



D-Optimal Design-Based Ultrasound-Assisted Extraction Optimization and Extensive Bio-Structural Analysis of Phenolic Compounds from Romanian Cornelian Cherry (*Cornus mas* L.) Genotypes

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Abstract

Cornelian cherry (*Cornus mas* L.) fruits are increasingly recognized for their rich phytochemical composition and health-promoting properties, which position them as promising candidates for nutraceutical and functional food applications. This study aimed to optimize ultrasound-assisted extraction (UAE) of phenolic compounds using a D-optimal design and to compare the phenolic profiles and bioactivities of ten Romanian genotypes (B351, F581, F2021, FXI1, M13, M16, MH41, MH51, SB3, and SB23). Optimal UAE conditions, 70% ethanol, 60 min, and 20% amplitude, yielded a TPC of optimized extract of 32.1 mg GAE/g and an antioxidant capacity (ABTS) of 80.2 mg TE/g lyophilized extract. Phytochemical analysis identified 295 phenolic compounds, with the optimized extract containing 1736.2 µg Eq/g dry matter, particularly enriched in flavanols (356.8 µg Eq/g) and other phenolics (853.2 µg Eq/g). Significant genotypic variability was observed: TPC ranged from 9.1 to 23.3 mg GAE/g extract, and antioxidant activities ranged from 20.9 to 47.5 mg TE/g (DPPH), 23.0 to 57.8 mg TE/g (FRAP), and 23.5 to 57.6 mg TE/g (ABTS). Enzyme inhibition assays revealed moderate inhibition of α-glucosidase (IC₅₀: 119.7–329.0 µg/mL), α-amylase (IC₅₀: 162.6–1447.6 µg/mL), and pancreatic lipase (IC₅₀: 1179.1–1824.0 µg/mL). Correlation analysis highlighted cyanidin, catechin, and oleuropein equivalents as key contributors to bioactivity. These results underscore the relevance of genotype selection and process optimization in maximizing the functional potential of *C. mas* extracts for the prevention of oxidative stress and metabolic disorders.

Keywords *Cornus mas* · Functional food · Phenolic profiling · Genotypes · UHPLC-HRMS

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Introduction

The genus *Cornus* L. (from the Cornaceae family) includes 65 species of trees, shrubs, and rare perennial herbs with woody rhizomes (Eyde, 1988; Morozowska et al., 2021; Seeram et al., 2002). *C. mas*, commonly known as cornelian cherry, originates from the Caucasus foothills, and it has spread to Turkey, Romania, Bulgaria, Italy, and Central Europe (Dinda et al., 2016). Cornelian cherry fruits have long been valued for their distinctive flavor, nutritional qualities, and medicinal applications (Martinović & Cavoski, 2020). Different studies have confirmed their antioxidant and anti-inflammatory properties, highlighting cornelian cherries potential to improve cardiometabolic risk factors through supplementation (Czerwińska et al., 2021; Dzydzan et al., 2022; Frumuzachi et al., 2024; Klymenko et al., 2021; Tenuta et al., 2023; Wójciak et al., 2022). In culinary, ripe fruits serve for various purposes, such as fresh consumption or in dishes like marmalade, jam, compote, syrup, juice, fruit yogurt, liquor, and wine (Brindza et al., 2007).

Cornelian cherry fruits are a rich source of biologically active compounds, including phenolic compounds, monoterpenes, organic acids, iridoids, and vitamin C (Aurori et al., 2023; De Biaggi et al., 2018; Drkenda et al., 2014; Okan et al., 2019). The main groups of phenolic compounds in these fruits are represented by anthocyanins (cyanidin 3-*O*-glucoside, cyanidin 3-*O*-robinobioside, cyanidin 3-*O*-rutinoside), flavonoids (quercetin, kaempferol, myricetin, and aromadendrin), phenolic acids (gallic, ellagic, chlorogenic acids), catechins, and tannins (Bayram & Arda Ozturkcan, 2020; Dinda et al., 2016). The total phenolic content (TPC) in *C. mas* fruits varies significantly due to several factors, including geographical origin, genotype, growth conditions, fruit ripeness, and extraction solvents. (Capanoglu et al., 2011; Celep et al., 2012; Dzydzan et al., 2019; Kucharska et al., 2011; Popović et al., 2012; Tarko et al., 2017; Yilmaz et al., 2009).

In terms of extraction, ultrasound assisted extraction (UAE) is an advanced method that enhances phytochemical recovery, often achieving higher yields compared to traditional extraction methods (Shen et al., 2023). For cornelian cherry fruits, UAE has been successfully applied to optimize phenolic and anthocyanin recovery under various conditions. For instance, a four-factor, three-level Box-Behnken Design (BBD) identified optimal conditions at 30 °C with 80% ethanol for 40 min (Dumitrașcu et al., 2019), while face-centered Central Composite Design (CCD) determined the optimal conditions as 80 °C, 28.2% ethanol, and 54.8 min, achieving significant extraction efficiency with a desirability function value of 0.916 (Vakula et al., 2019). Other techniques like ohmic heating-assisted

UAE, further improved extraction efficiency, with optimal conditions of 50 °C, a 40 V/cm electric field, and 60 min of sonication (Kutlu et al., 2021).

Despite prior research evaluating the phenolic compound composition of cornelian cherry and employing various methodologies to optimize UAE, there remains a gap in the application of the resource-efficient D-optimal design, a practical and precise model that minimizes experimental runs while maximizing parameter estimation. The D-optimal design is particularly advantageous when working under constraints such as a limited number of experimental runs, irregular experimental domains, or when incorporating both quantitative and qualitative factors (de Aguiar et al., 1995). Unlike CCD or BBD, which require a large and fixed number of runs and assume a symmetric experimental space, D-optimal design allows customization of the design space and the minimization of prediction variance by selecting the most informative experiments. This flexibility is especially beneficial when dealing with plant matrices of high compositional variability, such as *C. mas* genotypes, where each additional experiment translates into significant analytical and material costs. Additionally, existing studies have not thoroughly investigated the variability in phenolic compound profiles and bioactivities across multiple genotypes, particularly Romanian cornelian cherry fruits, nor have they employed advanced analytical techniques for extensive phenolic profiling like ultra-high-performance liquid chromatography and high-resolution mass spectrometry (UHPLC-HRMS). It is hypothesized that a comprehensive optimization might increase the yield of phenolic compounds without wasting too many resources and efforts, while different *C. mas* genotypes might differ in their phenolic compound composition and their extraction behavior.

Consequently, this study aimed at optimizing the UAE process using the D-optimal design of experiments (DoE) methodology. Subsequently, the optimized conditions were applied to ten distinct Romanian genotypes, followed by an in-depth structural and bioactivity characterization, focusing on phenolic compound profiles, in vitro antioxidant and enzyme inhibition activities, and correlating between phenolic compound composition and bioactive potential of the extracts.

Materials and Methods

Plant Material

Fresh plant material (*C. mas* fruits, dark and cherry-red color at full maturity), used for the D-optimal experimental design protocol of the study, was harvested in September 2022 from Mereșeni, Hâncești County, Republic

of Moldova (46°47'45.6"N 28°32'16.8"E), and stored in the herbarium of the Pharmaceutical Botany department, "Iuliu Hațieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania, under voucher specimen No. 66.1.1.1/09.2022. Fresh plant material from ten different genotypes (B351, F581, F2021, FXI1, M13, M16, MH41, MH51, SB3, and SB23), on which the optimal extraction conditions were applied, was also harvested in September 2022 from Măracineni, Argeș County, Romania, originating from plants collected from Romania's wild flora, but grown together at Research Institute for Fruit Growing Pitești—Măracineni (44°53'51.2"N 24°51'34.3"E). Fresh fruits from ten different genotypes were also stored in the herbarium of Pharmaceutical Botany department, voucher specimen No. 66.1.1.2–66.1.1.11/09.2022. After the harvest, all the plant materials were sorted, authenticated based on their botanical features, and stored at -20°C until the following extraction step.

Reagents

For the measurement of TPC, the following reagents and standards were utilized: Folin-Ciocalteu reagent, gallic acid, and sodium carbonate (Na_2CO_3). Rutin and aluminum chloride (AlCl_3) were employed in the assessment of total flavonoid content. The antioxidant capacity assays involved various reagents and standards, including ABTS^+ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium acetate (CH_3COONa), acetic acid (CH_3COOH), hydrochloric acid (HCl), ferric chloride hexahydrate ($\text{FeCl}_3 \times 6\text{H}_2\text{O}$), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), and Trolox.

Additionally, to evaluate the enzyme-inhibitory capacity of the samples, the following reagents and standards were used: α -D-glucoside glucohydrolase from *Saccharomyces cerevisiae* (24 units/mg protein), α -amylase from human saliva (2,000 units/mg protein), 3,5-dinitrosalicylic acid, acarbose, lipase from porcine pancreas (90 units/mg protein), *p*-nitrophenyl-butyrate, dimethyl sulfoxide (DMSO), 4-nitrophenyl- β -D-glucopyranoside (*p*-NPG), and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base). All reagents and standards were of analytical grade, and the products were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

For UHPLC-HRMS analysis, ethanol and acetonitrile were LC-MS grade, purchased from Sigma-Aldrich S.r.l. (Milan, Italy), while standards for the quantification of phenolic compound classes (ferulic acid, quercetin, catechin, cyanidin, luteolin, resveratrol, oleuropein) were purchased from Extrasynthese S.A. (Genay, France).

