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Quality by Design in optimizing the extraction of (poly)phenolic compounds from *Vaccinium myrtillus* berries

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Quality by Design in optimizing the extraction of (poly)phenolic compounds from *Vaccinium myrtillus* berries / L. Marzullo, O.O.. - In: JOURNAL OF CHROMATOGRAPHY A. - ISSN 0021-9673. - STAMPA. - 1677:(2022), pp. 1-12. [10.1016/j.chroma.2022.463329]

Availability:

The webpage <https://hdl.handle.net/2158/1277184> of the repository was last updated on 2025-01-26T18:37:14Z

Published version:

DOI: 10.1016/j.chroma.2022.463329

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(Article begins on next page)

Journal of Chromatography A

Quality by Design in optimizing the extraction of (poly)phenolic compounds from *Vaccinium myrtillus* berries --Manuscript Draft--

Manuscript Number:	JCA-22-772R1
Article Type:	Full length article
Keywords:	Anthocyanins; Experimental Design; Ultrasound-Assisted extraction; Mechanical Solvent Extraction; Microwave-Assisted Extraction
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Abstract:	Quality by Design was adopted for developing an effective extraction procedure of (poly)phenolic compounds from bilberry (<i>Vaccinium myrtillus</i> L.) fruits, using a pooled sample of berries from different regions of Ukraine. Mechanical solvent extraction, microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) were investigated by screening matrices. Extraction time (Time, from 5 to 15 min), organic solvent type (OS type, methanol, ethanol and acetone), organic solvent percentage (OS%, from 50% to 90%), sample/extractant ratio (S/E ratio, from 0.025 to 0.1 g mL⁻¹), and, only for MAE, extraction temperature (T, from 30 to 60 °C), were selected as critical method parameters (CMPs). The spectrophotometric assays total soluble polyphenols (TSP), total monomeric anthocyanins (TMA), and radical scavenging activity (evaluated by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), the 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid), and the ferric reducing antioxidant power methods) were chosen as critical method attributes (CMAs). The screening procedure allowed for selecting UAE and methanol, while the other CMPs underwent further optimization through Response Surface Methodology. Target values for TSP, TMA and DPPH were selected and the method operable design region (MODR) was defined by means of Monte-Carlo simulations. The optimized conditions, with the corresponding MODR intervals in bracket, were the following: (i) Time, 17 min (15-23 min); OS%, 56% (44-59%); S/E ratio, 0.030 (0.022-0.034) g mL⁻¹. Under these experimental conditions, CMAs values of the pooled sample were the following (n =3): TSP=4433±176 mg (+)-catechin eq/100 g dry weight (d.w.); TMA=3575±194 mg cyanidin-3-glucoside eq/100 g d.w.; DPPH=273±5 µg DPPH inhib./mg d.w. The optimized extraction method was tested for matrix effect (ME%) in the UHPLC-MS/MS analysis of 15 anthocyanins and 20 non-anthocyanins individual (poly)phenols commonly found in bilberries, as well as for luteolin, sinapic acid, and pelargonidin-3-glucoside, absent in this fruit and therefore added to the extracts as surrogate standards for evaluating apparent recovery (AR%). ME% was in any case ≤ 23% and AR% of the surrogate standards in the range 91-95%, confirming the very good performances of the optimized extraction method.
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DIPARTIMENTO
DI CHIMICA
"UGO SCHIFF"

EDITORIAL OFFICE
Journal of Chromatography A

Dear Prof. Hian Kee Lee,

I send you the revised version of the manuscript entitled "Quality by Design in optimizing the extraction of (poly)phenolic compounds from *Vaccinium myrtillus* berries" (original title: "Quality by Design in the optimization of the extraction of (poly)phenolic compounds from *Vaccinium myrtillus* L. berries: comparison of extraction methods and application to the analysis of Ukrainian bilberries"), for the resubmission to the Journal of Chromatography A.

First corresponding author and the submitter of the manuscript: Prof. Massimo Del Bubba, Department of Chemistry, University of Florence, Via della Lastruccia n. 3, 50019 Sesto Fiorentino (Florence), Italy. E-mail address: massimo.delbubba@unifi.it.

Second corresponding author: Prof. Sandra Furlanetto, Department of Chemistry, University of Florence, Via Ugo Schiff n. 6, 50019 Sesto Fiorentino (Florence), Italy. E-mail address: sandra.furlanetto@unifi.it.

In this revised version, we have replied to all the Editor and Reviewers comments. You will find point-by-point responses in the file "Replies to the Editor and Reviewers Comments", which has been uploaded during the resubmission of the manuscript.

I hope that the Editor and Reviewers will welcome this revised version.

I thank you in advance for your consideration and I send you my best regards.

Florence, 08/07/2022

Prof. Massimo Del Bubba


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Replies to the Editor and Reviewers Comments

Editor

1) *The title has repetitive elements and is unwieldy: It can be revised to Quality by Design in optimizing the extraction of (poly)phenolic compounds from Vaccinium myrtillus L. bilberries.*

Answer. As suggested by the Editor, the title has been modified to "Quality by Design in optimizing the extraction of (poly)phenolic compounds from *Vaccinium myrtillus* L. bilberries".

