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Use of a deep eutectic solvent-assisted matrix solid-phase dispersion extraction for phenolic compounds determination from pigmented wheat

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ABSTRACT

In this study, an innovative extraction method for the determination of phenolic compounds in pigmented wheat was developed and optimized. Using deep eutectic solvents (DESs), a greener alternative extraction procedure is offered to the conventional ones based on organic solvents. A matrix solid-phase dispersion (MSPD) extraction was performed and enhanced by the addition of DES in the homogenization step. In comparison with an ultrasound-assisted solid liquid extraction, the MSPD-DES technique allowed to obtain a higher extraction efficiency reducing organic solvent consumption and environmental impact. Although different DESs were tested, the one based on choline chloride and glycerol (1:2 molar ratio) showed the best extraction yield of phenolic compounds. A three-level four-factor Box-Behnken design optimization was performed: sample-to-dispersing material ratio, DES volume, extraction solvent volume, and number of cycles were evaluated. Under the optimal conditions a total phenolic content (TPC) of 3.562 ± 0.005 mg GAE/g was achieved. The AGREEprep, BAGI, and ComplexMoGAPI indicators confirmed the environmental sustainability of the proposed methodology. The qualitative profile of the extracted phenolic compounds was determined using high-performance liquid chromatography-photodiode array detector.

1. Introduction

As one of the most important crops worldwide, wheat is part of the daily diet of more than a third of the global population [1]. In addition to providing the main nutrients such as carbohydrates and proteins, wheat also contains a significant number of bioactive compounds such as flavonoids, carotenoids, phenolic acids, and anthocyanins [2].

Pigmented wheat has unusual genotypes compared to conventional ones. The term "pigmented" refers to the wheat's caryopsis (the outer part of the wheat) colours, which range from purple to blue to intense red [3]. This colour is due to the presence of anthocyanins.

In addition, pigmented wheat contains other groups of bioactive molecules such as phenolic acids and carotenoids [3]. Generally, all these substances are present in lower quantities in traditional wheat. The distribution of bioactive molecules varies in the wheat, decreasing from the outermost part to the inner part; also, the type of phytochemicals changes in the different wheat parts [4].

Bioactive compounds, in addition to their normal nutritional properties, have positive effects on human health [5]. Thanks to the higher content of antioxidants, this type of wheat is useful for reducing both oxidative stress and the risk of chronic diseases [6]. Due to this, there has been a great interest among customers in cereal-based products in recent years. Therefore, a profound development of research and experimentation activities aimed at increasing the content of nutritional substances in the caryopsis.

Phenolic acids are organic compounds belonging to the polyphenol family. They are divided into two main categories, according to their structure: benzoic acids and cinnamic acids [7]. Furthermore, phenolic acids are categorized into free and bound but the antioxidant capacity of these bioactive molecules is largely derived from bound phenolic [8].

Among phenolic acids present in wheat, ferulic acid is the most abundant and constitutes the principal antioxidant in purple-pigmented wheat [9]. Other important bioactive compounds present in pigmented wheat are gallic acid, p-coumaric, kaempferol, catechin and vanillic acid [10].

The traditional white wheat flour, where the bran is removed

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following refining processes, does not contain phenolic compounds [11].

The various types of pigmented wheat grains (black, red or purple) are influenced by several environmental and genetic factors that affect the amount of pigments present, such as anthocyanins, carotenoids or other phenolic compounds.

The genetics behind pigmentation in wheat are complex, with multiple genes involved in the anthocyanin biosynthetic pathway. Recent research has identified key regulatory genes, such as MYB and bHLH transcription factors, that modulate the expression of anthocyanin-producing enzymes in wheat grains [12].

This genetic understanding paves the way for targeted breeding efforts to develop pigmented wheat varieties that meet consumer demands for functional foods while maintaining desirable agronomic traits.

The main environmental factors affecting bioactive compounds in wheat grains are sunlight, temperature, soil quality and water stress [13].

Phenolic compounds extraction from wheat is increasingly being investigated as they are bioactive compounds with antioxidant properties and therefore with beneficial effects on human health [14].

Depending on whether they are in free or bound form, the extraction of phenols involves the use of different analytical techniques to optimize the recovery of these substances. In fact, while bound phenolic compounds require hydrolysis to break the interactions with polysaccharides or proteins, free phenolic compounds are generally soluble in organic solvents [15].

In this work, a traditional ultrasound-assisted extraction (UAE) was compared with a matrix solid-phase dispersion (MSPD) extraction. The latter one exploits the interaction between the sample and a dispersed solid phase, generally an adsorbent such as silica gel or Florisil® [16]. Mechanical crushing of the sample and dispersing material ensures an availability increase of analytes of interest, breaking the matrix itself.

Innovative applications of this extraction technique involve the addition of deep eutectic solvents (DESs) that can be used at different stages of the procedure [17]. DESs are neoteric solvents consisting of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), often in the form of salts capable of forming a stable liquid at room temperature thanks to the formation of strong non-covalent interactions, such as hydrogen bonds [18]. This phenomenon is due to a “deep” lowering of the real melting point of the mixture from the ideal one [19].

Several studies in the literature report how DESs can show great extractive abilities toward bioactive molecules when incorporated during the homogenization step of an MSPD [20]. Considering these assessments, this work aimed to design a green analytical method based on DESs for identifying phenolic compounds in pigmented wheat. Compared to simple UAE, the proposed MSPD with DESs added in the homogenization phase showed significant improvements in extraction yield.

High-performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA) was employed to determine the profile of phenolic compounds.

A complete optimization of the extraction procedure was performed, considering qualitative and quantitative aspects. Then, a four-factor three-level Box-Behnken Design (BBD) [21] was used for the further identification of optimal extraction conditions. The compliance of the method with the ten principles of green sample preparation [22] was also investigated calculating AGREEprep [23], blue applicability grade index (BAGI) [24] and complex modified green analytical procedure index (ComplexMoGAPI) [25] overall scores.

To the best of our knowledge, the present work is the first capable of extracting phenolic compounds from pigmented grains with the addition of a DES in the homogenization step and with the use of low amounts of organic solvents.