Extraction of Bioactive Compounds

Initially, the removal of stones from frozen cornelian cherry fruits was applied manually using a knife, followed by the manual grinding of the flesh and skin using a mortar and pestle until a paste consistency was achieved. Each experimental configuration included 2 g of cornelian cherry fruit samples with a solid-to-solvent ratio of 1/10. UAE was carried out using an SFX 150 Sonifier (Branson Ultrasonics Corporation, Brookfield, CT, USA). This equipment provides up to 150 watts of power at a frequency of 40 kHz. The SFX 150 Sonifier was equipped with a tapered microtip featuring a 3.2 mm diameter. During UAE, the microtip was precisely submerged 2 cm into the extract within a 50-mL capacity falcon test tube. Temperature control was achieved by using a probe to measure and maintain the temperature below 25°C , with an ice bath employed during sonication. A magnetic stirrer (VELP Scientifica S.r.l., Usmate Velate MB, Italy) operating at 300 rpm ensured uniform sample homogenization. For each determination, the total power used by the ultrasound (in watts) was noted, corresponding to the selected amplitude.

Following the completion of UAE, each resulting mixture underwent centrifugation at 8,000 rpm and 20°C for 10 min (Thermo Scientific Sorvall ST16R centrifuge, Thermo Fisher Scientific Inc., Rochester, NY, USA). After centrifugation, a vacuum filtration process was employed to ensure the clarity of the solution. The removal of ethanol was achieved through evaporation using a rotary evaporator (Heidolph 94200, Heidolph Scientific Products GmbH, Schwabach, Germany) under reduced pressure. Following this, the extract underwent freeze-drying using a vertical freeze dryer (BK-FD18S, Biobase International Co. Ltd, Jinan, China) and was stored in a desiccator at room temperature for subsequent analysis. The extraction of bioactive compounds from ten distinct cornelian cherry fruit genotypes was carried out using the same procedures while using the optimal extraction parameters.

Design of Experiment (DoE)

In this study, the MODDE 13.0 software (Sartorius Stedim Data Analytics AB, Umeå, Sweden) was employed for the DoE. This software facilitated the assessment of experimental variability and the identification of optimal experimental parameters. Specifically, a D-optimal design, generated by an automatic algorithm within MODDE, was selected due to its efficiency in minimizing the number of experimental runs while enabling reliable estimation of model parameters across complex design spaces. To optimize the extraction process of phenolic compounds three independent process variables were considered: ethanol concentration (0%, 35%, and 70%), extraction time (20, 40, and 60 min), and

ultrasound amplitude (20%, 40%, and 60%). These variables were selected based on prior research indicating that ethanol concentration, extraction time, and ultrasound amplitude are crucial factors in effectively extracting bioactive compounds from cornelian cherry fruits (Dumitraşcu et al., 2019; Enache et al., 2021; Vakula et al., 2019). Moreover, these values were selected to represent both practical and mechanistically relevant boundaries for UAE, ensuring sufficient coverage of the experimental domain.

Simultaneously, two response variables were considered: TPC, evaluated using the Folin-Ciocalteu assay, and total antioxidant capacity, evaluated using the ABTS⁺ assay. Following the implementation of the experimental runs provided by the software, data analysis was conducted by scrutinizing raw data, performing regression analysis, and interpreting the model. Subsequently, the MODDE optimizer and response contour plots derived from the determined model were utilized for the optimization phase (Eriksson et al., 2000; Sartorius Stedim Data Analytics AB, 2017).

TPC and Total Flavonoid Contents (TFC)

The TPC was determined using the Folin-Ciocalteu method, adapted for a SPECTROstar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany) (Babotă et al., 2022). In this method, diluted samples (20 µL) were mixed with 100 µL of diluted Folin-Ciocalteu reagent (1:9, v/v) and shaken vigorously. After 3 min, 80 µL of 1% Na₂CO₃ solution was added, and the mixture was incubated for 30 min at room temperature before measuring absorbance at 760 nm. For the UAE optimization phase, results were expressed as milligrams of gallic acid equivalents per milliliter (mg GAE/mL) of the resulting liquid extract, as each extract was analyzed in liquid form without undergoing lyophilization due to time and resource constraints. However, for the OCM and ten Romanian genotypes extracted under optimized conditions and subsequently freeze-dried, the results were expressed as mg GAE/g extract, to allow standardized comparison of phenolic composition on a dry weight basis. The TFC was assessed using the aluminum chloride colorimetric method (Babotă et al., 2022), where 100 µL of the sample was combined with 100 µL of 2% AlCl₃ solution in a 96-well microplate. Following a 15-min incubation in the dark at room temperature, absorbance was recorded at 420 nm. The results were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g).

UHPLC-HRMS Analysis of Phenolic Profile

The lyophilized *C. mas* fruit extracts (100 mg) were dissolved in 1 mL of 70% ethanol and then centrifuged at 6000 × g for 10 min at 4 °C. The obtained supernatants

were filtered using 0.22 cellulose-syringe filters and analyzed for untargeted phenolic profiling. This latter was done by HRMS using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific Inc., Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatograph (UHPLC) of the same supplier. All the details regarding UHPLC-MS/MS analysis (mass resolution of 70,000 FWHM at 200 m/z) are described in a previous study (Babotă et al., 2022). The raw data generated by HRMS was processed using the software MS-DIAL (version 4.90). Under the present experimental condition, a level 2 of confidence in annotation was reached via spectral matching against FoodDB and Phenol-Explorer's comprehensive databases, using a tolerance for mass accuracy of 5 ppm. Finally, all the phenolic compounds identified were grouped into different typical subclasses and then semi-quantified according to hydroalcoholic standard solutions of pure standard compounds analyzed under the same instrumental conditions. Overall, seven phenolic compounds, namely ferulic acid (phenolic acids), quercetin (flavonols), catechin (flavan-3-ols), cyanidin (anthocyanins), luteolin (flavones and other remaining flavonoids), resveratrol (stilbenes), and oleuropein (other remaining phenolics) were used for this purpose. The semi-quantitative results were expressed as µg of equivalents (Eq.)/g lyophilized extract (DM) (*n* = 3).

In vitro Antioxidant Potential Assays

The in vitro antioxidant potential of the optimized extract and ten cornelian cherry genotypes was evaluated using three complementary assays: ABTS, DPPH, and FRAP (Moldovan et al., 2023). Briefly, the ABTS assay involved preparing a radical solution with 7 mM ABTS⁺ and 2.45 mM potassium persulfate, incubating it in the dark for 16 h, and diluting it to an absorbance of 0.70 ± 0.02 at 734 nm. Samples (20 µL at a concentration of 1.5 mg/mL) were reacted with the radical solution (200 µL), and absorbance was measured after 30 min. The DPPH assay mixed 30 µL of the sample (1.5 mg/mL) with 270 µL of 0.004% DPPH methanol solution, and absorbance was measured after 30 min at 517 nm. For the FRAP assay, 175 µL of a reagent combining acetate buffer, TPTZ, and ferric chloride was prepared (0.3 M, 10 mM, and 40 mM, respectively, in a 10:1:1 ratio, v/v/v), and 25 µL of samples (1.5 mg/mL) were incubated for 30 min before measuring absorbance at 593 nm. For the UAE optimization step, antioxidant activity via the ABTS assay was expressed as milligrams of Trolox equivalents per milliliter (mg TE/mL) of liquid extract, as the extracts were not lyophilized. In contrast, the results for the OCM and ten genotypes, extracted under optimized conditions and subsequently freeze-dried, were expressed as mg TE/g extract to allow

comparisons on a dry weight basis. Antioxidant activity was quantified similarly as mg TE/g extract across DPPH and FRAP assays.

Enzyme Inhibitory Activity

The inhibitory effects of the optimized extract (OCM, obtained from cornelian cherry fruits collected from Moldova) and extracts from ten Romanian cornelian cherry genotypes were evaluated against α -glucosidase, α -amylase, and pancreatic lipase through specific in vitro assays (Babotă et al., 2022; Chelladurai & Chinnachamy, 2018; Moldovan et al., 2023). Briefly, extracts (50 μ L, 62.5–1,000 μ g/mL) were incubated for 15 min with 50 μ L of α -glucosidase enzyme in phosphate buffer (pH 6.8) at 37 °C, followed by the addition of 50 μ L of 10 mM substrate (*p*-NPG) and 10 more min of incubation. Absorbance was measured at 400 nm, with acarbose used as the positive control. Results were expressed as IC₅₀ values (μ g/mL). For the α -amylase inhibition assay, 100 μ L of extracts (23.43–3,000 μ g/mL), dissolved in sodium phosphate buffer (pH 6.9), were incubated with 100 μ L of α -amylase enzyme (1 mg/mL) for 10 min, followed by the incubation with 100 μ L of starch solution (1% [*w/v*]) at 37 °C for another 10 min. The reaction was stopped using 200 μ L of DNSA color reagent (96 mM DNSA, 2 M sodium potassium tartrate tetrahydrate, and 2 M NaOH), boiled (5 min at 100 °C), and, after the samples cooled down, absorbance was measured at 540 nm. Acarbose served as the positive control, and IC₅₀ values (μ g/mL) were calculated. For the pancreatic lipase inhibition assay, 40 μ L of extracts (375–3,000 μ g/mL) were incubated for 15 min with 40 μ L of enzyme in 100 mM Tris–HCl buffer (pH 7.0) containing CaCl₂, followed by the addition of 20 μ L *p*-NPB substrate and further incubation for 15 min. Absorbance was measured at 405 nm, and IC₅₀ values (μ g/mL) were determined using orlistat as the positive control.