2) *The iTenticate similarity index of 32% is rather high. It should be reduced to ca. 20%.*

Answer. We agree that 32% is a rather high level of similarity and that a level of about 20% could be a good target for reducing this similarity index. However, it is not easy for us to identify the parts of the manuscript that generate this rather high level of similarity. Moreover, we do not have any software that makes this similarity control. What we can state is that no plagiarism was made and that the content of our manuscript is original and never published before, not even in part. We hope that during the revision, the changes made have involved a reduction of the similarity index.

3) *The abbreviation for dry weight should be explicitly defined in the abstract.*

Answer. The abbreviation for dry weight (d.w.) has been introduced in the abstract, as suggested by the Editor.

4) *'detection limits (LODs)' ? - Please match the wording of the abbreviation to the full term.*

Answer. The term Detection Limit has been abbreviated in "DL" throughout the manuscript, as recommended by the Editor.

5) *References: Journal titles are reported in an inconsistent manner. The proper abbreviations in the style of this journal should be used. The correct abbreviation of the journal Trends in Analytical Chemistry, is Trends Anal. Chem.*

Answer. The style of the references has been modified according to JCA Guide for Authors.

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Reviewer #1:

1) *The authors developed an efficient extraction method of (poly)phenolic compounds from bilberry by means of an analytical QbD framework. The QbD approach has proved to be of great use for the optimization of analytical procedures. It is an innovative application in this field with great advantages in terms of time and resources saving. The extraction conditions optimized in this work for bilberry are potentially suitable to be extended to the extraction of other small fruits, which are very rich in anthocyanins. Generally, the manuscript is well organized and contains sufficient scientific information. To my mind, this work is of interest and helpful for related study in this filed. Therefore, I recommend publishing this manuscript in Journal of Chromatography A after major revision.*

Answer. We would like to thank Reviewer #1 for her/his very positive comments on our manuscript.

2) *The limitation of the method should also be clearly described in the manuscript.*

Answer. We are not sure that we have correctly interpreted the comment and we apologize if we are wrong. We believe to understand that the Reviewer #1 asks for eventual limitations of the extraction approach we selected based on the application of the QbD protocol. Of course, each approach has its own limitation. However, each approach can be put into practice in a way that minimizes its drawbacks. The ultrasound-assisted extraction can be performed using a tip system or a bath system (the one used in our research), with the latter being less expensive and able with a single apparatus to perform multiple extractions simultaneously, thus increasing the analytical throughput. Hence, we honestly believe to have reduced the limitations of the UAE approach. We have tried to clarify this further point in lines 516-519 of the revised version, changes tracked.

3) *The mechanical solvent extraction, MAE and UAE methods were investigated by screening matrices. As know, the high-speed counter current chromatography is also widely applied for anthocyanins extraction. The performance of the developed should be carefully compared with these extraction methods in the introduction.*

Answer. We agree with the Reviewer #1 that counter current chromatography (CCC) has been applied for the isolation/concentration of anthocyanins from liquid extracts



obtained from different vegetal matrixes. We did not mention this technique because we focused our attention on the extraction methods devoted to the recovery of anthocyanins from solid matrixes. It is not easy to compare the extraction efficiency of CCC with extraction methods applied to the solid matrix, since CCC is a further purification step after the extraction of the solid matrix and not an alternative extraction method to be applied on the solid materials. However, we have added in the Introduction a sentence citing this technique, to increase the amount of valuable information provided by this section (lines 85-87 of the revised version, tracked changes), also providing a reference (new number 13 of the reference list).

4) *The author should also carefully check the format requirement of references.*

Answer. The style of the references has been checked and modified when necessary, according to JCA Guide for Authors.

Reviewer #2:

1) *The authors presented a novel approach to determine the optimum conditions for the extraction of anthocyanins in bilberries after a design of experiments. Their contribution is very valuable to the scientific community, however a minor suggestions are given in order to be accepted for publication.*

Answer. We would like to thank Reviewer #2 for her/his very positive comment.

2) *Page 2. Abstract. Lines 44 - 47. CMA abbreviation was not introduced. Also, please include the recoveries for each compound in the optimized selected method. The description of other analytical parameters from the method validation are desirable.*

Answer. CMA abbreviation has now been introduced in the abstract. Moreover, a new paragraph has been added, containing information on matrix effect and recovery of analytes.

3) *Page 5. Paragraph starting in line 103. Although it is usual to introduce the goals of the work in the last paragraph of the introduction, in your case there are a lot of details that belong to the methodology. To be organized, please just briefly resume what are your goals and why are you using your approximation to the proposed problem and move the methodology details to the experimental sections.*



Answer. We would like to thank the Reviewer #2 for her/his remark. The methodological details to which the Reviewer referred were moved from the "Introduction" to the "Materials and methods", and the latter section reorganized accordingly.

4) *Page 7. Line 167. Did you mean 9000 rpm instead of 9000xg?*

Answer. We would like to thank the Reviewer #2 for this remark. The values of 9000 actually refers to rpm and corresponds to about 8500xg. The text has been changed accordingly.