2. Materials and methods

2.1. Chemicals

Solvents and reagents employed for the extraction procedure and for HPLC–PDA analyses were acetonitrile (ACN; ≥ 99.9 %), methanol (MeOH; ≥ 99.9 %), ethanol (EtOH; ≥ 99.8 %), hexane (≥ 99.8 %), water (HPLC-MS grade), formic acid (≥ 95 %) and Florisil®, all obtained from Sigma-Aldrich (Milan, Italy). Gallic acid, ferulic acid, kaempferol, kaempferol 3-O-rutinoside, protocatechuic acid and catechin standards were also purchased from Sigma-Aldrich (Milan, Italy). An analytical balance (Mettler Toledo MS105DU, Capacity 120/42 g; resolution 0.1/0.01 mg) was used to weight the standard powders. Subsequently, 1 mg mL^{-1} stock solutions were prepared in MeOH. All the solutions were stored at −80 °C.

As far as concern the preparation of DESs, betaine, choline chloride (ChCl), glycerol (Gly) and ethylene glycol (EG) were provided by Carlo Erba (Milan, Italy).

Finally, the chemicals used for the Folin-Ciocalteu test — gallic acid (97.5 %), sodium carbonate (>99.5 %), and Folin-Ciocalteu reagent — were also purchased from Sigma-Aldrich (Milan, Italy).

2.2. Samples

Triticum turgidum L. subsp. durum genotype, named 15,124, derived from a breeding programme between the elite cultivar Primadur and the pigmented cultivar T1303 (USDA code PI 352,395), was used in the current study.

2.3. Preparation of DESs

HBAs and HBDs used for the DES screening are reported in Table 1. Mixtures of HBA and HBD were heated to 80 °C until a colorless liquid was obtained. After that, the DESs were left to cool to room temperature before being used for the extraction procedure.

2.4. UAE and MSPD extraction procedures

A UAE procedure was developed and compared with an MSPD.

For both the extraction procedures, wheat was ground before starting to reduce the sample size and to increase its surface area. A defatting phase was carried out by adding 1 mL of hexane to 0.1 g of previously ground wheat, to eliminate the lipid component of the matrix [26].

UAE of phenolic compounds from pigmented wheat was carried out using the following extraction procedure: once the hexane was removed, 1 mL of an extraction solvent consisting of MeOH-water (80:20 v/v) [3] was added to the sample. Subsequently, the mixture was subjected to ultrasonic treatment in a bath (Elmasonic Series S; Elma Schmidbauer GmbH, Singen, Germany) for 10 min at room temperature.

The MSPD procedure was conducted as follows: 0.1 g of defatted wheat was grinded in a mortar with 0.3 g of Florisil® as sorbent phase (sample-to-dispersing material ratio: 1:3, w/w), with the further addition of 87.65 μL of ChCl-Gly (1:2) DES. The resulting powder was used to pack an empty 6-mL SPE cartridge, and the analytes elution was carried out by adding 1.42 mL of a MeOH-water (80:20 v/v) mixture. At the end, both the UAE and MSPD extracts were filtered with 0.2 μm Whatman nylon membrane filter. Extracts were subjected to a Folin-Ciocalteu assay to determine the total phenolic content (TPC) and to HPLC-PDA

Table 1
HBA, HBD, and molar ratio of DESs used during the MSPD extraction procedure.

DES	HBA	HBD	MOLAR RATIO
ChCl-Gly	Choline chloride	Glycerol	1:2
ChCl-EG	Choline chloride	Ethylene glycol	1:2
Bet-Gly	Betaine	Glycerol	1:2

analysis for the qualitative identification of phenolic compounds.

2.5. *Determination of total phenolic content (TPC)*

To determine the TPC of the pigmented wheat, the indirect colorimetric assay of Folin-Ciocalteu [27] was performed.

Simply, 20 μL of sample extracts were added to 1580 μL of water. The blank was prepared in 1600 μL of water. Then 100 μL of Folin-Ciocalteu reagent was added. Subsequently the mixture was stocked at room temperature in the dark for 8 min. Then 300 μL of sodium carbonate solution (0.2 g/mL) was added and incubated for 2 h in a dark environment at room temperature. After this, 200 μL of each extract were taken and tripled into the 96-well Greiner plate.

The absorbance was measured with the Infinite M200 PRO Tecan microplate spectrophotometer (Tecan Trading AG, Switzerland) at 765 nm.

Gallic acid was used as the standard to prepare a calibration curve in the range of 0–2000 μg mL^{-1}. The concentrations of phenolic compounds were reported as mg of gallic acid equivalents (mg GAE) per gram of dry weight of pigmented wheat.

2.6. *OFAT optimization of the extraction procedure*

In the optimization phase of the extraction procedure, both the type of solvent and the extraction technique were evaluated through a one-factor-at-a-time (OFAT) optimization. TPC determination was used as response.

Firstly, a UAE extraction procedure was developed, and a series of experiments were performed to select the best extraction solvent to use.

UAE was conducted as follows: 1 mL of MeOH-water (80:20, v/v) was added to 0.1 g of ground and defatted wheat in a 15 mL centrifuge tube and the mixture was placed in an ultrasonic bath for 10 min at room temperature. The same UAE already mentioned was replicated using another extraction solvent, consisting of ethanol:water:formic acid (97:2:1 v/v/v). Maintaining the same extraction conditions, an MSPD procedure was carried out as follows: a quantity of 0.1 g of defatted pigmented wheat was grinded in a mortar with 0.4 g of Florisil® and *An spe* cartridge was packed with the resulting powder. Then, 1 mL of MeOH-water (80:20, v/v) was used for analytes elution.

TPCs of both UAE and MSPD extracts were evaluated by using the Folin-Ciocateau spectroscopic assay, reading the absorbance at 765 nm.

To increase the MSPD extraction yield, an important qualitative parameter was optimized, the choice of DES type to be added during the homogenization phase, among ChCl-Gly, ChCl-EG and Bet-Gly, all in a 1:2 molar ratio.

Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA) was used for all calculations. GraphPad Prism 10.0 software was used to perform one-way ANOVA (analysis of variance) and Tukey's multiple comparisons tests. p-values of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were considered significant.

2.7. *Box–Behnken experimental design and data analysis*

For the experimental design of the extraction of phenolic compounds from pigmented wheat, the statistical model of Box-Behnken Design (BBD) [21] was used. Specifically, sample-to-dispersant ratio, volume of DES, volume of extraction solvent and number of extraction cycles were selected as factors to optimize. Each factor was evaluated at three levels: low (−1), central (0) and high (+1), allowing linear, quadratic and interaction effects to be assessed. Each variable was codified according to the following equation:

$$x_i = \frac{x_i - x_0}{\partial x}$$

where x_i is the coded value, X_i is the uncoded value of the variable, X_0 is the value of X_i at the central point, and ∂x is the step change.

