG standard in HPB-FLUODES and set 2 was built with mushroom samples extracted as described in Section 2.3. The concentration studied levels ranged from 20-500 mg L-1 for sets 1 and 2, and were analyzed by Fluorometer and LC-UV. Fig. S1 shows an LC-UV chromatogram of ERG in MeOH, HPB-FLUODES, and mushroom samples.

The experimental F value was calculated to test the linearity of the calibration curve, as recommended by IUPAC [21]. The LOD and LOQ of the calibration curves were evaluated, as recommended by IUPAC [21], as follows:

$$\mathrm{LOD},\ \mathrm{LOQ} = \frac{3.3,\ 10\ \mathrm{Sy/x}}{\mathrm{A}}\sqrt{1 + \mathrm{h0} + 1/\mathrm{I}}$$

Where A is the slope of the univariate calibration graph, I is the number of calibration samples, h0 is the null hypothesis, and Sy/x is the residual standard deviation. The accuracy was assessed by comparing the predicted RFU values against the RFU values obtained in set 2.

3. Results and discussion

Experimental variables that affect the analytical responses of the ERG were evaluated: HPB-FLUODES composition, sample mass/volume ratio, sonication time and extraction temperature. These studies were carried out by modifying one variable at a time, while keeping the remaining constant.

The RFU value was used to evaluate the impact of experimental conditions on the analytical signal of ERG. To optimize each variable, the relative response (RR (%)) for ERG was considered. The highest RFU was selected at 100% of the analytical signal. Consequently, the RR was calculated as follows: $RR\ (\%) = A_{ERG,j}/A_{ERG,j\ max} \times 100$, where $A_{ERG,j\ max}$ is the maximum analytical signal (RFU value) of ERG obtained in a specific assay (j) and $A_{ERG,j}$ is the analytical signal (RFU) of ERG obtained in the specific assay (j) at different levels of the variables under study.

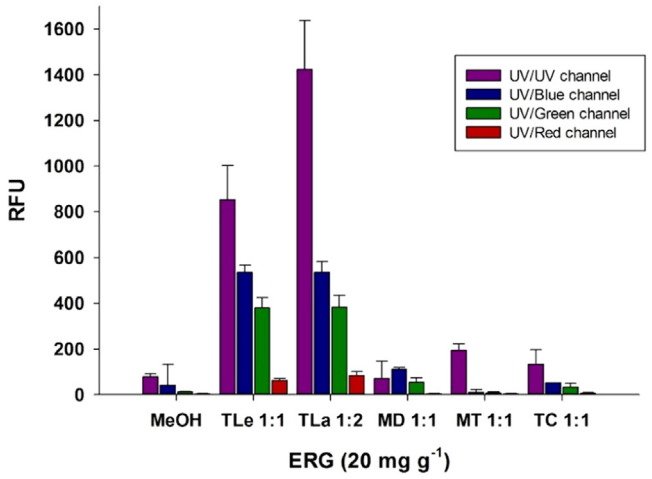


Fig. 1. Comparison of the fluorescence behavior of ERG (20 mg g^{-1}) measured in different systems (methanol and HPB-FLUODES). The bars correspond to: UV/UV channel (violet bars), UV/Blue channel (blue bars), UV/Green channel (green bars), and UV/Red channel (red bars).

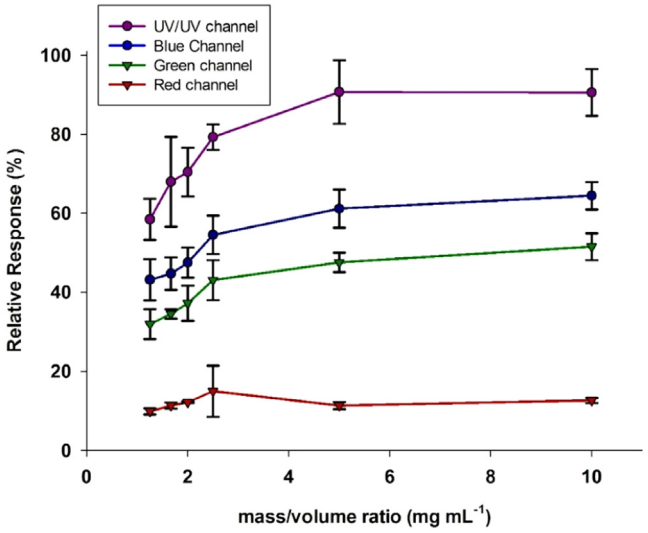


Fig. 2. Effect of the mass/TLa 1:2 ratio on the relative response (%) of ERG. UV/UV channel (violet line), UV/Blue channel (blue line), UV/Green channel (green line), and UV/Red channel (red line).