Statistics and Correlation Analysis

All assays were conducted in triplicate, and the outcomes were presented as the mean $\pm$ standard deviation. Correlation analysis, including correlograms, Pearson's correlation coefficients, and a *p*-value matrix (with significance set at *p* < 0.05), for various phenolic classes and biological activities, was carried out using R-studio software (version 4.1.3). Statistical analyses associated with the experimental design were directly conducted in MODDE through replicate plot inspection and regression analysis. Additional statistical assessments were carried out using GraphPad Prism 9.

Results and Discussion

This study used thawed frozen fruits stored at –20 °C for all experiments to ensure consistent conditions across ultrasound-assisted extraction. While freezing and thawing are known to facilitate phenolic compound extraction, as demonstrated in prior research on blueberries and chokeberries (King et al., 2022; Nowak et al., 2019), this investigation focused on the impact of UAE rather than the effects of freezing–thawing. Previous studies have shown that freezing–thawing reduces berry hardness and induces colour changes but does not significantly alter phenolic extraction when combined with ultrasounds. By standardizing storage and preparation conditions, this study isolated the influence of extraction methods on phenolic compound recovery.

DoE and Experimental Model Fitting

The assessment of the generated D-optimal design involved the consideration of specific criteria, with two statistical parameters serving as indicators: the condition number and G-efficiency. The condition number, reflecting the design's symmetry and sphericity, is calculated as the ratio between the largest and smallest values of the variability matrix. Ideally, a condition number close to 1 signifies orthogonality, but as generated designs are inherently imperfect, this parameter may vary, with a maximum value of 8 considered acceptable for efficient optimization. Conversely, the G-efficiency parameter (expressed as a percentage) assesses a design's performance relative to a fractional factorial design, and its value should ideally exceed 60–70%.

The D-optimal design employed in this optimization study comprised three quantitative factors: ethanol concentration (0, 35, and 70%), extraction time (20, 40, and 60 min), and ultrasound amplitude (20, 40, and 60%). Regarding the response variables, the study considered two factors: TPC, assessed through the Folin-Ciocalteu method, and antioxidant capacity (assessed through the ABTS assay) (Supplementary material 2, Table 1). TPC served as a key indicator of the overall concentration of phenolic compounds, while the ABTS assay specifically measured the total antioxidant capacity. The ABTS assay was selected for the optimization model because of its broad reactivity profile, including both hydrogen atom and electron transfer mechanisms, its applicability to hydrophilic and lipophilic compounds, and its documented reproducibility and sensitivity in evaluating complex plant extracts (Lang et al., 2024; Thaipong et al., 2006). Thus, the design included a total of 14 and 15 experimental runs in the case of TPC, and ABTS, respectively, with four replicates conducted at the center point to estimate process reproducibility. Randomization of experimental

Table 1 Summary of predicted and experimentally determined total phenolic content (TPC) and ABTS values acquired for the OCM extract prior to the freeze-drying stage

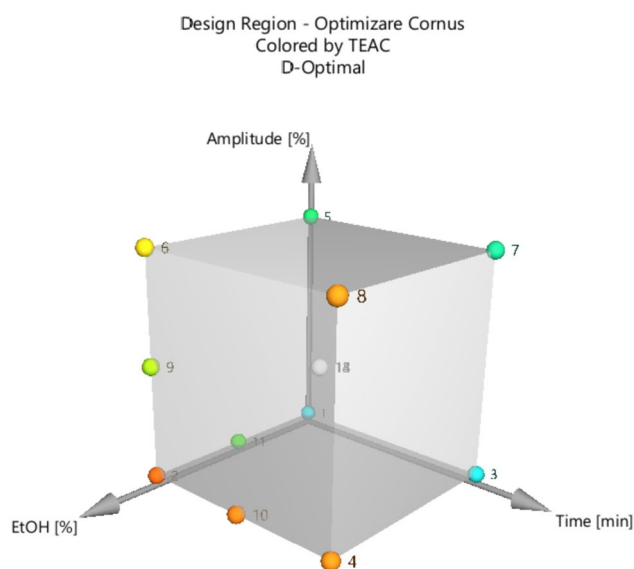
Experimentally measured	OCM						
	TPC (mg GAE/mL)	0.41 ± 0.01					
	ABTS (mg TE/mL)	0.99 ± 0.03					
Predicted	Min	Target	Max	DoE	Predicted	Prob- ability of Failure	Recovery
TPC (mg GAE/mL)	0.30	0.45	0.50	0.41		4.50%	95.50%
ABTS + (mg TE/mL)	0.60	0.85	1.00	0.84		9.70%	90.30%

Findings are presented as the mean ± standard deviations based on three parallel measurements

runs was implemented to minimize the potential impact of systematic errors.

In the present design, the condition number had a value of 1.5, and the G-efficiency was 56.18%, indicating a certain degree of deviation from perfect orthogonality in the generated D-optimal design. While the condition number of 1.5 suggests a level of asymmetry and non-ideal sphericity, it remains within an acceptable range for efficient optimization processes. However, the G-efficiency parameter of 56.18% indicates that the design's performance falls slightly below the ideal threshold of 60–70% when compared to a fractional factorial design. This signifies that the generated D-optimal design may not fully exploit the potential efficiency achieved by a perfect orthogonal design. In this case, the optimization process involved a trade-off between the two response variables, TPC and ABTS. In this study, the correlation between TPC and ABTS, along with the variability in their responses, played a crucial role in determining the efficiency of the experimental design. The aim was to identify conditions that simultaneously enhance both TPC and TEAC values, achieving a balance that aligns with the overall objectives of obtaining cornelian cherry extracts with optimal bioactive potential. Even though the G-efficiency parameter, which assesses the design's performance relative to a fractional factorial design, falls slightly below the ideal threshold of 60–70%, the design remains valuable for exploring and optimizing the extraction parameters in the study. The graphical transposition of the DoE matrix is presented in Fig. 1.

Once all the experimental runs were executed, the obtained responses were centralized and entered into the design worksheet, facilitating the characterization of the experimental data through multiple linear regression (MLR). The initial evaluation focused on two significant statistical parameters: R^2 , which signifies the model's goodness of fit, and Q^2 , which indicates the predictive power or goodness of prediction of the model. Additionally, the model's reproducibility was assessed by considering the values from the four replicates, along with the evaluation of the relative standard

**Fig. 1** Graphical representation of the Design of Experiment (DoE) matrix (D-optimal). Each numerical representation corresponds to a specific experimental ID setup, as outlined in Table 1

deviation (RSD) and the validity of the model. The value for each parameter can be observed in Supplementary material 2, Table 2.

For a highly effective model, R^2 and Q^2 values should ideally approach 1, and a valid model typically maintains a difference between these two parameters that does not exceed the range of 0.2–0.3. Simultaneously, a reproducibility threshold exceeding 0.5 is indicative of a valid model (Eriksson et al., 2000). In the current experimental design, the TPC parameter exhibited R^2 and Q^2 values of 0.944 and 0.802, respectively, with a difference of 0.142 between them. Model validity and reproducibility were reported as 0.829 and 0.898, respectively. The ABTS parameter, on the other hand, demonstrated R^2 and Q^2 values of 0.915 and 0.671, with a difference of 0.244. Model validity and reproducibility for ABTS

Table 2 Semi-quantitative analysis resulting from the UHPLC-HRMS, considering the phenolic classes detected in the different *C. mas* genotypes