The parameter that was considered as a response of the statistical model was the TPC.

The four-factor statistical model with three levels each showed five replicates at the central point to ensure extraction reproducibility.

The following second-degree polynomial equation correlating the independent variables with the response can be expressed as:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{11} x_2 + \beta_{22} x_{22} + \beta_{33} x_{23} + \beta_{44} x_{24}$$

where Y is the response, x_1, x_2, x_3, x_4 are the independent variables, β_0 is the model intercept coefficient; β_1, β_2, β_3, β_4 are the linear coefficients, β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are the interaction coefficients, and β_{11}, β_{22}, β_{33}, β_{44} are the quadratic coefficients. R^2 and R^{2}adj were considered to evaluate the efficiency of the model. Experimental design, mathematical model, and optimization were performed through the R Studio software using the "rsm" package [28].

2.8. *HPLC–PDA analysis of phenolic compounds*

The phenolic profile of the extract obtained was defined by chromatographic analysis coupled to PDA spectrophotometry using a Vanquish™ UHPLC System from Thermo Fisher Scientific (Waltham, Massachusetts, USA) equipped with a binary pump, an autosampler, a column oven, and a degasser.

An Ascentis® C18 column, 15 cm * 4.6 mm, 3 μm particles (Merck KGaA, Darmstadt, Germany), at the temperature of 40 °C was used for the analysis. The mobile phase (A, water 1 % aqueous formic acid and B, acetonitrile) flow rate was 1 mL min^{-1}. The studied compounds were separated using the following gradient: t_0 = 30 % B, $t_0 - t_{10}$ = 100 % B, $t_{10} - t_{20}$ = 100 % B. The injection volume was 2 μL. Data were acquired using a PDA with a wavelength detection of 280 nm for the extracts. Qualitative analysis was performed considering retention time of available standard molecules and comparison with data present in literature.

2.9. *Sustainability evaluation*

Sustainability of the optimized method was evaluated by calculating AGREEprep, BAGI and ComplexMoGAPI overall scores.

AGREEprep software [23] evaluates the procedure's adherence to the ten GSP principles [22]. The AGREEprep tools encompass a series of principles, including a preference for in situ sample preparation (1), the use of safer solvents and reagents (2), the employment of sustainable, reusable, and renewable materials (3), the minimization of waste (4) and sample amounts, chemicals, and materials (5), the maximization of productivity (6), the integration of steps and the promotion of automation (7), the reduction of energy consumption (8), the selection of the most sustainable post-sample preparation analytical configuration (9), and the implementation of safe procedures for the operator (10). Each principle is rated on a scale from 0 (lowest) to 1 (highest) and each category has a default weight in the calculation of the overall score. The bars show the criteria and are colored according to the criterion's positive or negative influence (from red for low values to green for high values). Each criterion is assigned a length based on its assigned weight. The center shows the overall score (ranging from 0 to 1), with a color ranging from red for low sustainability to green for ideal results.

BAGI [24] metric tool assesses how well an analytical method meets specified criteria and the overall score was also calculated. The system is based on ten main attributes, including the type of analysis (1), the number of analytes determined simultaneously (2), the analytical technique and instrumentation required (3), the number of samples that can be processed simultaneously (4), sample preparation (5), the number of samples that can be analyzed per hour (6), the type of reagents and materials used in the analytical method (7), the need for preconcentration (8), the degree of automation (9), and the sample quantity

(10). The BAGI pictogram uses four blue shades to indicate compliance levels: white (no compliance), light blue (low compliance), blue (medium compliance), and dark blue (high compliance). The result is a star-shaped pictogram, in which each point corresponds to an indicator of the method with the final score ranging from 25 (worst) to 100 (best) reported in the center.

Finally, ComplexMoGAPI [25] overall score was evaluated. This diagram shows an evolution of the original ComplexGAPI diagram, with its multi-sectional geometric shape and advanced scoring system. The pictogram has colored polygonal segments representing stages of the analytical process.

The sample preparation involves collection (1), preservation (2), transport (3), and storage (4). The type of method (5), scale of extraction (6), solvents or reagents used (7), and any additional treatment (8) are also specified. Reagents and solvents are considered in terms of amount (9), health hazard (10), and safety hazard (11). Instrumentation includes energy use (12), occupational hazard (13), waste generated (14), waste treatment (15), and quantification (16). Yield (I) and temperature/time conditions (II) are considered. The relation to green chemistry is evaluated by the number of principles met (III). Reagents and solvents are further assessed for health (IVa) and safety hazards (IVb), while instrumentation considers technical setup (Va), energy (Vb), and occupational hazard (Vc). Workup and purification cover processing of the final product (VIa) and its purity (VIb) are also evaluated.

Each segment of the pictogram is assigned a color based on the environmental performance of that stage: green signifies high sustainability and adherence to green chemistry principles, yellow moderate impact, and red poor performance or high hazard. An oval mark at the core differentiates methods that permit both qualitative and quantitative determinations. Additionally, a numerical score derived from all evaluated parameters may be displayed to quantify the overall greenness of the procedure. However, by generating an easy-to-read percentage score, shown at the bottom of the graph, this index makes it possible to evaluate the overall environmental sustainability of an entire analytical method, from sampling to final instrumental determination.

3. Results and discussion

3.1. Screening of the extraction solvent

To determine the most suitable solvent for extracting phenolic compounds from pigmented wheat, a screening of extractive solvents was performed. The aim was to maximize the efficiency of extraction for the target compounds.

The UAE procedure was conducted as follows: 1 mL of an extraction solvent was added to 0.1 g of grinded and defatted wheat, and the mixture was placed in an ultrasonic bath for 10 min at room temperature.

Two different extraction solvents were tested: a mixture of MeOH and water (80:20 v/v) and a mixture found in the literature [29] composed of ethanol, water and formic acid (97:2:1 v/v/v). The mixture reported by Gozzi et al. [29] used ethanol as it is a less toxic solvent than MeOH, with the addition of formic acid that exploits the synergistic properties of water and MeOH.

As shown in Fig. 1, MeOH:water (80:20, v/v) provided a higher extraction yield with a statistically significant difference if compared to the EtOH:water:formic acid mixture. This can be explained by the fact that MeOH:water mixture offered a higher overall polarity than ethanol-water-formic acid, making it more effective for the extraction of different classes of phenolic molecules. In addition, the MeOH-water mixture did not require additional acidification, simplifying the extraction process and reducing the need for purification steps.