3.1. Effect of the hydrophobic FLUODES

In this section, the fluorescent behavior of the ERG in the eutectic media was evaluated. Thus, five HPB-FLUODES composed of thymol (T), menthol (M), camphor (C), L (+) lactic acid (La), levulinic acid (Le), and dodecanol (D) were chosen. **Fig. S1** shows the fluorescence of selected HPB-FLUODES. To the best of our knowledge, this is the first time that the intrinsic fluorescence of hydrophobic NADES is described. The five HPB-FLUODES with and without 20 mg g^{-1} of ERG were tested by fluorescence at the four channels of excitation/emission (UV/UV, UV/Blue, UV/Green, and UV/Red). An ERG standard in methanol was prepared to validate the ERG fluorescence without the influence of the HPB-FLUODES. Thus, the RFU obtained for the combination HPB-FLUODES-ERG was subtracted from the RFU for the HPB-FLUODES. The assay demonstrated the exceptional enhancement of the fluorescence intensity observed for ERG in the eutectic systems against its signal in methanol, as can be seen in Fig. 1. The RFU of ERG measured in TLa 1:2 media was the highest value within the five assayed HPB-FLUODES, and this solvent was selected for further experiments, obtaining an improvement factor of 1825%.

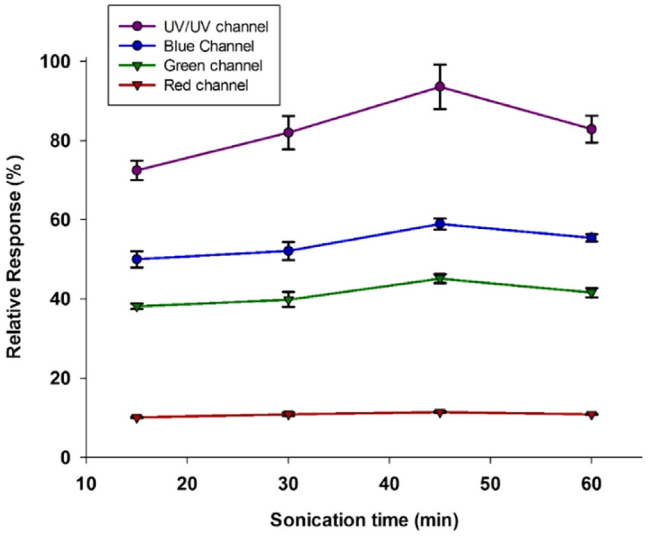


Fig. 3. Effect of the sonication time (min) on the relative response (%) of ERG. Lines correspond to: UV/UV channel (violet line), UV/Blue channel (blue line), UV/Green channel (green line), and UV/Red channel (red line).

3.2. Effect of the mass/volume ratio

The volume of the extracting solvent is a critical variable in the development of analytical methodologies. The overall knowledge is that the solvent volume must be sufficient to ensure that the matrix is entirely immersed [20]. Generally, a higher ratio of solvent volume to solid matrix may be effective in conventional extraction methods [20]. However, in miniaturized strategies, a higher ratio may yield lower recoveries, which may be due to inadequate stirring of the solvent [21]. Based on these considerations, the effect of the sample mass/HPB-FLUODES ratio was studied within the range of 1.25-10 mg mL^{-1}. Fig. 2 shows the RR (%) for ERG at different mass/TLa 1:2 ratios measured at the four channels. It was observed that the highest RR (%) for ERG was achieved with 2.5 mg mL^{-1}. Mass/HPB-FLUODES ratios higher than 2.5 mg mL^{-1} did not show improvements in the analytical response ($p > 0.05$). Indeed, for the red channel, the analytical signal decreased. Thus, 2.5 mg in 1 mL of HPB-FLUODES TLa 1:2 were selected as the optimum value for further assays.