Genotype	Cluster (HCA)	Anthocyanins	Flavanols	Flavonols	Other flavonoids	Other phenolics	Phenolic acids	Stilbenes	Total phenolic content
B351	1	274.1 ± 5.0 ^e	161.5 ± 1.8 ^a	61.6 ± 19.8 ^b	133.1 ± 10.5 ^{de}	1532.7 ± 43.3 ^{cd}	298.1 ± 7.2 ^b	108.4 ± 3.9 ^{ab}	2569.5
F581	1	266.8 ± 4.6 ^e	194.4 ± 8.1 ^a	63.6 ± 2.6 ^b	142.8 ± 15.8 ^e	1458.7 ± 73.3 ^c	303.4 ± 62.8 ^b	142.3 ± 8.7 ^c	2572
F2021	2	126.4 ± 4.6 ^b	310.7 ± 7.6 ^d	52.8 ± 13.1 ^{ab}	106.0 ± 1.0 ^{abc}	1318.9 ± 8.3 ^b	290.6 ± 17.7 ^b	113.1 ± 2.0 ^b	2318.5
FX11	2	181.1 ± 16.1 ^{cd}	275.4 ± 19.8 ^c	56.3 ± 5.0 ^b	120.5 ± 3.3 ^{cd}	1763.6 ± 45.2 ^g	322.1 ± 39.6 ^{bc}	107.1 ± 6.1 ^{ab}	2826.1
M13	2	91.2 ± 5.3 ^a	175.4 ± 15.4 ^a	36.3 ± 4.3 ^a	93.6 ± 7.6 ^a	1148.8 ± 33.1 ^a	207.8 ± 9.4 ^a	99.0 ± 8.8 ^{ab}	1852.1
M16	1	200.5 ± 47.4 ^d	181.0 ± 24.5 ^a	58.0 ± 11.8 ^b	131.8 ± 7.1 ^{de}	1527.2 ± 8.5 ^{cd}	316.5 ± 8.4 ^{bc}	117.6 ± 4.2 ^b	2532.6
MH41	2	155.6 ± 5.6 ^{bc}	268.8 ± 20.2 ^c	58.5 ± 7.9 ^b	96.2 ± 5.9 ^{ab}	1664.0 ± 81.9 ^{ef}	332.8 ± 8.6 ^{bc}	116.3 ± 4.6 ^b	2692.2
MH51	1	211.2 ± 5.2 ^d	171.6 ± 8.3 ^a	57.7 ± 10.8 ^b	163.8 ± 12.8 ^f	1580.4 ± 28.1 ^{de}	367.6 ± 14.9 ^c	84.8 ± 39.3 ^a	2637.1
SB3	2	142.7 ± 12.4 ^b	162.2 ± 15.3 ^a	106.6 ± 1.6 ^c	111.1 ± 16.8 ^{abc}	1757.7 ± 90.7 ^{fg}	370.5 ± 54.3 ^c	114.0 ± 11.9 ^b	2764.8
SB23	2	130.0 ± 8.2 ^b	236.4 ± 30.8 ^b	60.0 ± 7.1 ^b	112.9 ± 2.4 ^{bc}	1722.9 ± 39.1 ^{fg}	327.2 ± 18.8 ^{bc}	100.8 ± 5.5 ^{ab}	2690.2
<i>Significance</i>		**	**	**	**	**	**	*	

The results are expressed as mean values (µg phenolic equivalents/g lyophilized extract) ± standard deviation ($n = 3$). Different superscript letters within each column indicate significant differences according to ANOVA and Duncan post hoc test ($p < 0.05$). ** = $p < 0.01$; * = $p < 0.05$

were recorded at 0.776 and 0.882, respectively. These results suggest that the developed model for total TPC and total ABTS is reasonably effective, with high R^2 values indicating a strong fit and substantial predictive power. Importantly, the differences between R^2 and Q^2 for both parameters fall within the optimal range of 0.2–0.3, suggesting a good balance between model fit and predictive capability. Additionally, the model's validity is supported by acceptable values, indicating its general reliability. Reproducibility values exceeding 0.5 for both TPC and ABTS affirm the consistency of the model. Furthermore, a comparable statistical profile has been noted in prior successful optimization investigations. For example, Nicolescu et al. (2022) applied a D-optimal DoE to optimize UAE of phenolic compounds from *Rosa canina* L. pseudo-fruits, yielding values of 0.986 for R^2 , 0.917 for Q^2 (with a difference of 0.069), and 0.951 for reproducibility. These results indicated a statistically robust and valid model.

Nonetheless, in a systematic review of 60 studies optimizing phenolic compound extraction processes, the majority employed BBD or CCD, particularly in ultrasound- and microwave-assisted extractions (Szopa et al., 2024). These designs are well-established but often

require more experimental runs and can be less efficient in identifying optimal conditions when multiple constraints or factor interactions are involved. The presented application of a D-optimal design represents a more resource-efficient alternative, allowing for flexible modelling with fewer runs while maintaining strong predictive power, particularly relevant when experimental resources are limited or when applying complex extraction systems.

Effects of Process Variables on the Quantities of TPC and ABTS Activity

In Fig. 2 are represented the scaled and centered coefficient plots of the process parameter influence and the summary of fit plots for the optimization model. The regression models obtained for TPC and ABTS displayed a model's goodness of fit (R^2) of 0.944 and 0.915, respectively (Supplementary material, Table 2), suggesting that only 0.056 and 0.085 of the variation of TPC and ABTS, respectively, cannot be explained by the present model. The most substantial observed influence, evident for both TPC and antioxidant capacity assessed through ABTS, was associated with the solvent concentration, a crucial factor influencing the extraction of phenolic compounds from *C. mas* fruits in this study.

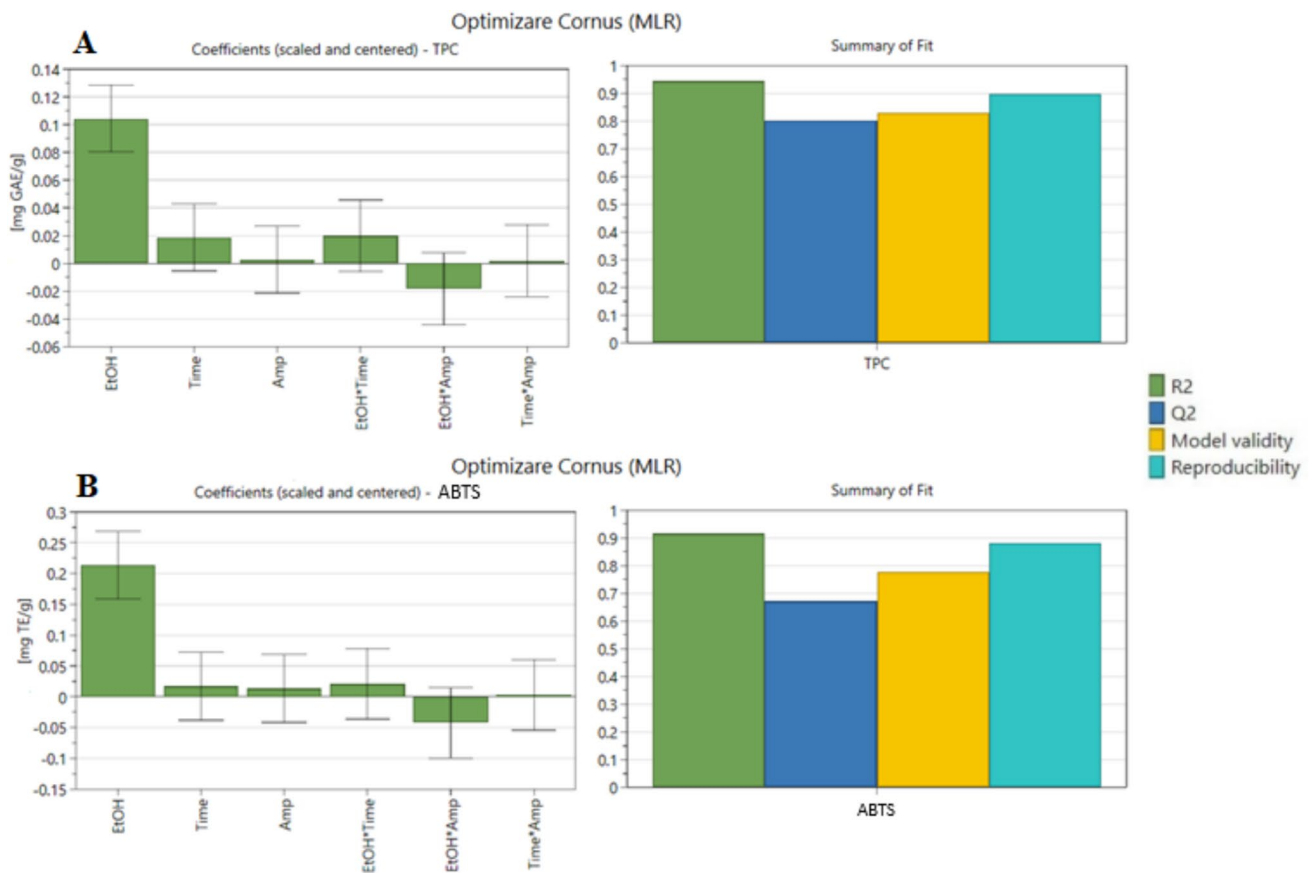


Fig. 2 Regression analysis and interpretation of the optimization model for the ultrasound-assisted extraction process: a scaled and centered coefficient plot illustrating the influence of process param-

eters and a summary of fit plot for the optimization model. **A** Total phenolic content optimization; **B** Antioxidant capacity assessed through ABTS assay optimization

However, no other factor has been identified as statistically significant in the current model, with *p*-values being higher than 0.05 for time, amplitude, and all the interaction factors.