3.2. Choice of the extraction procedure

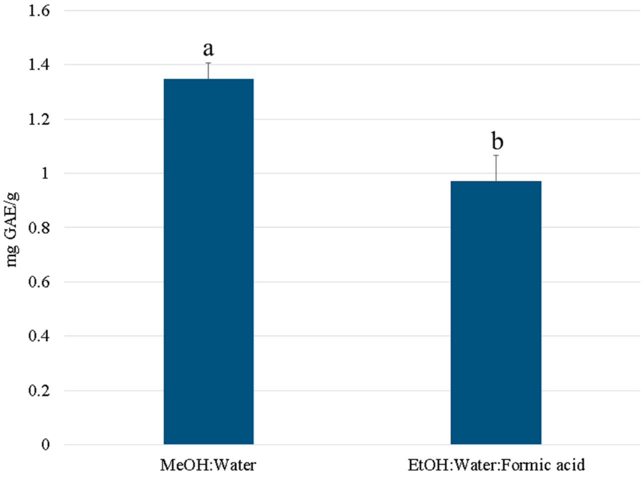


Fig. 1. Screening of the extraction solvent.

To further increase the extraction yield, an MSPD extraction procedure was applied. The same extraction solvent optimized with the UAE procedure was used as eluent phase during the MSPD extraction.

As shown in Fig. 2, MSPD proved to be the best extraction technique in terms of TPC from pigmented wheat. The histograms show that the quantity of phenolic compounds extracted with a traditional UAE was (1.39 ± 0.04) mg GAE/g of dry weight while with the MSPD procedure the extraction yield reached a TPC of (2.13 ± 0.04) mg GAE/g of dry weight.

Other studies have shown TPCs comparison between the white and colored lines of wheat [26,30].

In a comparative study of colored grain lines to assess both TPC and antioxidant capacity conducted by Sharma et al. [31], the TPC of the various wheat samples ranged from 0.57 to 3.28 mg GAE/g. The analyses highlighted the considerable variation in TPC between the blue, purple, and black colour lines and compared with traditional wheat. Data showed significantly higher TPC (> 3.28 mg GAE/g) in all colored lines than white grain lines (> 1.63 mg GAE/g).

The potential of MSPD-based techniques for extracting phenolic compounds from a wide range of matrices has been demonstrated in several studies, confirming the versatility of this method compared with conventional extraction procedures. For example, Fariñas-Mera et al. [32] investigated the use of MSPD to recover antioxidant polyphenols from apple pomace. Employing sand as the dispersing agent and an ethanol/water mixture (50:50, v/v) as the eluent, they achieved a TPC of

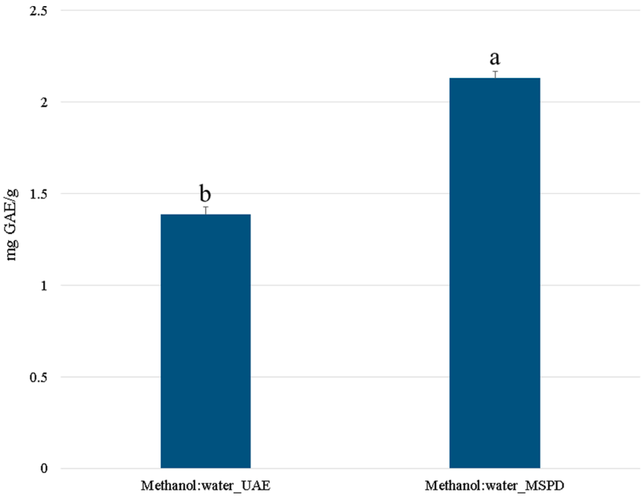


Fig. 2. Comparison between MSPD and UAE using the same extraction solvent MeOH-water (80:20 v/v).

14.61 ± 3.87 mg GAE/g dry weight—about 66.5 % of the yield obtained by conventional solvent extraction (CSE)—while using less solvent and requiring only 30 min of extraction at room temperature. Likewise, Awlqadr et al. [33] summarized MSPD applications in plant materials such as Lemon Balm (*Melissa officinalis*), emphasizing that the method provides high recovery of phenolics with minimal solvent consumption and energy demand. Further evidence comes from vortex-assisted MSPD using a ChCl:Lactic acid (2:1) solvent system applied to *Helichrysum arenarium* L. extracts, which yielded a TPC of 38.34 ± 0.09 mg GAE/g dry weight—higher than that obtained by UAE with a MeOH/water (80:20, v/v) system (32.73 ± 0.52 mg GAE/g dry weight)—while substantially reducing the environmental footprint [34].

3.3. Screening of the best DES for the MSPD extraction procedure

During MSPD, Florisil® was mixed with the solid sample. This dispersant facilitates the breakdown of matrix structure, making target compounds more accessible by increasing the extraction yield as shown in Fig. 2.

In the study of Ivanović et al. [34], the use of DESs during the MSPD extraction procedure was already analyzed; however, an innovative aspect of the MSPD procedure concerns the use of a volume of DES added in the mortar during the homogenization phase.

After demonstrating that MSPD was the most efficient technique, one more qualitative parameter was studied: the choice of DES to be added during the homogenization phase. To perform this evaluation, 0.1 g of defatted pigmented wheat was put in contact with 0.4 g of Florisil® and 50 µL of different DESs. The obtained mixture was used to pack an empty SPE cartridge and a volume of 1 mL of MeOH-water (80:20, v/v) was used to elute analytes.

Fig. 3 reports the effect of different DESs on the extraction efficiency of TPCs. The use of green solvents has proved to be an important choice in terms of increasing the extraction yield. The best results were obtained using ChCl-Gly. Indeed, DESs can form non-covalent bonds with phenols such as hydroxybenzoic acids, and flavonoids, increasing their extraction efficiency [35].

3.4. Box–Behnken design and data evaluation

To minimize the number of experiments and to perform the BBD through a multivariate optimization, the parameters that most influence the effectiveness of the MSPD were identified.

For this reason, the following parameters have been selected: sample-to-dispersing material ratio (w/w), volume of DES, extraction solvent volume and number of extraction cycles. Each factor was made to vary in a range that has an upper, lower and middle value. For the elution of analytes usually a single elution cycle is performed, but in some cases, multiple cycles may be required to achieve a higher recovery of analytes.