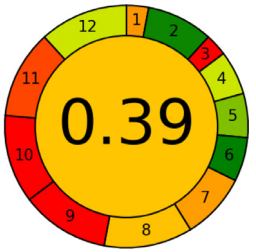
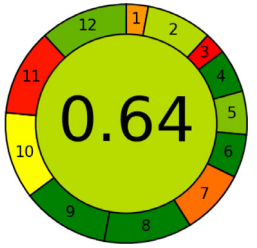
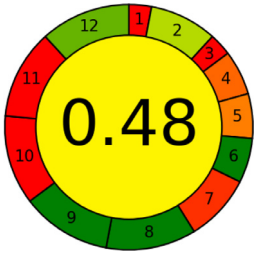
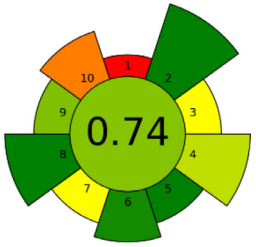
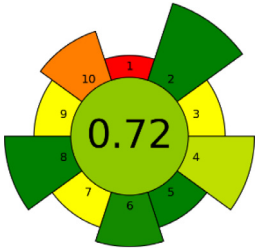
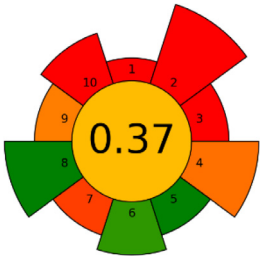
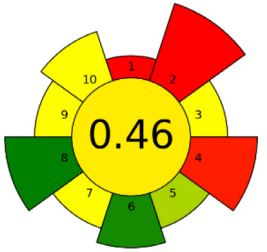
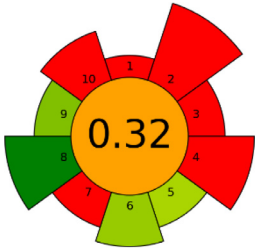
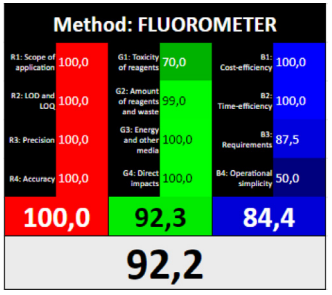
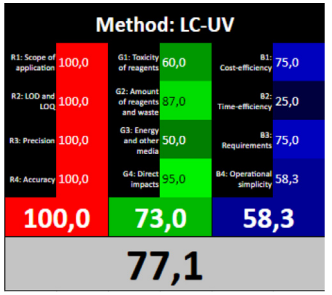
3.3. Sonication time effect

Diverse physical and chemical phenomena including agitation, vibration, shockwaves, and cavitation are responsible for the sonication effect [22]. The bubble cavitation also disrupts the saturated boundary layer surrounding the particles, thus allowing fresh solvent to reach the particle surface, favoring the mass transference of the analytes to the medium and thus improving the efficiency of the UAE technique [22]. Thus, the sonication time was studied within 15-60 min. Fig. 3 shows that the highest RR (%) was observed at 45 min of sonication for the UV/UV, UV/Blue, and UV/Green channels. Values higher than 45 min did not improve the signals ($p > 0.05$). For UV/Red channel, the signal was not significantly different in the studied range ($p > 0.05$).

3.4. Extraction temperature effect

Temperature is another variable that influences the extraction, as elevated values usually enhance the extraction resulting in an increased

Table 2
Comparison of analytical methodologies through the greenness assessment.

	This work		[27]	[16]	[28]
Extraction solvent type	HPB-FLUODES TLa 1:2		Hexane	1.5 M KOH in Ethanol (96% purity)	Methanol:Dichloromethane
Solvent volume (mL)	1		12.5	33.33	75
Mass of mushroom (g)	0.0025		0.1	1	1
Instrumental Analysis	Fluorescence	LC	UPLC-MS	Fluorescence spectroscopy	Square wave voltammetry
Green assessment of the methodology[1]					
Green assessment of the sample preparation[2]					
White Analytical Chemistry[3]			-	-	-

[1] Score description: (1) Sampling procedure, (2) Amount of sample, (3) Position of the analytical device, (4) Number of steps in the sample preparation procedure, (5) Degree of automation and miniaturization, (6) Use of derivatization agents (7) Amount of waste, (8) Number of analytes in a single run and sample throughput (9) Use of enegy, (10) Sources of the reagents, (11) Use of toxic reagents or solvents, and (12) Threats which are not avoided in the methodology.