Ethanol–water mixtures are widely recognized for their synergistic effect: water enhances the swelling of plant tissues and facilitates diffusion, while ethanol disrupts interactions between phenolics and the plant matrix (Şahin & Şamli, 2013). In the present study, 70% ethanol consistently yielded the highest TPC and ABTS values, aligning with previous studies demonstrating optimal phenolic and flavonoid recovery at similar concentrations from various botanical matrices, including spruce bark, *Euonymus alatus* (Thunb.) Sieb, and cotton flowers (Dong et al., 2022; Ghitescu et al., 2015; Yang & Zhang, 2008). Moreover, the increase in ethanol concentration exhibited a positive quadratic impact on the extraction of total anthocyanin content, emphasizing the crucial role of the solvent in extracting phenolic compounds from cornelian cherry (Dumitraşcu et al., 2019). Therefore, the concentration of ethanol in the solvent mixture played a pivotal role in influencing the extraction efficiency of

phenolic compounds and antioxidant capacity from *C. mas* fruits.

To improve visualization of the influences of independent variables on TPC and antioxidant capacity, response surface plots were generated (Fig. 3). As could be observed from the visual analysis of surface plots, they are consistent with multiple regression analysis, showing the significant influence of ethanol concentration on evaluated dependent parameters. Notably, any significant influence of either the extraction time or ultrasound amplitude, or the interaction between ethanol concentration and time, ethanol concentration and amplitude, or time and amplitude during the optimization process, have not been observed. This lack of significant influence or interaction between independent factors could be attributed to the preprocessing step undertaken before the extraction process. The cornelian cherry fruits were manually grounded, incorporating both the flesh and skin, using a mortar and pestle until a paste consistency was achieved. However, most of the time, the efficiency of extraction is influenced by the granulometry of the vegetable material used (Fujioka et al., 2016). Finely ground material (as used

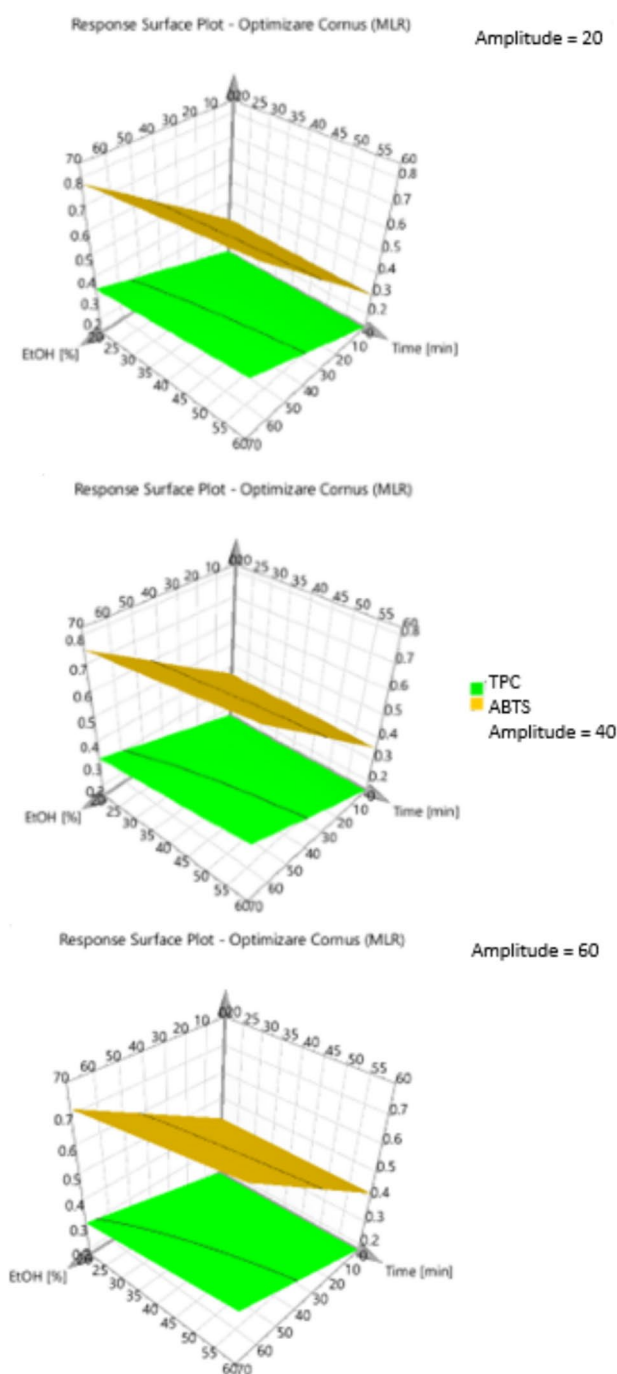


Fig. 3 Surface plots representing the variation of TPC (mg GAE/mL) and ABTS (mg TE/mL) values as a function of ethanol concentration and time, at three different amplitudes

in the current experimental approach) allows for more effective penetration of ultrasound waves and greater exposure of bioactive compounds to the solvent. In contrast, coarsely ground or unprocessed material may impede the uniform transmission of ultrasound energy and hinder the release of targeted compounds (Rivera-Mondragón et al., 2019).

The manual grinding of cornelian cherry fruits could have resulted in the partial destruction of cellular walls in the vegetable material. Consequently, the subsequent application of ultrasounds, which are known for their ability to disrupt cell walls and release bioactive compounds, may have been limited in its impact due to the preexisting disruption caused by the manual grinding process.

Therefore, the extraction process was primarily driven by the variations in ethanol concentration, emphasizing that, in this particular experimental approach where vegetable material was pre-grounded, the variations in time and amplitude did not contribute significantly to the observed outcomes compared to the decisive impact of ethanol concentration. As such, the observed increase in the yield of total phenolic compounds and antioxidant capacity with the rise in ethanol concentration from 0 to 70% supports the crucial role of ethanol. This increase is attributed to enhanced mass transfer between the liquid and solid phases, leading to improved solubility of polar phenolic compounds. Overall, these findings highlight the singular importance of ethanol concentration in optimizing the extraction of bioactive compounds from *C. mas* fruits in this specific context.

Optimization Process

While UAE can serve as an effective method for acquiring plant extracts with increased levels of bioactive phytochemicals, its efficacy is contingent upon the appropriate combination of extraction parameters. Without optimized extraction conditions, UAE may yield low extraction efficiency, potentially even lower than traditional extraction methods (Shen et al., 2023). Moreover, improper conditions have the potential to adversely impact the physicochemical properties and biological activities of bioactive components. For example, high-frequency ultrasound has the capacity to generate numerous free radicals, break down phenolic compounds, and diminish their biological activity (Shen et al., 2023).

Using the MODDE optimizer function, which is capable of determining an experimental setpoint for optimizing the dependent variables, the goal was to maximize the process parameters. In doing so, a range for desired TPC values (minimum 0.3 mg GAE/mL, maximum 0.5 mg GAE/mL, and a target value of 0.45 mg GAE/mL) and ABTS values (minimum 0.6 mg TE/mL, maximum 1.0 mg TE/mL, and a target value of 0.85 mg TE/mL) was specified, taking into consideration that the experimental determinations ranged from 0.13 to 0.41 mg GAE/mL for TPC and 0.27 to 0.83 mg TE/mL for ABTS (Supplementary material 2, Table 1). The predicted and experimentally measured values of TPC and ABTS for the optimized sample (OCM) are provided in Table 1.

Following the outcomes of the experimental design, potential optimal values for considered parameters were

identified as follows: 70% ethanol concentration, 60 min of extraction, and 20% ultrasound amplitude (equivalent to an average power of 3.97 W), with predicted TPC and ABTS values for the optimal extract of 0.41 mg GAE/mL and 0.84 mg TE/mL, respectively. Employing the same extraction process but with the optimized extraction parameters, the anticipated data for the resulting OCM extract was validated. The experimentally measured TPC and ABTS values for OCM were 0.41 GAE/mL and 0.99 mg TE/mL, respectively, for aqueous extracts and 32.1 mg GAE/g and 2.5 mg QE/g extract, respectively, for lyophilized ones (Supplementary material 1). The optimization process demonstrated an overall recovery of 95.5% and 90.3% for TPC and ABTS +, respectively. Therefore, it could be stated that the optimization process was successful in identifying and validating conditions that maximized the extraction efficiency, as evidenced by the close agreement between the predicted and experimentally measured values for TPC and ABTS. The high overall recovery percentages further support the effectiveness of the optimized parameters in obtaining the desired bioactive components from the *C. mas* fruits.

The reported results from the literature showed that extracts obtained through other emerging technologies, such as microwave or ohmic-heating-assisted methods, yielded comparable or higher phenolic contents under different operational conditions. For example, Kutlu et al. (2021) reported a TPC yield of 7.52 mg GAE/g dry matter using ohmic-heating-assisted ultrasound extraction of phenolic compounds from cornelian cherry, which was 13% and 37% higher than ultrasound and maceration alone, respectively; however these extraction methods involved higher equipment complexity and electric field exposure (Kutlu et al., 2021). Similarly, microwave and ohmic-heating-assisted microwave extractions achieved a TPC of 27.7 and 29.4 mg GAE/g, respectively, reducing extraction time by over 90% compared to maceration, but requiring multi-step thermal and electrical input (Kutlu et al., 2021).