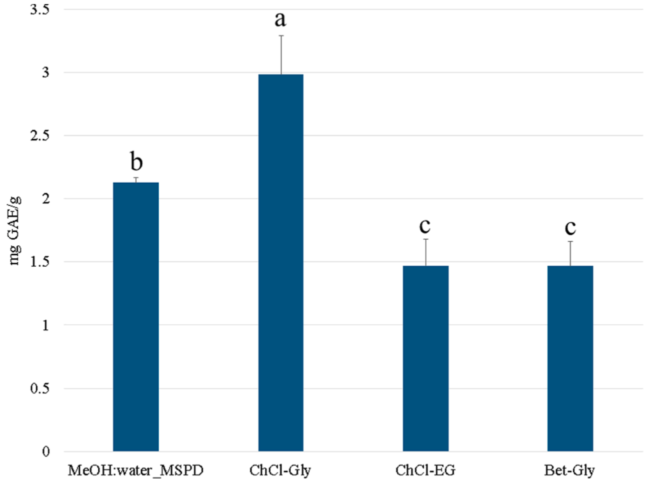


Fig. 3. Evaluation of the best DES able to improve the extraction yield.

To establish the optimal extraction conditions for phenolic compounds in pigmented wheat, TPC was selected as the response parameter. Table 2 shows selected variable symbols and values in coded and uncoded forms.

A total of twenty-nine experiments were performed through the statistical model, and they are reported in Table 3.

As shown in Table 3, five replicates were carried out at the central point (running order 4, 5, 19, 21, 24) and the TPC values obtained varied from 1.916 mg GAE/g to 2.541 mg GAE/g.

A variability percentage of 94.26 % (R^2: 0.9426) can be explained by the predicted model, while an acceptable correlation between the predicted and the experimental responses was shown by the R^2_{adj} coefficient, whose value was 0.8851.

The coefficients of the statistical model and their significance are shown in Table 4.

The p value ≤0.001 for the intercept, x1, x3 and x4 show that they are the most statistically significant. A $p \leq 0.01$ was observed for x1:x4, demonstrating that it is highly statistically significant. A $p \leq 0.05$ was observed for x1:x3, x1ˆ2 and x2ˆ2. With a p-value of 0.058, the variable x2 has a less significant effect. At the end, no statistical significance ($p >$ 0.1) was observed for x2:x3, x2:x4, x3:x4, x3ˆ2 and x4ˆ2.

The model shows that the variables x1, x3, and x4 have a large impact on extraction yield, as well as some interactions (x1:x3 and x1:x4) and quadratic terms (x1ˆ2 and x2ˆ2).

Fig. 4 shows response surface plots of extraction yield.

Figs. 4a, 4b and 4c show that increasing the sample-to-dispersing material ratio does not result in significant changes in the extraction yield. The same trend was seen for the volume of the extraction solvent (Figs. 4a, 4d and 4e), as the response surfaces show a slight curvature, but the effect does not seem as pronounced compared to other graphs. From Figs. 4c, 4e and 4f, the decrease in yield with the variable "Cycle" suggests that many cycles might reduce the efficiency of the process. Nevertheless, it is important to emphasize that increasing the volume of DES (Figs. 4b, 4d and 4f) has a strong positive impact on the extraction yield, confirming the innovative aspect carried forward in the present work.

The optimal extraction conditions resulted to be: 1:3 as sample-to-dispersing material ratio, 87.65 µL as DES volume, 1.42 mL as extraction solvent volume and 1 as extraction cycles number.

To confirm the extraction yield predicted by the statistical model, additional experiments were performed using the optimal conditions set. The TPC predicted by the model of 3.7 mg GAE/g of pigmented grains was confirmed experimentally by performing the optimized MSPD procedure and obtaining a TPC of (3.562 ± 0.005) mg GAE/g of dry weight.

3.5. HPLC–PDA extracts qualitative analysis

The phenolic profile of the extract obtained was determined by chromatographic analysis combined with UV–Vis spectrophotometry following experimental conditions reported in paragraph 2.8. To identify the analytes within the extract, literature data, and retention time were considered. In addition, the extract was spiked separately with

Table 2
Coded levels and symbols for the chosen variables.

Variables	Symbol		Coded levels		
	Uncoded	Coded	−1	0	+1
Sample-to-dispersant ratio (w/w)	SD	x1	2	4	6
Extraction solvent (mL)	Solvent	x2	1000	1500	2000
DES (µL)	DES	x3	0	50	100
Cycles	Cycles	x4	1	2	3

Table 3
Twenty-nine runs proposed by the Box–Behnken Design.

Run.order	Std.order	SD	Solvent	DES	Cycles	Experimental	Predicted	Difference
1	3	2	2000	50	2	1.633	1.776	8.7
2	17	2	1500	0	2	0.816	0.692	−15.2
3	6	4	1500	100	1	3.188	3.672	15.2
4	29	4	1500	50	2	2.008	2.266	12.9
5	28	4	1500	50	2	2.341	2.266	−3.2
6	5	4	1500	0	1	1.008	1.112	10.3
7	7	4	1500	0	3	0.758	0.418	−44.8
8	9	2	1500	50	1	3.333	3.263	−2.1
9	22	4	2000	50	1	2.044	2.137	4.5
10	21	4	1000	50	1	2.672	2.304	−13.8
11	23	4	1000	50	3	1.794	1.556	−13.3
12	1	2	1000	50	2	2.316	2.494	7.7
13	20	6	1500	100	2	2.025	2.004	−1.1
14	13	4	1000	0	2	0.388	0.668	72.2
15	18	6	1500	0	2	0.450	0.675	49.9
16	10	6	1500	50	1	1.508	1.266	−16.1
17	8	4	1500	100	3	2.375	2.416	1.7
18	14	4	2000	0	2	0.755	0.610	−19.2
19	25	4	1500	50	2	1.916	2.266	18.3
20	19	2	1500	100	2	4.291	3.921	−8.6
21	26	4	1500	50	2	2.541	2.266	−10.8
22	15	4	1000	100	2	3.138	3.283	4.6
23	11	2	1500	50	3	1.016	1.259	23.9
24	27	4	1500	50	2	2.525	2.266	−10.2
25	16	4	2000	100	2	2.833	2.553	−9.9
26	12	6	1000	50	2	1.200	1.202	0.2
27	2	6	1500	50	3	1.250	1.320	5.6
28	4	6	2000	50	2	1.166	1.132	−2.9
29	24	4	2000	50	3	0.711	0.934	31.4

Table 4
Box-Behnken design regression coefficients and their significance.