[2] Score description: (1) Sample preparation placement, (2) Use of hazardous materials, (3) Sustainability and renewability of materials, (4) generation of waste, (5) Size economy of the sample, (6) Sample throughput, (7) Integration and automation, (8) Energy consumption, (9) Post-sample preparation configuration for analysis, and (10) Number of distinct hazards of chemical.

[3] Score description: (R1) Scope of application, (R2) LOD and LOQ, (R3) Precision, (R4) Accuracy, (G1) Toxicity of reagents, (G2) Amount of reagents and waste, (G3) Energy and other media, (G4) Direct impacts, (B1) Cost-efficiency, (B2) Time-efficiency, (B3) Requirements, and (B4) Operational simplicity.

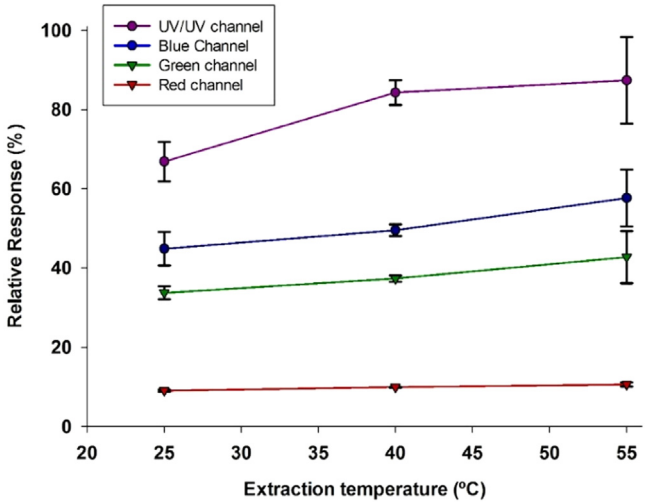


Fig. 4. Effect of the extraction temperature (°C) on the relative response (%) of ERG. Lines correspond to: UV/UV channel (violet line), UV/Blue channel (blue line), UV/Green channel (green line), and UV/Red channel (red line).

diffusivity of the solvent into the internal parts of the matrix under high temperatures boosting desorption of the components from the active sites of the matrix [5]. In this sense, the extraction temperature was studied within 25-55°C. Fig. 4 shows the RR % for the ERG at different extraction temperatures for the four channels. Within the results, the highest RR % was achieved at 40°C for the UV/UV, UV/Blue, and UV/Green channels. For temperatures higher than 40°C, the responses were not significantly different than 40°C ($p > 0.05$). However, for the UV/Red channel, there were no significant differences across all temperatures assayed (($p > 0.05$). Therefore, 40°C was selected as the working extraction temperature.

3.5. Analytical performance and application to mushroom samples

The present work represents the first application of HPB-FLUODES for ERG extraction and determination from food samples taking advantage of the fluorescence enhancement provided by NADES. The analytical methodology was studied in terms of linear range, linearity, accuracy, recovery, precision (relative standard deviation, RSD), and limits of detection and quantification (LOD and LOQ) and validated against LC.

The calibration data were fitted using a linear equation for each channel as follows: UV/UV channel (y=19.04x+279.85), UV/Blue channel (y=13.29x+85.61), UV/Green channel (y=10.62x+116.63), and UV/Red channel (y=1.92x+33.22). The linearity was evaluated by calculating the statistical F value showing good values (Fexp<Fcrit=1.96) within the range of 0.29-200000 μg g^{-1} (dw) for the UV/UV channel. Based on these considerations and the obtained results, the UV/UV channel was selected to quantify the ERG due to the best slope that presents the calibration curve equation and will be used for further calculations.

The LOD and LOQ were calculated according to IUPAC and were 0.09 μg g^{-1} (dw) and 0.29 μg g^{-1} (dw) for LOD and LOQ, respectively. These values are in concordance with the limit content of 15 mg g^{-1} (dw) of ERG proposed by Kadakal [23] and de Sio [10] as the limit of acceptability in tomato products.