5) *Page 18. Line 450. Which test did you use for Boxplot comparisons, Mann-Whitney, Kruskal-Wallis...?*

Answer. We thank the Reviewer #2 for this remark. Actually, in the text this information was missing, while it is reported in the caption of Supplementary Fig. S3. We used Games-Howells test, since this is the only one available test in our software that provide comparison of means without assuming equal variances within the investigated groups. This information is now added in the text in paragraph 3.3, lines 510-511 of the revised version, changes tracked.

6) *Page 20. Line 500. Although there are no certified materials for your analytes in berries, a possibility is to evaluate accuracy may be the comparison of the same sample with a reference extraction method (e.g., Soxhlet extraction) instead of assuming a median level from your set of experiments. Maybe you can develop such comparison and completely validate your method.*

Answer. The Reviewer is right in pointing out an issue regarding the median level, which has not been properly explained in the manuscript and was effectively not clear. In the text of the original manuscript it was reported: "The target values for the three selected CMAs (i.e. TSP, TMA, and DPPH) were set by considering overall the results....The thresholds for the CMAs were fixed at the median level of the BBD experimental data (**Table 1**), i.e. $TSP \geq 4100$ mg CAT eq/100 g d.w; $TMA \geq 3400$ mg CYA-3-GLU eq/100 g d.w.; $DPPH \geq 260$ μ g DPPH inhib./mg d.w." The median level for TSP, TMA and DPPH was not assumed as a comparison to evaluate accuracy. The median was calculated on the values measured when running the RSM experimental plan, referring to different UAE experimental conditions. The median level was assumed as threshold in order to give to all the CMAs the same importance when calculating the MODR and a sentence has been added in the text to explain our choice (lines 523-524 of the revised versions, changes tracked). Other mathematical



thresholds could be selected, as the 75% quartile, to stress the optimization of the CMAs trying to approach to higher values. Nevertheless, the selection of the median was one of the few possibilities for being able to define a MODR. In this regard, it should be pointed out that QbD process is very restrictive, because the MODR is identified not only on the basis of the calculated predicted results, but also considering the model error. For this reason, the space of the MODR is limited with respect to the space of the sweet spot. Hence, stricter requirements, such as the 75% quartile, or a higher number of CMAs, possibly could not lead to the possibility of identify the MODR, just for mathematical constraints.

Regarding the use of Soxhlet extraction, for sure it is a valid reference extraction method for the extraction of thermally stable hydrophobic compounds in various matrixes, using low-boiling point solvents. However, the solvents of choice, widely adopted for polyphenol extraction from berries (and many other fruits) are mixtures of water and methanol, or ethanol or acetone. The high boiling points of these extraction mixtures and the thermal susceptibility of target analytes (this consideration was reported in the original version of the manuscript at the end of paragraph 3.3) make the Soxhlet not suitable for our application. For example, when MAE was used, the maximum temperature adopted was 60°C, which is well below the boiling temperature of our extraction solvent mixtures. Hence, with this temperature, Soxhlet cannot be used. Moreover, besides the considerations regarding the Soxhlet method, it should be noted that for the extraction of polar phenolic compounds such as anthocyanins, it is not appropriate to speak about reference extraction methods, since the three methods tested are widely used and therefore, in a sense, all three are reference extraction approaches. This consideration was present in the Introduction section of the originally submitted paper (lines 103-104).

7) Page 22. Lines 542 - 544. *I think the information regarding recoveries and matrix effect is valuable to be included in the abstract as they reflect easily how your method performed.*

Answer. We would like to thank the Reviewer #2 for her/his valuable comment. As specified above, the information has been added in the abstract as suggested by the Reviewer.

8) *All Figures. You should state the units of the time variable in all graphs even when it is declared in the graph description.*

Answer. The units of the "time" variable are in minutes in all the Figures. According to the Reviewer's request, all the figures are now showing the time unit.

- First comprehensive study of an extraction process by using Quality by Design
- Tuning was based on assays for (poly)phenols and antiradical/antioxidant activity
- Mechanical, microwave-assisted, and ultrasonic-assisted extractions were studied
- Ultrasounds and methanol/water 56/44 (v/v) gave the best extraction of anthocyanins
- The use of this method can be extended to other anthocyanin-rich small fruits

Quality by Design in optimizing the extraction of (poly)phenolic compounds from *Vaccinium myrtillus* berries