Variables	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	2.2662	0.147993	15.3129	3.87E-10	***
x1	−0.483833	0.095529	−5.0648	0.0001725	***
x2	−0.197167	0.095529	−2.0639	0.0580708	.
x3	1.139583	0.095529	11.9292	1.01E-08	***
x4	−0.487417	0.095529	−5.1023	0.000161	***
x1:x2	0.16225	0.165461	0.9806	0.3434357	
x1:x3	−0.475	0.165461	−2.8708	0.012334	*
x1:x4	0.51475	0.165461	3.111	0.007663	**
x2:x3	−0.168	0.165461	−1.0153	0.3271681	
x2:x4	−0.11375	0.165461	−0.6875	0.5030152	
x3:x4	−0.14075	0.165461	−0.8507	0.4092751	
x1ˆ2	−0.285433	0.129933	−2.1968	0.0453744	*
x2ˆ2	−0.329683	0.129933	−2.5373	0.0236924	*
x3ˆ2	−0.157808	0.129933	−1.2145	0.2446332	
x4ˆ2	−0.203808	0.129933	−1.5686	0.1390699	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

each standard compound.

Fig. 5 shows a HPLC-PDA chromatogram (λ = 280 nm) of the pigmented wheat extract.

Three different phenolic acids have been tentatively identified: gallic acid derived from benzoic acid, ferulic acid and protocatechuic acid derived from cinnamic acid. In addition, among the class of flavonoids were identified catechin (flavanols) and kaempferol and kaempferol 3-o-rutinoside which belong to the flavonols, another subclass of flavonoids.

3.6. *Greenness evaluation*

AGREEprep, BAGI and ComplexMoGAPI overall scores were calculated and are shown in Fig. 6.

The overall AGREEprep score was 0.6. *Ex situ* sample preparation (criterion 1), use of MeOH (criterion 2) and amount of waste generated (criterion 4) influenced the environmental impact. Although some of the materials used are sustainable or renewable, these are not reusable, further limiting the "greenness" of the method (criterion 3). However, the small sample quantities (criterion 5), the ability to process many samples per hour (criterion 6), the low number of steps (criterion 7) with almost no energy consumption (criterion 8), the use of HPLC-PDA (criterion 9) and the limited number of chemical hazards (criterion 10) made the method very efficient.

The BAGI score of 77.5 showed that the chosen analytical method has an excellent degree of practicability. Even though DES must be synthetized in lab (criterion 7), the MSPD method (criterion 5) allowed quantitative analysis (criterion 1), the use of HPLC-PDA (criterion 3) and small sample quantities (criterion 10), ensuring multiple compounds to be analyzed simultaneously (criterion 2) without any preconcentration (criterion 8). In addition, many samples can be analyzed per hour (criteria 4 and 6) with the presence of partial automation (criterion 9).

ComplexMoGAPI tool showed that the method led to a good operational profile with low energy consumption and favorable experimental conditions (room temperature, <1 h). Other favorable sustainability aspects concerned the scale of microextraction and the absence of preservation and storage steps. Critical environmental and safety issues were related to the use of MeOH, the release of vapors and the lack of waste treatment. However, the method satisfied most green chemistry principles.

3.7. *Comparison with other methods in the literature*

Table 5 shows the comparison between UAE and MSPD for phenolic compounds extraction from different matrices, including apple pomace, *Helichrysum arenarium* L., and *Triticum turgidum* subsp. *durum*, also evaluating sustainability using the AGREEprep tool.

As far as concern apple pomace [32], UAE provided a TPC of 21.96 ± 0.82 mg GAE/g, which is higher than the 14.61 ± 3.87 mg GAE/g TPC obtained with MSPD. However, MSPD showed greater sustainability (AGREEprep = 0.66) than UAE (0.62), thanks to lower solvent consumption and milder extraction conditions.

For *Helichrysum arenarium* L [34]., NADES-assisted MSPD produced a higher TPC (38.34 ± 0.09 mg GAE/g) than UAE (32.73 ± 0.52 mg GAE/g) and a higher environmental profile (0.68 vs. 0.44).