A recovery study of ERG was performed using mushroom samples spiked with ERG standard at levels of 20 and 80 mg g^{-1}, resulting in recovery values of 99.9-104% with RSD<2.9%. Moving to sample analysis and in order to demonstrate the feasibility of the proposed methodology as proof of concept for food analysis, ERG was detected in mushroom samples at concentration levels of 94.57±5.12 mg g^{-1}.

The methodology was validated against LC-UV analysis (**Fig. S2**). The calibration data was fitted using a linear equation resulting in y=3181.2x-5764.9. The linearity was evaluated by calculating the statistical F value showing good values (Fexp<Fcrit=1.96) within the range of 0.037-200000 μg g^{-1}. The LOD and LOQ were calculated according to IUPAC and were 0.012 μg g^{-1} (dw) and 0.037 μg g^{-1} (dw), respectively.

3.6. Comparison with other analytical methodologies through the greenness assessment

The present methodology was compared with other analytical strategies reported in the literature for determining ERG in fungal samples. Table 2 summarizes the green assessment using AGREE® [24] and AGREEprep® [25] for selected reports. The references of each score for both AGREE are found at the end of the table.

AGREEprep® Calculator metric tool provides prominence to sample preparation and is based on ten environmental impact categories calculated to 0-1 scale sub-scores and then used to calculate the final assessment score. Each score is related to the solvents, materials and reagents, waste generation, energy consumption, sample size, and throughput. On the other hand, AGREE® calculator metric tool is focused on the entire methodology and is based on the 12 categories of the Principles of Green Analytical Chemistry. Nature and volume of reagents, generated waste, energy requirements, and the number of procedural steps, miniaturization, and automation are considered.

The proposed methodology based on ERG extraction using HPB-FLUODES and determination by Fluorescence resulted in the greenest sample preparation due to the small size of the sample (0.0025 g instead of 1 g), the use of bio-based solvents, and the small volume of HPB-FLUODES. The resulting sample throughput obtained for the proposed methodology was 2.4 samples per minute. Besides, the instrumental technique used in the present work is simple, fast, low-input energy and low-cost analytical instrument. It is possible to observe that the two methodologies based on the use of NADES are the greenest. However, the size of the sample, the volume of the solvent and the instrumental detection defined the final result.

Nowak et al. presented a new approach called "White Analytical Chemistry (WAC)" [26], which considers, unlike AGREE, the analytical parameters, the animals for bioassays, and genetically modified organisms. Table 2 summarized the results of WAC principles. The analytical parameters (red principle) show similar results for both fluorescence and LC-UV analysis. The green evaluation shows that the fluorescence analysis is greener due to lower energy consumption, reagent expenditure, and waste generation. Regarding the principles categorized as blue, it is also observed that the fluorescence-based method presents a better time-efficiency and cost-efficiency relationship.

Likewise, **Table S2** shows the reports obtained from both software's for the developed methodology and those methodologies reported for comparison, and the scores obtained from 12 principles of WAC.

4. Conclusions

In this study, hydrophobic fluorescent natural deep eutectic solvents (HPB-FLUODES) were explored as fluorescent probes to enhance the fluorescent detection of ERG in food samples. HPB-FLUODES TLa 1:2 was applied for the easy extraction and subsequent detection of ERG by fluorescence using Portobello mushroom samples.

The developed methodology was fast, sensitive, and more ecological than others reported in the literature. It was possible to improve the ERG signal by 1825% against the methanol medium, becoming the present methodology a powerful tool to detect fungal contamination in food samples. In addition, high throughput of 2.4 samples per minute was obtained. The analytical figures of merit obtained were LOD and LOQ of 0.09 μg g^{-1} and 0.29 μg g^{-1}, respectively; linearity in the range of 0.29-200000 μg g^{-1} at the UV/UV channel and RSD<2.9%. The greenness of the proposed methodology was evaluated using the AGREE, AGREEprep

software and WAC, obtaining scores of 0.86 and 0.92, and 92.2%, respectively for fluorescence analysis. The field of analytical sensors could greatly profit from the possibility of fine-tuning of target analytes spectral behavior in eutectic systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2022.100026.

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