It is worth mentioning that while the optimization process successfully enhanced TPC and antioxidant capacity, it primarily focused on overall extract phenolic compound quantity without directly quantifying the extraction efficiency of specific phenolic compounds. The goal was to optimize the total phenolic yield assessed as TPC, which, despite its known limitations, such as non-specificity and susceptibility to interference (Pérez et al., 2023), remains a practical and widely used proxy for estimating total extractable phenolic compounds. In contrast, optimizing individual compound recovery would have required detailed phytochemical profiling, which involves significantly higher demands in terms of time, analytical complexity, and consumable resources. Additionally, the UAE process was validated at the laboratory scale using a relatively low ultrasound amplitude (20%), corresponding to a power input of 3.97 W. While

this ensured energy efficiency and minimized compound degradation, it may pose limitations for industrial translation. Scaling up UAE systems often involves higher power inputs, continuous flow setups, and specialized sonotrodes (Capaldi et al., 2024). Nonetheless, the simplicity of the optimized protocol, relatively short time, moderate solvent concentration, and mild amplitude, offers a feasible starting point for pilot-scale adaptation. Further techno-economic evaluations and trials in scaled extraction systems are warranted to assess its industrial viability.

Preliminary TPC and TFC and In Vitro Antioxidant Activity of Cornelian Cherry Fruits Genotypes

In the assessment of the bioactive potential of cornelian cherries, a preliminary assessment was conducted to evaluate the TPC and TFC across various genotypes of cornelian cherry fruits. This initial investigation sought to provide insight into the phenolic and flavonoid composition of cornelian cherries, laying the foundation for further detailed analyses. Different genotypes were included in the study, and the extraction protocol applied for each sample followed validated optimal conditions and lyophilization processes, and the TPC and TFC of obtained extracts were measured using established methodologies. The preliminary results are presented in Supplementary material 1. The values for ten different genotypes ranged from 9.1 to 23.3 GAE/g extract in TPC assay and from 0.5 to 1.6 mg QE/g extract in TFC assay.

Regarding the antioxidant activity, the present study employed well-established ABTS, DPPH, and FRAP assays, recognized for their widespread use in the analysis of phenolic compounds. These assays, known for their convenience, high-throughput nature, and independence from specialized equipment, operate as "fixed-time" assays with reaction times spanning from 12 to 30 min. The obtained results are presented in Fig. 4. In contrast to the OCM extract, which exhibited antioxidant activities of 58.2, 89.7, and 80.2 mg TE/g lyophilized extract in DPPH, FRAP, and TEAC assays, respectively, the antioxidant activity of ten different cornelian cherry fruit genotypes varied significantly, with notable disparities among the genotypes. The lowest antioxidant activity across all the conducted assays was identified in the F581 genotype, while the highest antioxidant activities were observed in the cases of F2021 and M16 in DPPH and FRAP assays, and for F2021 in the TEAC assay. Specifically, in the DPPH assay, the antioxidant activity of the tested extracts ranged from 20.9 to 47.5 mg TE/g lyophilized extract, in FRAP from 23.0 to 57.8 mg TE/g lyophilized extract, and in TEAC from 23.5 to 57.6 mg TE/g lyophilized extract.

Scientific literature reports different results. For instance, in the assessment of cornelian cherry cultivars and genotypes

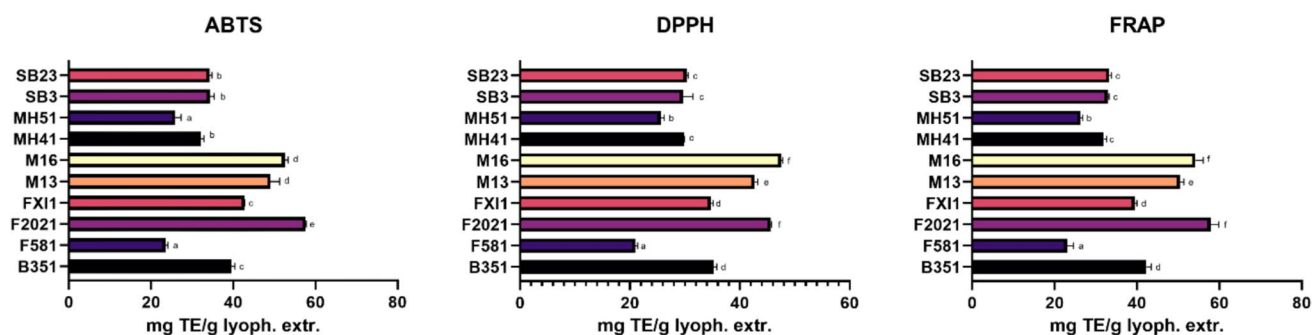


Fig. 4 In vitro antioxidant activity of cornelian cherry fruits genotypes. To assess statistically significant differences between the different genotypes, a one-way analysis of variance (ANOVA) was carried

out, followed by Tukey's Honest Significant Difference (HSD) post hoc test. Different letters represent the presence of a statistically significant difference ($p < 0.05$) between different genotypes

from Montenegro, the local cultivar 'Kosten 1' showed the highest antioxidant activity in the DPPH assay, with a value of $19.0 \mu\text{mol TE/g}$. A local genotype ('CT02') exhibited the highest antioxidant activity in ABTS and FRAP assays, with values of $14.7 \mu\text{mol TE/g}$ and $59.5 \mu\text{mol Fe}^{2+}/\text{g}$, respectively, followed closely by the local cultivar 'Kosten 1'. Conversely, introduced cultivars 'Krupnoplodni NS' and 'Lukyanovsky' displayed lower antioxidant activities, with values of 6.2 and $9.9 \mu\text{mol TE/g}$ for the DPPH assay, and $4.4 \mu\text{mol TE/g}$ for the ABTS assay, respectively (Martinović & Cavoski, 2020). On the other hand, Klymenko et al. (Klymenko et al., 2021) conducted an assessment of the antioxidant activity of fruits from five *C. mas* cultivars ('Eckzotychnyi', 'Koralovyi Marka', 'Koralovyi', 'Uholok' [black fruit], and 'Yantarnyi' [yellow fruit]), two *C. officinalis* genotypes ('Co-01' and 'Co-02'), and two interspecific hybrids (*C. mas* $\times$ *C. officinalis*, CmCo-01 and CmCo-02') collected in Ukraine. The DPPH radical scavenging capacity of each *C. mas* cultivar extract ranged from 6.7 to $77.3 \mu\text{mol TE/g}$, *C. officinalis* extracts varied from 17.8 to $39.7 \mu\text{mol TE/g}$, and *C. mas* $\times$ *C. officinalis* extracts ranged from 20.8 to $45.5 \mu\text{mol TE/g}$. Additionally, the antioxidant capacity of *C. mas* cultivar extracts, as measured by the ABTS assay, ranged from 13.5 ('Koralovyi') to $60.0 \mu\text{mol TE/g}$ ('Uholok'). For *C. officinalis*, the range was 31.6– $36.7 \mu\text{mol TE/g}$, and for *C. mas* $\times$ *C. officinalis*, it was 42.2– $44.5 \mu\text{mol TE/g}$ (Klymenko et al., 2021).

Profiling and Variability of *C. mas* Extracts by UHPLC-HRMS

The profiling of phenolic compounds in *C. mas* genotypes has revealed substantial diversity in phenolic content and composition, which can be contextualized by hierarchical clustering analysis (HCA) and the characterization of an optimized extract. Overall, 19 anthocyanins, 13 flavanols, 22 flavonols, 86 other flavonoids (flavones and remaining flavonoids), 91 other phenolics (including lignans,

lower-molecular-weight phenolics, and tyrosol derivatives), 57 phenolic acids, and 7 stilbenes were identified, accounting for a total of 295 compounds. All the information regarding the annotated compounds is available in Supplementary material 1. The optimized extract (OCM) displayed a concentrated phenolic profile with a TPC of $1736.2 \text{ g equivalents/g DM}$ (Supplementary material 2, Table 3). The extract was particularly enriched in flavanols ($356.8 \text{ g equivalents/g DM}$) and other phenolic compounds ($853.2 \text{ g equivalents/g DM}$), which are likely contributors to its bioactivity. OCM captured a broad spectrum of phenolic compounds, particularly compounds with like flavanols, anthocyanins, and stilbenes. These classes of compounds are well-documented in the literature for their multiple biological effects. For instance, flavanols such as catechin and epicatechin contribute to antioxidant and anti-inflammatory effects, while anthocyanins like cyanidin-3-galactoside and pelargonidin-3-glucoside have shown antidiabetic and anticancer activities (Sevindik et al., 2024). Stilbenes and ellagic acid derivatives identified in *C. mas* are also associated with antiproliferative effects on various cancer cell lines, including colon and breast adenocarcinoma models (Šavikin et al., 2009; Tiptiri-Kourpeti et al., 2019). Moreover, several phenolic acids, such as gallic acid, caffeic acid, and chlorogenic acid, exhibit strong antimicrobial and enzyme inhibitory activities, including α -glucosidase and acetylcholinesterase inhibition (Dzydzan et al., 2019; Milenković-Andjelković et al., 2015).