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Abstract

Quality by Design was adopted for developing an effective extraction procedure of (poly)phenolic compounds from bilberry (*Vaccinium myrtillus* L.) fruits, using a pooled sample of berries from different regions of Ukraine. Mechanical solvent extraction, microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) were investigated by screening matrices. Extraction time (*Time*, from 5 to 15 min), organic solvent type (*OS type*, methanol, ethanol and acetone), organic solvent percentage (*OS%*, from 50% to 90%), sample/extractant ratio (*S/E ratio*, from 0.025 to 0.1 g mL⁻¹), and, only for MAE, extraction temperature (*T*, from 30 to 60 °C), were selected as critical method parameters (CMPs). The spectrophotometric assays total soluble polyphenols (TSP), total monomeric anthocyanins (TMA), and radical scavenging activity (evaluated by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), the 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid), and the ferric reducing antioxidant power methods) were chosen as critical method attributes (CMAs). The screening procedure allowed for selecting UAE and methanol, while the other CMPs underwent further optimization through Response Surface Methodology. Target values for TSP, TMA and DPPH were selected and the method operable design region (MODR) was defined by means of Monte-Carlo simulations. The optimized conditions, with the corresponding MODR intervals in bracket, were the following: (i) *Time*, 17 min (15-23 min); *OS%*, 56% (44-59%); *S/E ratio*, 0.030 (0.022-0.034) g mL⁻¹. Under these experimental conditions, CMAs values of the pooled sample were the following (*n*=3): TSP=4433±176 mg (+)-catechin eq/100 g dry weight (d.w.); TMA=3575±194 mg cyanidin-3-glucoside eq/100 g d.w.; DPPH=273±5 µg DPPH inhib./mg d.w. The optimized extraction method was tested for matrix effect (ME%) in the UHPLC-MS/MS analysis of 15 anthocyanins and 20 non-anthocyanins individual (poly)phenols commonly found in bilberries, as well as for luteolin, sinapic acid, and pelargonidin-3-glucoside, absent in this fruit and therefore added to the extracts as surrogate standards for evaluating apparent recovery (AR%). |ME%| was in any case ≤ 23%

48 and AR% of the surrogate standards in the range 91-95%, confirming the very good performances of the
49 optimized extraction method.

50

51 *Keywords:* Anthocyanins; Experimental Design; Ultrasound-Assisted extraction; Mechanical Solvent
52 Extraction; Microwave-Assisted Extraction

1 Introduction

(Poly)phenols possess important health promoting and protecting properties, which are directly related to their strong antioxidant and antiradical activities [1, 2]. Recent studies point out the potential of a (poly)phenol-rich diet to control chronic inflammation associated with various conditions, like cardiovascular diseases, diabetes and cancer [3-6].

Bilberry (*Vaccinium myrtillus* L.) fruits contain a wide variety and high concentrations of (poly)phenolic compounds, particularly anthocyanins [7, 8] and therefore they are extensively used for the formulation of supplements and drugs for the treatment of different diseases, such as minor venous circulatory disturbances, mild diarrhoea, and minor inflammations of the oral mucosa [9].

The development of effective extraction procedures for the antioxidant compounds contained in the berries' matrix has proven to be a challenge, mainly due to their thermal instability, high structural diversity, and powerful antioxidant activity. These characteristics, while beneficial in medicinal use, make them able to readily react with co-extracted matrix components, ultimately losing their ability to act as radical scavengers [10]. Mechanical solvent extraction (MSE) is an established and widely used procedure for the extraction of bioactive compounds from natural plant materials. While undoubtedly having a simple experimental setup and low energy consumption, this technique finds its main disadvantage in the time span required for extraction and in the amount of solvent employed for subsequent extractions. More recently, both microwave-assisted extraction (MAE) [10] and ultrasonic-assisted extraction (UAE) [11, 12] have been evaluated as potential alternatives to MSE. Moreover, when large-scale isolation of anthocyanins is required (e.g. for clinical trials), post extraction techniques, such as counter current chromatography is applied [13]

The mechanism and dynamics of solvent extraction are well known, with extraction efficiency rapidly reaching a natural plateau after a few subsequent extractions. Ultrasound and microwave assisted applications push extraction efficiency forward through different energy-delivery routes [14].

77 Ultrasounds work through cavitation, producing peak point-variations of pressure, which result in small
78 implosions that locally heat up and finely mince the solid structure of the plant material, enhancing the
79 solid's surface area, and favouring a more intimate and efficient contact between the solvent and the
80 extractant. Microwaves work by assisting the diffusion of target analytes from the solid matrix to the
81 solvent bulk, making molecular dipoles rotate, thus heating up the solution from the inside, and creating
82 a heating gradient that has its peak in the bulk of the solution, extending outward. The combination of
83 this heat gradient with a soaked solid matrix causes both diffusive and heat-driven motions to be aligned
84 in the same direction, ultimately aiding analytes desorption [15].

85 Several Design of Experiments driven optimizations have been carried out in different papers,
86 encompassing several fields of application for both MAE [16-18] and UAE [19-22], highlighting the
87 efficiency of these techniques in increasing the extraction yield of (poly)phenols from the investigated
88 matrices. In particular, Routray and co-workers [17] evidenced that MAE resulted in higher extraction
89 yields than the 24-h mechanical extraction procedure, whereas Xu et al. [22] proved that (poly)phenols
90 extraction yield increased by the application of a multi-frequency ultrasound procedure instead of a
91 single-frequency one. However, to our knowledge, a comprehensive Quality by Design (QbD) approach
92 to the development of these extraction procedures, particularly regarding the extraction of (poly)phenols
93 from a vegetal matrix, has not yet been investigated. QbD has been firstly introduced by the US Food
94 and Drug Administration [23] as a new and rational way to integrate quality into products and product
95 manufacturing in the pharmaceutical field. The application of this quality paradigm is also the subject of
96 the Q8(R2) Guideline by the International Council for Harmonisation (ICH) [24] and is therein defined
97 as “a systematic approach to development that begins with predefined objectives and emphasizes product
98 and process understanding and process control, based on sound science and quality risk management”.