Finally, for *Triticum turgidum* subsp. *durum* (this work), an increase

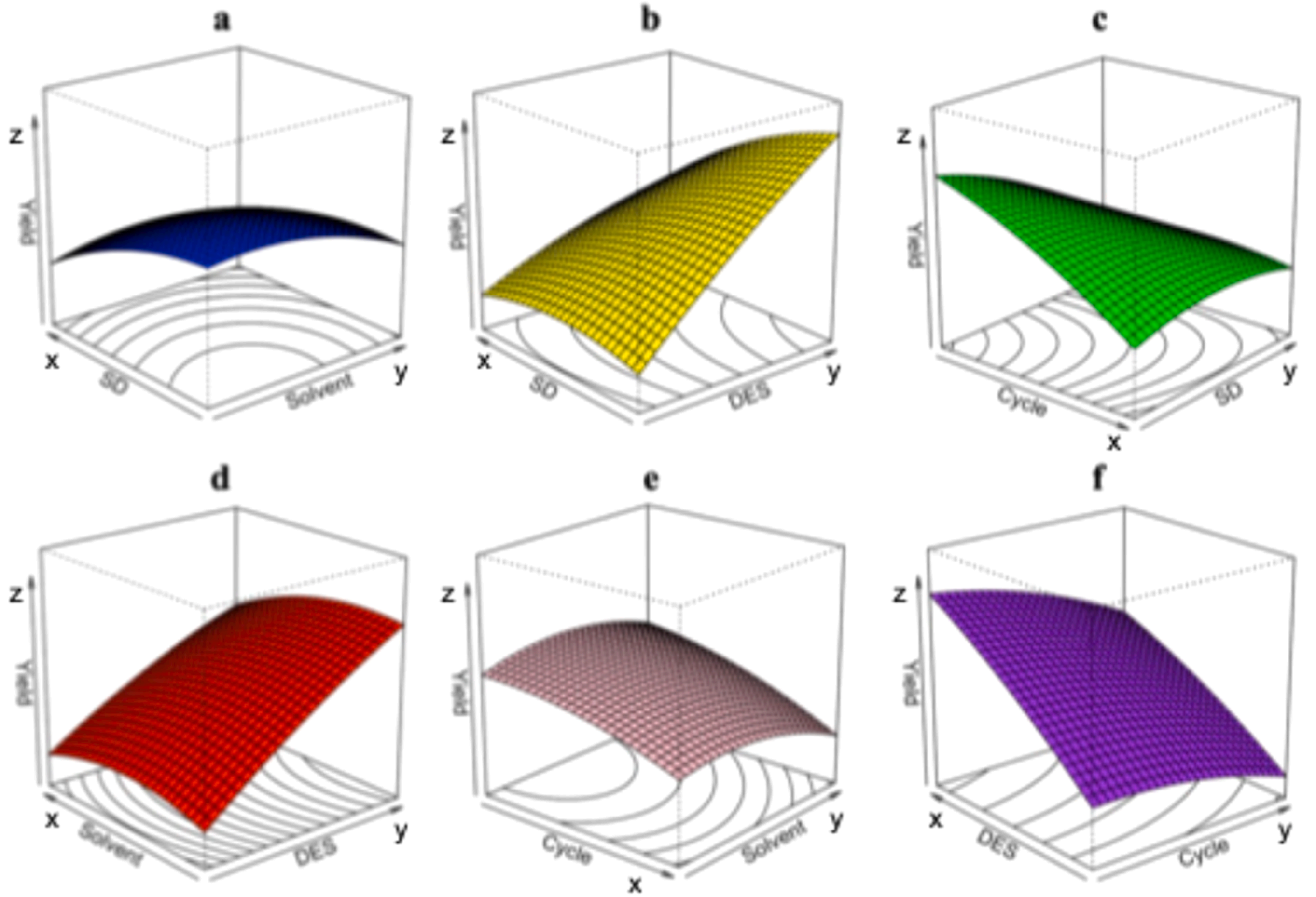


Fig. 4. Response surface plots of extraction yield as affected by SD ratio and solvent volume keeping DES volume at 50 μL and cycles at 2 (a); SD ratio and DES volume keeping solvent volume fixed at 1.5 mL and cycles at 2 (b); cycles and sample-to-dispersing material ratio keeping solvent volume fixed at 1.5 mL and DES volume at 50 μL (c); solvent and DES volumes keeping SD at 1:4 and cycles at 2 (d); cycles and solvent volume keeping SD at 1:4 and DES volume at 50 μL (e); DES volume and cycles keeping SD at 1:4 and solvent volume at 1.5 mL (f).

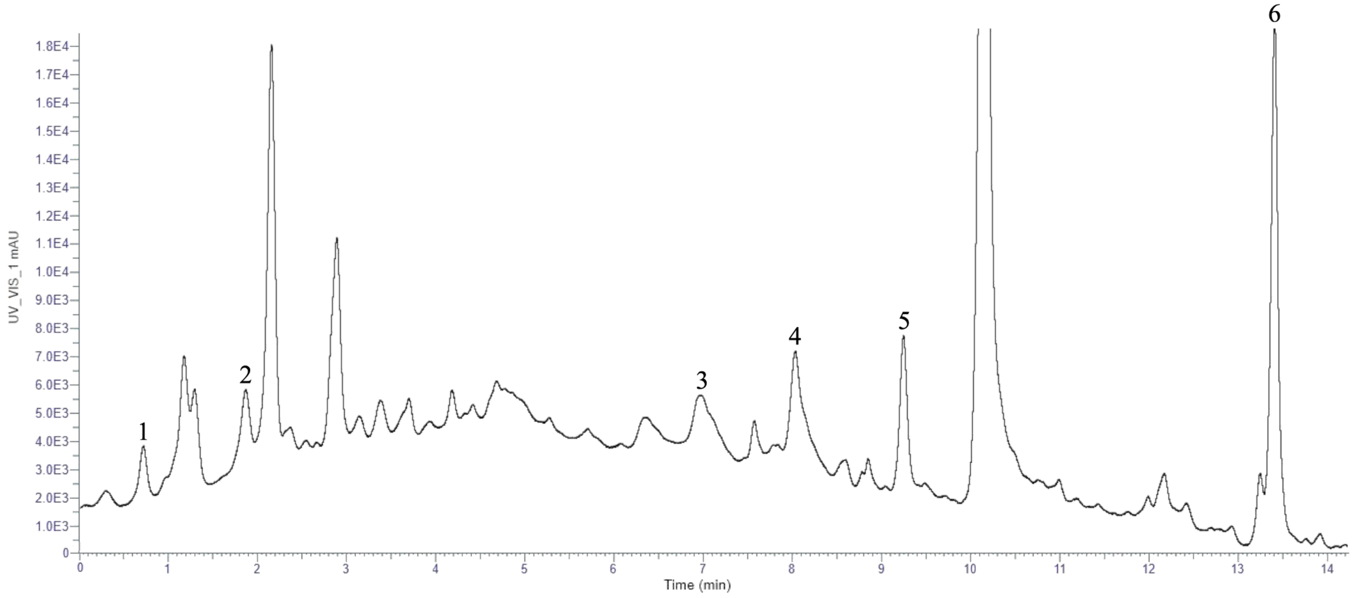


Fig. 5. HPLC-PDA chromatogram of pigmented wheat extract at 280 nm. Peaks correspond to the following phenolic compounds: 1) Gallic acid, 2) Protocatechuic acid, 3) Kaempferol, 4) Catechin, 5) Kaempferol 3-o-rutinoside, 6) Ferulic acid.