The UHPLC-HRMS analysis (Table 2) showed significant variability ($p < 0.05$) in phenolic compound profiles among the different *C. mas* genotypes. Phenolic acids and other phenolics were the most abundant classes of phenolic compounds across all genotypes. For example, MH51 and SB3 recorded high values for phenolic acids, reaching 367.6 and $370.5 \text{ g equivalents/g DM}$, respectively, whereas FXI1 had $1763.6 \text{ g equivalents/g DM}$ in the "other phenolics" category. Anthocyanins and flavonols, while less

Table 3 The In vitro anti-enzymatic activity of cornelian cherry fruit genotypes, expressed as IC₅₀ values (μg/mL)

Bioassay	Genotype										OCM	Reference inhibitor
	B351	F581	F2021	FXII	M13	M16	MH41	MH51	SB3	SB23		
α-Glucosidase inhibition	273.87 ± 7.60 ^b	278.97 ± 11.58 ^b	119.71 ± 6.23 ^f	173.84 ± 11.24 ^d	170.29 ± 11.37 ^d	147.48 ± 1.96 ^e	270.02 ± 12.16 ^b	329.01 ± 3.23 ^a	200.31 ± 7.02 ^c	275.03 ± 10.90 ^b	125.05 ± 4.94 ^f	Acarbose: 52.04 ± 2.30 [*]
α-Amylase inhibition	737.28 ± 35.68 ^e	807.42 ± 24.00 ^d	162.69 ± 10.52 ^k	576.27 ± 10.88 ^g	387.96 ± 7.42 ^h	266.33 ± 10.37 ⁱ	1447.65 ± 122.91 ^a	892.11 ± 3.33 ^c	689.22 ± 36.73 ^f	1126.22 ± 38.92 ^b	191.25 ± 13.65 ^j	Acarbose: 21.28 ± 1.42 [*]
Lipase inhibition	1780.89 ± 100.66 ^{ab}	1329.37 ± 100.13 ^d	1468.41 ± 132.53 ^{bcd}	1818.81 ± 102.45 ^a	1518.93 ± 204.34 ^{bc}	1824.08 ± 122.33 ^a	1560.49 ± 19.69 ^c	1624.40 ± 177.54 ^{ab}	1594.89 ± 78.55 ^{bc}	1179.13 ± 145.25 ^d	1196.82 ± 108.67 ^d	Orlistat: 68.47 ± 2.98 [*]

Statistical differences were evaluated by implementing one-way ANOVA, followed by Tukey's HSD post hoc test ($p < 0.05$). Different lower-case letters indicate significant differences among cornelian cherry fruit extracts, while the presence of asterisk (*) indicates a significant difference between reference inhibitor and the evaluated extracts

abundant than phenolic acids, showed notable differences. For instance, B351 exhibited the highest anthocyanin levels (274.1 g equivalents/g DM), whereas M13 had lowest levels. Flavanols were particularly increased in genotypes from Cluster 2, such as F2021 (310.7 g equivalents/g DM), while the lowest levels were notice in B351 (161.5 g equivalents/g DM). The hierarchical clustering grouped genotypes into two clusters, reflecting phenolic similarities. Cluster 1 genotypes, including B351 and M16, displayed distinct phenolic profiles compared to Cluster 2 genotypes like F2021 and MH41. This clustering highlights phenolic class-based differentiation. Moreover, the variability in phenolic compounds underscores the influence of genetic factors on phenolic biosynthesis.

The unsupervised heatmap analysis provided a clear visual differentiation of phenolic profiles among the genotypes (Fig. 5). The clustering corroborates the statistical significance of phenolic variation and reveals phenolic class-driven groupings. In particular, Cluster 1 was characterized by higher anthocyanin and flavonol levels but moderate amounts of phenolic acids, while Cluster 2 showed elevated flavanols (mainly methylated forms of epicatechin and epigallocatechin) and phenolic acids (mainly isoferulic acid 3-glucuronide) levels, highlighting their potential as rich sources of these compounds. Among the most abundant anthocyanins there were cyanidin 3-glucoside and petunidin 3-galactoside (for anthocyanins), while kaempferol, myricetin, and quercetin were the most abundant flavonols.

The significant variability observed across genotypes can be attributed not only to genetic diversity but also to environmental and agronomic influences on phenolic biosynthesis. Although all genotypes were cultivated under uniform horticultural conditions at the Mărăcineni research station, their wild origins from distinct Romanian regions suggest underlying genetic adaptations to local environments, including differences in soil composition, altitude, and climatic stressors. Such variability has been similarly reported in studies conducted in Turkey, Serbia, and Iran, where genotype-specific differences in phenolic content, antioxidant capacity, and morphological traits were linked to both inherent genetic profile and ecological growing conditions (Bijelić et al., 2011; Moradi et al., 2019; Yilmaz et al., 2009). For example, Yilmaz et al. (2009) found that genotype 44–18, grown in the Eastern Anatolia region, exhibited the highest TPC (74.8 mg GAE/g DW) and antioxidant activity, suggesting strong genotype × environment interactions (Yilmaz et al., 2009). Likewise, in Serbia, Bijelić et al. (2011) reported mesocarp phenolic contents ranging widely from 35.63 to 126.53 mg/100 g fruit among genotypes, with strong correlations to fruit size and altitude (Bijelić et al., 2011). These findings align with our data, where significant differences in TPC and antioxidant capacity were observed across the ten Romanian genotypes. Additionally, long-term

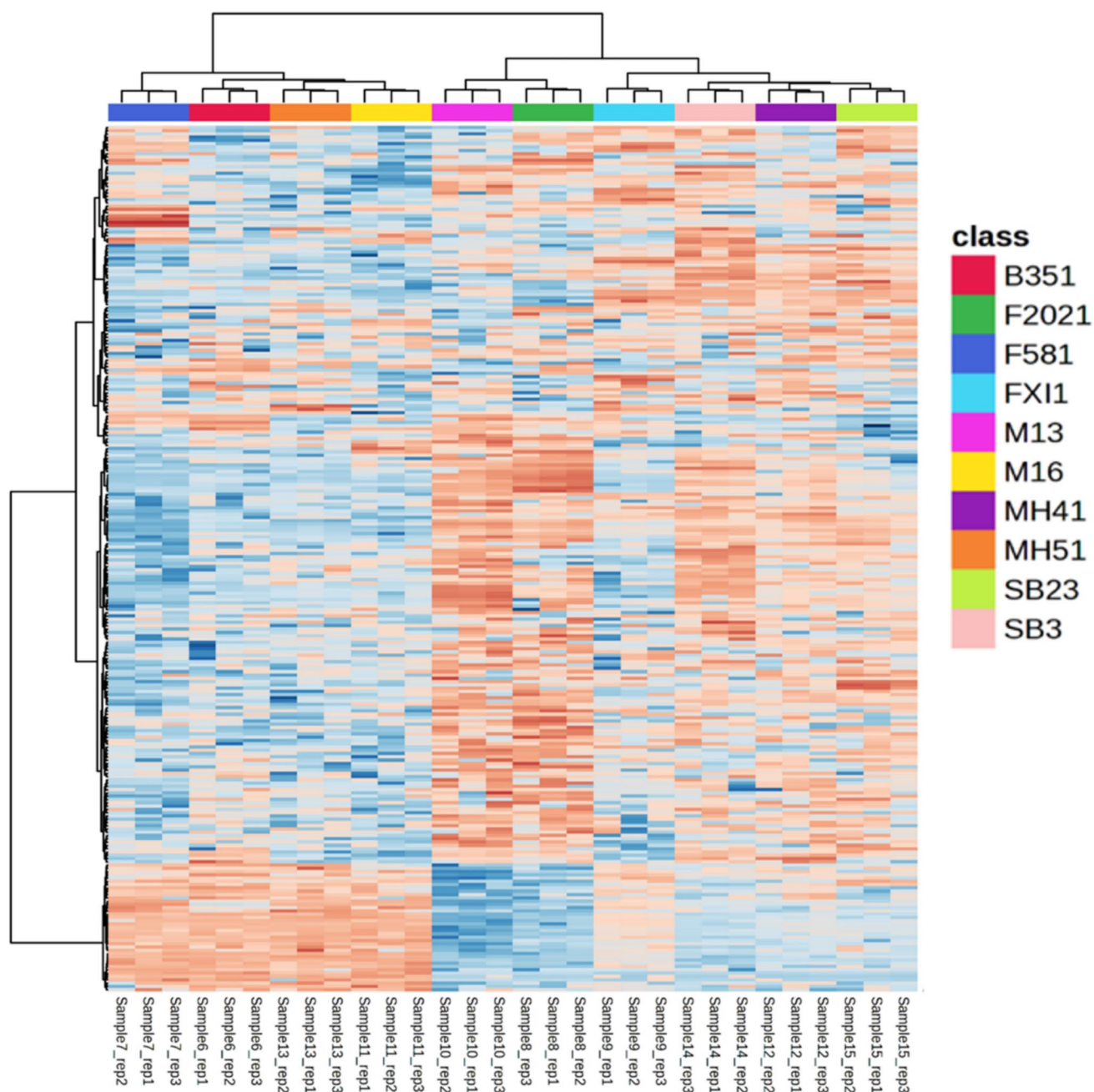


Fig. 5 Unsupervised hierarchical clustering heat-map built considering the phenolic profiling of the different *C. mas* genotypes

open-pollination and limited domestication in wild populations, as observed by Cornescu and Cosmulescu (2017) in Romanian accessions, have contributed to maintaining high levels of heterozygosity and phenotypic plasticity. Together, these findings emphasize the value of targeted genotype selection in functional food development and the importance of conserving genetic resources for future breeding programs. Nonetheless, the present study demonstrates that *C. mas* genotypes offer a rich phenolic profile with significant diversity, making them a valuable resource

for functional and nutraceutical applications. The optimized extract (OCM) further consolidates the phenolic richness and bioactive potential of *C. mas*.