99 While these texts focus on pharmaceutical product development, the application of QbD has also been
100 extended to different fields; notably the analytical method development in drug analysis [25-30], food

analysis [31] and environmental analysis [29, 32]. Using a risk-based and systematic strategy, QbD steers process development by building quality into the process itself, through the definition of process goals and the specification of a method operable design region (MODR), while also bringing deeper process understanding. The MODR is a multidimensional region, which ensures that critically chosen responses are included within predefined limits with an acceptable level of probability [25, 33].

In this study, a systematic evaluation of the three most used extraction alternatives, MSE, MAE and UAE, was performed employing an analytical QbD (AQbD) approach to maximize the extraction efficiency of phenolic compounds from a pooled sample of bilberries collected in different regions of Ukraine. The three extraction procedures were firstly investigated through screening matrices. On the basis of screening results, UAE was selected for further optimization by Response Surface Methodology (RSM) through Box-Behnken Design (BBD), leading to the definition of the MODR, calculated by Monte-Carlo simulations [34], and to the selection of an optimum working point. Finally, the optimized UAE extraction was applied to the UHPLC-MS/MS determination of individual (poly)phenolic compounds in the pooled sample, thus obtaining for the first time data on bilberry from this European region.

2 Materials and methods

2.1 Chemicals and reagents

Reference standards were supplied as follows: cyanidin-3-galactoside (CYA-3-GAL), cyanidin-3-glucoside (CYA-3-GLU), cyanidin-3-arabinoside (CYA-3-ARA), delphinidin-3-glucoside (DEL-3-GLU), delphinidin-3-galactoside (DEL-3-GAL), delphinidin-3-arabinoside (DEL-3-ARA), pelargonidin-3-glucoside (PEL-3-GLU), malvidin-3-glucoside (MAL-3-GLU), malvidin-3-arabinoside (MAL-3-ARA) and malvidin-3-galactoside (MAL-3-GAL) by Extrasynthese (Genay, France); peonidin-3-glucoside (PEO-3-GLU), peonidin-3-galactoside (PEO-3-GAL), peonidin-3-arabinoside (PEO-3-ARA) and petunidin-3-glucoside (PET-3-GLU) by Polyphenols Laboratories AS (Sandnes, Norway);

petunidin-3-arabinoside (PET-3-ARA) and petunidin-3-galactoside (PET-3-GAL) by MEDChem Express (New Jersey, USA); gallic acid (GAC), caffeic acid (CAF), p-coumaric acid (COU), sinapic acid (SIN), ferulic acid (FER), salicylic acid (SAL), chlorogenic acid (CHL), neochlorogenic acid (NEO), cryptochlorogenic acid (CRY), esculetin (ESC), scopoletin (SCO), (+)-catechin (CAT), epicatechin (EPI), luteolin (LUT), myricetin (MYR), phloretin (PHL), phloridzin (PHZ), quercetin (QUE), quercetin-3-rhamnoside (QUE-3-RHA), quercetin-3-glucoside (QUE-3-GLU), quercetin-3-galactoside (QUE-3-GAL) and quercetin-3-rutinoside (QUE-3-RUT) by Sigma–Aldrich (St. Louis, MO, USA). LC–MS grade methanol and water were obtained from J.T. Baker (Deventer, The Netherlands). HPLC-grade methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Laboratory reagents adopted were: formic acid, sodium fluoride, Folin–Ciocalteu reagent and sodium carbonate by Merck (Darmstadt, Germany); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), ABTS, and DPPH radical by Sigma–Aldrich (St. Louis, USA); acetic acid and hydrochloric acid (36-37%) by J.T. Baker (Center Valley, PA, USA); iron (III) chloride hexahydrate by Panreac (Barcelona, Spain); potassium chloride and sodium acetate trihydrate by Carlo Erba (Milan, Italy). Nylon membranes (porosity 0.2 μm) for the filtration of the bilberry extracts before UHPLC analysis were obtained from VWR International (Radnor, PA). Milli-Q water was taken from a Milli-Q system supplied by Millipore (Billerica, MA).

2.2 Stock solutions

Stock solutions were prepared for all phenolic standards at 2 mg mL⁻¹ in methanol and kept in the dark at -18°C for long-term storage. A working solution was prepared as a standard mix of all investigated compounds, at a concentration of 10 ppm in methanol, and stored under the same conditions.

2.3 Sample origin

Fully ripe *V. myrtillus* berries were harvested between July and August 2019 in different regions of Ukraine traditionally involved in the harvest of spontaneous bilberry (see Supplementary **Table S1** for

149 full details). A sample pool of the different harvested fruits was obtained by grinding equal amounts of
150 each individual sample by a hand blender in an ice bath.