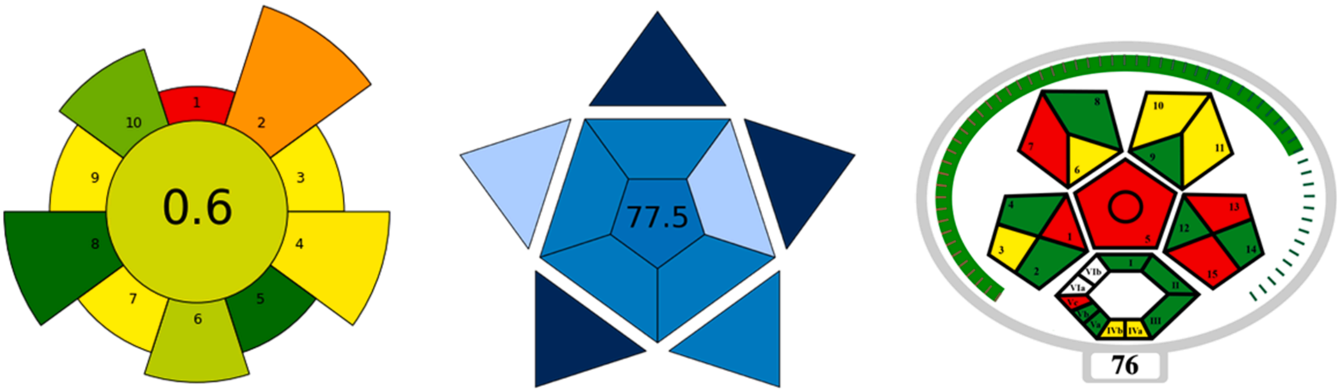
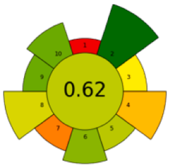
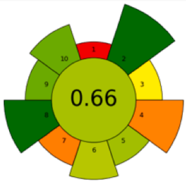
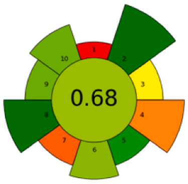
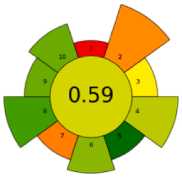
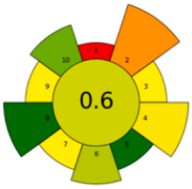


Fig. 6. AGREEprep (on the left), BAGI (in the center) and ComplexMoGAPI (on the right) pictograms of the resulting method.

Table 5
Comparison between UAE and MSPD for phenolic compounds extraction from different matrices.

Matrix	UAE		MSPD		Ref.
	TPC (mg GAE/g)	AGREEprep	TPC (mg GAE/g)	AGREEprep	
Apple pomace	21.96±0.82		14.61±3.87		[32]
Helichrysum arenarium L.	32.73±0.52		38.34±0.09		[34]
Triticum turgidum subsp. durum	1.39±0.04		3.562±0.005		This work

from 1.39 ± 0.04 mg GAE/g (UAE) to 3.562 ± 0.005 mg GAE/g (MSPD) was observed, with a similar sustainability (0.59 vs. 0.60).

A comprehensive analysis of the available data suggests that MSPD yields results are comparable to or superior to those of UAE in numerous matrices.

As shown in Table 6, extraction efficiency and environmental impact of different methods for the TPC determination from *Triticum turgidum* subsp. durum matrices were evaluated.

Three of the methodologies reported are based on SLE, employing different types of solvents: a mixture of MeOH, acetone and water (7:7:6 v/v/v), 80 % ethanol and pure MeOH. A ternary mixture was selected by Yilmaz et al. [36], showing a relatively low TPC (0.45 ± 0.08 mg GAE/g) in comparison to the higher values obtained by Kouider et al. [37] using ethanol (1.82 ± 0.23 mg GAE/g). The use of only MeOH, performed at 50 °C, gave an even higher result (3.43 ± 0.14 mg GAE/g) [38], but with a big impact on the environmental sustainability, as reported by the AGREEprep score of 0.4.

This work represents a novel approach based on an MSPD. Even in this study, the extraction is performed using MeOH as extraction solvent but mixed with a percentage of water and enriched with a DES based on ChCl and Gly (1:2 molar ratio). This novel approach not only provides the highest TPC among all the techniques reported (3.562 ± 0.005 mg GAE/g) but is also a sustainable method, as indicated by the AGREEprep (score of 0.6), combining both extraction efficiency and environmental friendliness.

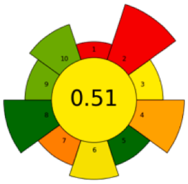
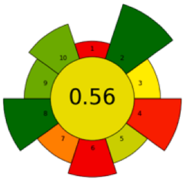
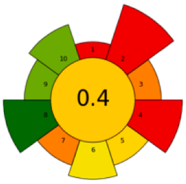
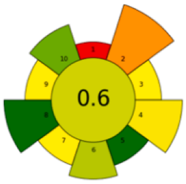
4. Conclusions

In this research, a novel miniaturized MSPD extraction approach was developed to extract phenolic compounds from pigmented wheat. The extraction was supported by the addition of a DES during the homogenization phase.

The presence of an added DES in the homogenization step allowed to promote the extraction yield by limiting the volume of eluent phase as much as possible. In fact, works in the literature either do not have the same extraction efficiency obtained in the present work or use high volumes of organic solvents.

The presence of DES had a positive impact on the matrix and

Table 6
Comparison of the proposed method with those reported in the literature.

Matrix	Extraction Technique	Solvent	Quantity of matrix	Solvent volume	Extraction time	Extraction temperature	TPC (mg GAE/g dry weight)	AGREEprep	Refs.
Triticum turgidum subsp.durum	SLE	MeOH/ acetone/water (7:7:6, v/v/v)	0.1 g	7.5 mL	1 h	RT	0.45±0.08		[36]
Triticum turgidum subsp.durum	SLE	80 % (v/v) ethanol	1.5 g	30 mL	24 h	RT	1.82±0.23		[37]
Triticum turgidum Desf.	SLE	MeOH	5 g	50 mL	1 h	50 °C	3.43±0.14		[38]
Triticum turgidum L. subsp. durum	MSPD	MeOH:water (80:20, v/v) + ChCl-Gly (1:2 molar ratio)	0.1 g	1.42 mL	30 min	RT	3.562±0.005		This work

promoted greater contact with the dispersant phase. ChCl-Gly-based DES (1:2 molar ratio), chosen between three DESs, showed a better extraction ability can be explained by the more complex structure of glycerol, which can exhibit many -OH groups that can interact with phenolic compounds via hydrogen bonds.

The sustainability of the technique was also confirmed by the AGREEprep, BAGI and ComplexMoGAPI analyses.

In conclusion, the present work represents an innovative technique for phenolic compounds extraction from pigmented wheat, approaching the requirements of green chemistry.

CRediT authorship contribution statement

Susanna Della Posta: Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Vittoria Terrigno:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Maria Chiara Frondaroli:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **Laura De Gara:** Writing – review & editing. **Chiara Fanali:** Writing – review & editing, Writing – original draft, Supervision.

Declaration of competing interest

The Authors C. Fanali and S. Della Posta, given their role as Guest Editor and Member of the Advisory Board, respectively in J. Chromatography Open, had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor.

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Data availability

Data will be made available on request.

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