In Vitro Anti-Enzymatic Activity of Cornelian Cherry Fruits Genotypes

The inhibitory effects of OCM and ten different genotype extracts of cornelian cherry fruits were assessed against α -glucosidase, α -amylase, and pancreatic lipase using

different in vitro methods. These enzymes play crucial roles in the digestion and absorption of carbohydrates and lipids, making them key targets for managing blood glucose levels and body weight (Tucci, 2010). Lowering the postprandial glucose uptake through the inhibition of carbohydrate-hydrolyzing enzymes in the small intestine is one of the treatment strategies for the management of type 2 diabetes. In the present study, the IC_{50} values obtained for acarbose, a commercial inhibitor of α -glucosidase and α -amylase, were 52.0 and 21.2 $\mu\text{g/mL}$, respectively. Compared with the acarbose, cornelian cherry extracts were less effective in inhibiting α -glucosidase activity, with the IC_{50} values ranging from 119.7 (F2021) to 329.0 $\mu\text{g/mL}$ (MH51), as presented in Table 3. Similarly, compared with the acarbose, cornelian cherry extracts were less effective in inhibiting α -amylase activity, with the IC_{50} values ranging from 162.6 (F2021) to 1447.6 $\mu\text{g/mL}$ (MH41).

Present data are of interest when compared with the ones reported in the literature. Dzydzan et al. (2022) studied the influence of red and yellow cornelian cherry fruit extracts on α -glucosidase activity from *Saccharomyces cerevisiae*. The IC_{50} values for red and yellow fruit extracts were 25.6 and 28.4 $\mu\text{g/mL}$, respectively. These fruit extracts, both red and yellow, demonstrated superior inhibitory potential against the enzyme compared to a purified loganic acid extract from cornelian cherry fruits. In a separate investigation by Tenuta et al. (2023), various *C. mas* fruit extracts displayed IC_{50} values ranging from 30.4 to 312.4 $\mu\text{g/mL}$, with one sample exhibiting higher activity than acarbose (IC_{50} of 35.50 $\mu\text{g/mL}$). Additionally, Blagojević et al. (2021) evaluated extracts from cornelian cherry fruits of three cultivars (Ukrainian cultivars 'Elegantnyi', 'Semen', 'Svetlyachok') and four selections ('Apatinski rani', 'Bačka', 'KIC1', 'R1'). The IC_{50} values of the studied extracts ranged from 0.2 to 0.3 mg/mL , indicating a robust inhibitory potential against the α -glucosidase enzyme, with up to ten times better α -glucosidase inhibition activity than the commercial inhibitor (IC_{50} value of 1.8 mg/mL). On the other hand, Shishehbor et al. (2016) assessed the hypoglycemic activity of hydroalcoholic extract obtained from fresh cornelian cherry fruits, revealing IC_{50} values of 6.8 mg/mL , which is significantly higher than those reported in our study, indicating a low inhibition potential. Regarding the inhibition of α -amylase activity, in the study by Tenuta et al. (2023), the IC_{50} values of *C. mas* fruit extracts against α -amylase ranged from 208.2 to 953.5 $\mu\text{g/mL}$. The authors concluded that, in general, *C. mas* fruit extracts exhibit higher activity as α -glucosidase inhibitors compared to α -amylase inhibitors. Similarly, Blagojević et al. (2021) reported IC_{50} values for different cornelian cherry genotypes ranging from 3.5 mg/mL ('Bačka') to 13.8 mg/mL ('KIC1'). The findings presented

herein align with the literature, indicating that, indeed, the α -amylase inhibition activity of *C. mas* fruit extracts is less potent than their α -glucosidase inhibition activity. Contrarily, the pancreatic lipase inhibitory potential of all studied extracts was found to be modest. The SB23 extract exhibited the highest pancreatic lipase inhibitory activity (IC_{50} 1179.1 $\mu\text{g/mL}$), while the M16 extract demonstrated the least activity (IC_{50} 1824.0 $\mu\text{g/mL}$). In contrast, Świerczewska et al. (2019) reported strong pancreatic lipase inhibitory effects for all tested *C. mas* fruit extracts, showing IC_{50} values ranging from 15.2 to 112 $\mu\text{g/mL}$. Similarly, Tenuta et al. (2023) observed potent lipase inhibition by *C. mas* fruit extracts, with IC_{50} values ranging from 74.3 to 400.5 $\mu\text{g/mL}$. This discrepancy in lipase inhibition underscores the variability in enzyme inhibitory effects observed across different studies, which could be attributed to differences in extraction methods, sample preparation, or assay conditions.

Correlations

The Pearson's correlation analysis provided insightful relationships between the phenolic subclasses and the bioactivities of the different fruit extracts. The results are presented in Supplementary material 2, Table 4. "Other phenolics" demonstrated the strongest negative correlation with TEAC ($r = -0.76$), DPPH ($r = -0.72$), and FRAP ($r = -0.79$), suggesting their prominent role in modulating these antioxidant activities. Anthocyanins and flavanols also showed notable correlations. Anthocyanins negatively correlated with TEAC ($r = -0.67$) and DPPH ($r = -0.62$), while flavanols positively correlated with TEAC ($r = 0.63$) and FRAP ($r = 0.61$). This dual behavior underscores the nuanced interactions of phenolics with antioxidant mechanisms. Phenolic acids exhibited moderate negative correlations with all three assays, indicating their consistent but lesser impact on antioxidant activities compared to other phenolic classes. Regarding enzymatic inhibitions, anthocyanins ($r = 0.59$) and "other phenolics" ($r = 0.51$) were strongly correlated with glucosidase inhibition, suggesting their bioactivity in regulating glucose metabolism. Only "other phenolics" showed a significant positive correlation ($r = 0.62$), pointing to their selective role in inhibiting amylase activity. Anthocyanins ($r = 0.44$) and "other phenolics" ($r = 0.39$) also displayed moderate correlations, indicating potential implications in lipid metabolism and obesity management. Flavonols and stilbenes did not show significant correlations with any bioactivity, suggesting that their impact on the analyzed activities is minimal or operates through mechanisms not captured by the performed assays.

The class of “other phenolics” consistently showed strong correlations across multiple bioactivities, underscoring its versatility in influencing both antioxidant and enzymatic activities. While both anthocyanins and flavanols demonstrated significant correlations, their effects varied across bioactivities, highlighting their selective action. The absence of significant correlations for flavonols and stilbenes suggests their limited direct contribution to the measured activities, warranting further investigation into their potential synergistic roles or impact on other bioactivities. These correlations provide valuable insights into how phenolic subclasses contribute to the bioactivities of fruit extracts, paving the way for targeted utilization in nutraceutical and functional food applications.

Conclusions

The present study provides a comprehensive analysis of the optimization, profiling, and bioactivity assessment of phenolic compounds extracted from *C. mas* fruits, highlighting their potential for functional food and nutraceutical applications. Using the D-optimal design methodology, UAE conditions were successfully optimized. UHPLC-HRMS profiling identified substantial diversity in the phenolic composition across different Romanian *C. mas* genotypes. A total of 295 compounds were annotated, with phenolic acids, anthocyanins, and flavanols being prominent contributors to the bioactive potential of the extracts. Hierarchical clustering analysis revealed genotype-specific differences, emphasizing the influence of genetic and environmental factors on phenolic biosynthesis. The bioactivity assays underscored the antioxidant and enzyme inhibitory potential of *C. mas* extracts. Genotypes varied significantly in their ability to scavenge free radicals and inhibit key metabolic enzymes, such as α -glucosidase, α -amylase, and pancreatic lipase. These findings demonstrate the bioactive richness of *C. mas* fruits, with potential implications for managing oxidative stress, type 2 diabetes, or obesity. Furthermore, the observed variability among genotypes offers opportunities for selective breeding to enhance specific bioactive compounds. Future research should focus on validating the extraction protocol at industrial scale and developing functional food formulations enriched with the optimized extract, aiming to enhance nutritional value and deliver measurable health benefits in vivo and under real food matrix conditions. Additionally, given the pronounced genotype-dependent variability in phenolic composition and bioactivity, selecting high-performing genotypes is essential to maximize the functional potential and consistency of the final product. Overall, the present study advances the understanding of *C. mas* as a valuable source

of bioactive compounds, supporting its inclusion in dietary interventions and functional food formulations aimed at improving health outcomes.

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Data Availability Data of interest could be made available on request.

Declarations

Competing interest The authors declare no competing interests.

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