151 2.4 Design of the experiments and extraction conditions

152 In the screening step, the pool was extracted with MSE, MAE, and UAE, investigating the following
153 critical method parameters (CMPs): (i) “Extraction Time” (Time), (ii) “Organic Solvent type” (OS type),
154 (iii) “Organic Solvent percentage” (OS%), (iv) “Sample/Extractant ratio” (S/E ratio, g mL⁻¹), and, only
155 for MAE, (v) “Extraction Temperature” (T). For all extraction methods, 0.250 g aliquots of dry weight
156 (d.w.) material were extracted using mixtures of water and organic solvent (i.e. methanol, ethanol or
157 acetone) under the following range of experimental conditions (see Supplementary **Table S2**): organic
158 solvent percentage 50-90%, extraction time 5-15 min, extractant volume 2.5-10 mL, temperature (for
159 MAE only) 30-60 °C. In all cases, 10 mM NaF was added to the extractant to inactivate polyphenol
160 oxidase. After extraction, the obtained mixture was centrifuged at 8500xg for 3 min and the supernatant
161 recovered. The procedure was repeated twice, and the extracts were analysed separately.

162 For each extraction approach, a specific number of experiments was performed as described in the
163 Supplementary **Table S3**, **Table S4**, and **Table S5** for MSE, MAE, and UAE, respectively. MSE was
164 carried out by magnetic stirring at room temperature in flat bottom glass vials of proper capacity. MAE
165 was performed through an Ethos 1 Microwave Digester supplied by Milestone s.r.l. (Sorisole, Italy),
166 while UAE was performed using a Bandelin Sonorex Digitech Type DT512H (Bandelin electronic,
167 Berlin, Germany) ultrasonic bath. In the final optimized conditions, UAE was employed for the
168 extraction of 0.250 g aliquots of d.w. material for 17 min with 8.3 mL of methanol 56%, corresponding
169 to a Sample/Extractant ratio of 0.030 g mL⁻¹. The MODR corresponded to the following interval:
170 Extraction Time, 15-23 min; Organic Solvent percentage, 44-59%; Sample/Extractant ratio, 0.022-0.034
171 g mL⁻¹.

The critical method attributes (CMAs) used as tools for comparing the extraction efficiency of the three methods were represented by a series of macroparameters measured by spectrophotometric assays, in order to profile the (poly)phenolic content and the antioxidant capacity of the extracts. These included total soluble polyphenols (TSP), total monomeric anthocyanins (TMA), and radical scavenging activity, the last one evaluated by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays.

2.5 Spectrophotometric assays

2.5.1 Analysis of total soluble polyphenols

TSP were spectrophotometrically determined [35] as follows: 25-200 μ L of the extract (depending on the estimated concentration of (poly)phenols in the extract) were mixed with Milli-Q water and 200 μ L of Folin-Ciocalteu reagent to achieve in any case a final volume of 9.4 mL. After 3 min of incubation in the dark, 400 μ L of an aqueous solution saturated with sodium carbonate were added and the mixture obtained was dark incubated for 1 h; afterwards the absorbance was measured at 740 nm and (poly)phenol concentration calculated on the basis of a CAT calibration curve; accordingly, the results were expressed as mg of CAT eq/100 g of d.w. berries.

2.5.2 Analysis of total monomeric anthocyanins

TMA were measured by the following procedure [36]. Aliquots of 50-500 μ L of bilberry extract were diluted in solutions at pH=1.0 (KCl 1.86 g + 6 mL HCl 37%; final volume 1 L Milli-Q water) and pH=4.5 (anhydrous CH_3COONa 32.81 g + 20 mL HCl 37%; final volume 1 L Milli-Q water), to obtain a final volume of 10 mL. The absorbance (*Abs*) of both solutions were measured at 520 and 700 nm and the quantity ΔAbs was calculated according to Equation (1).

$$\Delta Abs = (Abs_{pH=1.0}^{520\text{ nm}} - Abs_{pH=1.0}^{700\text{ nm}}) - (Abs_{pH=4.5}^{520\text{ nm}} - Abs_{pH=4.5}^{700\text{ nm}}) \quad (1)$$

Similarly, ΔAbs values were also calculated for different concentrations of CYA-3-GLU, used as reference standard, and plotted as a function of CYA-3-GLU concentrations. The best equation fitting

the experimental points was calculated by the least square method, thus obtaining a linear calibration curve. TMA in the extracts were finally calculated using this calibration curve as mg of CYA-3-GLU eq/100 g of d.w. berries.

2.5.3 DPPH radical scavenging activity assay

Radical scavenging activity (RSA) was spectrophotometrically measured [37] using the free radical DPPH, according to the following procedure. Five different volumes of each extract (5-100 μ L and 10-200 μ L, for the first and second extraction aliquot respectively) were added to 5 mL of a 0.1 mM DPPH solution in methanol. For each extract, a blank was performed by replacing the extract with Milli-Q water. The decrease in absorbance at 517 nm was monitored every 30 min up until 360 min of the incubation in the dark when the reaction reached a plateau. For each concentration tested, the reaction kinetics was plotted. The percentage of DPPH neutralized at the steady state (%DPPH) was determined by using Equation (2):

$$\%DPPH = 100 \times \frac{(Abs_0^E - Abs_{ss}^E) - (Abs_0^B - Abs_{ss}^B)}{Abs_0^E} \quad (2)$$

where Abs_0^E , Abs_{ss}^E , Abs_0^B , Abs_{ss}^B are the absorbance values of the extract (E) and of the blank (B) at time zero (0) and at the steady state (ss).

The DPPH inhibition percentages obtained were plotted as a function of the corresponding amount of bilberry (d.w.) in the five samples analysed and the best equation fitting the experimental points was calculated by the least square method. IC50 was defined as the concentration of the sample (mg of fruit, d.w.) in 1 mL of reaction mixture, necessary to decrease the initial DPPH concentration (which was kept constant in all the experiments) by 50%; therefore, higher IC50 values correspond to lower RSA values. The RSA of samples (RSA_{sample}) can be derived from IC50 values (μ g d.w./ μ mol DPPH solution) determined on bilberry extracts ($IC50_{sample}$) by using Equation (3), allowing for obtaining a parameter

218 that increases with the rise in antiradical activity and that is expressed as µg DPPH inhibited by 1 mg of
219 d.w. berry sample.

$$220 \quad RSA_{sample} = \frac{MW_{DPPH}}{IC50_{sample}} \quad (3)$$

221 2.5.4 ABTS radical scavenging activity assay

222 RSA was spectrophotometrically measured using the free radical ABTS [38], according to a procedure
223 similar to that described for DPPH. ABTS was converted to its radical cation by addition of 175 µL of
224 140 mM potassium persulfate to 9.825 mL of 7 mM ABTS (both solutions in Milli-Q water), followed
225 by incubation in the dark for 12-16 h. The obtained solution was diluted 1/100 (v/v) in buffer solution at
226 pH=4.5 (anhydrous CH₃COONa 32.81 g + 20 mL HCl 37%; final volume 1 L MilliQ water) and used
227 for the analyses. As the method is very sensitive, ABTS-RSA was performed using 5-25 µL or 20-100
228 µL of diluted extracts (dilution ratio 5-10 times). The absorbance was measured at 734 nm. All
229 measurements and calculations were carried out as described for DPPH.

230 2.5.5 FRAP antioxidant activity assay

231 FRAP reagent [39] was prepared with 10 volumes of 300 mM acetate buffer pH=3.6 (anhydrous
232 CH₃COONa 1.9 g + CH₃COOH 99% 16 mL; final volume 1 L Milli-Q water), 1 volume of 10 mM TPTZ
233 in 40 mM HCl and 1 volume of 20 mM FeCl₃. Aliquots of 25-500 µL of a properly diluted extract (to
234 obtain an absorbance value included in the calibration line) were added to 5 mL of FRAP reagent pre-
235 warmed at 37°C. After 4 hours of incubation in the dark at 37°C, absorbance was read at 593 nm. Results
236 are expressed as mmol of Trolox equivalents per g of d.w. berries.

237 2.6 UHPLC-MS/MS instrumentation and analysis

238 Pooled sample extracts were filtered before UHPLC-MS/MS analysis on 0.22 µm nylon membranes, and
239 further diluted 1/1000 (v/v). Analysis of the individual compounds was performed through an UHPLC
240 system, hyphenated with an electrospray ionization (ESI) source and a tandem (MS/MS) mass

spectrometer, working as triple quadrupole. The UHPLC system (Shimadzu, Kyoto, Japan) consisted of a low-pressure gradient quaternary pump Nexera X2 LC-30AD, a CTO/20AC thermostatted column compartment, a SIL-30AC autoinjector, a DGU-20A 5R degassing unit and a CBM-20A module controller. The detector was a 5500 QTrapTM hybrid triple quadrupole-linear ion trap mass spectrometer (Sciex, Ontario, Canada) through a Turbo VTM interface equipped with an ESI probe.

The chromatographic separation was performed on a Phenomenex Kinetex XB-C18 chromatographic column (150×2.1 mm i.d., particle size 2.6 µm) equipped with a guard column using the same stationary phase. Chromatographic analysis of the non-anthocyanin (poly)phenols was performed at 50 °C, eluting the injected volumes with water/formic acid 99.9/0.1 (v/v) (eluent A) and methanol/formic acid 99.9/0.1 (v/v) (eluent B), according to the following gradient: 0-3 min isocratic 2% (eluent B), 3-4 min linear gradient 2-15% (eluent B), 4-27 min linear gradient 15-60% (eluent B), 27-29 min linear gradient 60-95% (eluent B), 29-33 min isocratic 95% (eluent B). Chromatographic analysis of the anthocyanins was performed at 50 °C using water/formic acid 95/5 (v/v) (eluent A) and methanol/formic acid 95/5 (v/v) (eluent B), according to the following gradient: 0.1-10 min linear gradient 7-17% (eluent B), 10-20 min linear gradient 17-22% (eluent B), 20-22 min linear gradient up to 95% (eluent B), 22-27 min isocratic 95% (eluent B). Flow rate and injection volume were set at 0.3 mL min⁻¹ and 5 µL respectively, for both chromatographic runs.

Target compound MS/MS analysis was carried out using the Multiple Reaction Monitoring mode (MRM) using the ESI source in negative or positive ionization mode, depending on the analyte considered. Source dependent parameters were optimized through flow injection analysis, at optimal LC flow and mobile phase composition. For the negative ionization mode, ESI (-), optimal source parameters were set as follows: curtain gas, 30; CAD gas, medium; temperature, 600 °C; gas 1, 50; gas 2, 65; interface heater on; ion spray voltage, -4500 V. For the positive ionization mode, ESI (+), optimal source parameters were: curtain gas, 30; CAD gas, medium; temperature, 600 °C; gas 1, 40; gas 2, 65; interface

265 heater on; ion spray voltage, 5500 V. For each investigated compound, the most intense transition current
266 was used for its quantification and the second most intense one for confirming its identification [40].
267 Compound dependent parameters were optimized by direct infusion of properly diluted target analyte
268 standard solutions and are reported in Supplementary **Table S6**.

269 2.7 UHPLC-MS/MS method

270 The analytical performance of the UHPLC-MS/MS method here adopted for the quantification of the
271 individual (poly)phenols was checked in terms of: (i) instrumental detection limits (DLs), (ii)
272 instrumental limit of quantification (LOQ), (iii) instrumental repeatability, and (iv) linearity range. These
273 data are shown in Supplementary **Tables S7** and **S8** for positively and negatively ionisable compounds,
274 respectively.

275 DLs were the considered as the concentration corresponding to a signal-to-noise ratio of three and were
276 evaluated through successive injections of standard solutions at increasing dilutions. The linearity of the
277 method was tested measuring standard solutions at seven different concentration levels (LOQ – 5 – 25 –
278 50 – 100 – 250 – 500 ppb) in triplicate. Intraday repeatability was measured as RSD of five different
279 injections of standards at 25 ppb and 250 ppb concentration levels in LC-MS water. Spiked standards for
280 apparent recovery (AR%) evaluation were LUT and SIN for negative ionization, and PEL-3-GLU for
281 positive ionization. The AR% were evaluated to account for the goodness of the whole extraction and
282 analysis procedure, by spiking aliquots of dried bilberry powder with these standards and subjecting them
283 to the optimised extraction procedure, as to obtain a theoretical extract concentration of 100 ppb for each
284 standard. Matrix effect (ME%) was investigated by spiking the extracts with different concentrations of
285 target analytes (5, 25, 100, 250 and 500 ppb) and analysing them by UHPLC-MS/MS, together with the
286 non-spiked ones. For each compound, the chromatographic areas found in the spiked extracts were
287 subtracted from those of the non-spiked ones and the resulting differences were plotted as a function of
288 the spiked concentrations. The best curve fitting the experimental points was calculated by least square

289 method and its slope (s_{matrix}) was compared with the one of the corresponding calibration curve in water
290 ($s_{solvent}$). ME% values were calculated according to the following equation (4) [7].

$$291 \quad ME\% = \left(\frac{s_{matrix}}{s_{solvent}} \cdot 100 \right) - 100 \quad (4)$$

292 2.8 Softwares

293 Chromatograms were elaborated using Sciex Analyst software (version 1.6.2). Peak attribution and
294 quantitative determination were performed using MultiQuant software version 3.0.2 (Sciex).

295 NemrodW software (NemrodW, LPRAI sarl, Marseille, France) was used to set up the screening matrices
296 used during the screening phase and for the related data elaboration. MODDE 13 software (Sartorius
297 Stedim Data Analytics AB-Umetrics, Umeå, Sweden) was employed to plan BBD selected for RSM, to
298 perform the relative data treatment and to define the MODR by means of Monte-Carlo simulations. The
299 target for defect per million opportunities (DPMO) [41] was fixed at 10,000, corresponding to a risk of
300 error of 10%.

301 3 Results and discussion

302 An AQbD procedure was followed for the optimization of the extraction conditions of (poly)phenolic
303 compounds. This procedure consisted in (i) the definition of the Analytical Target Profile (ATP), (ii) the
304 selection of CMAs, (iii) the identification of CMPs, (iv) the screening phase, (v) RSM optimization and
305 MODR definition. The selected knowledge space was investigated separately for MSE, MAE, and UAE
306 through three distinct screening experimental designs. The most performing extraction technique was
307 then selected for RSM optimization and MODR definition, and a convenient working point was finally
308 defined for the extraction of the target compounds from the fruit matrix.

309 3.1 Analytical target profile and critical method attributes

310 The establishment of the ATP represents the first step of an AQbD process. Delineating an ATP consists
311 in selecting a set of criteria that encompasses which performance characteristics the developed procedure

312 should exhibit, as well as the means to measure such characteristics [24, 33]. The purpose of optimizing
313 the extraction procedure of (poly)phenols from bilberries was to achieve the best possible extraction
314 efficiency within the limits of a reasonable extraction time, with low solvent consumption, ease of
315 handling and cost efficiency.

316 A series of readily available and widely employed assays was selected for evaluating performance
317 variations during each extraction procedure by tracing the (poly)phenolic contents, as well as the
318 antiradical/antioxidant activity of the final extracts.

319 Since bilberry is a fruit well known for its large content of anthocyanins, but also of non-anthocyanin
320 (poly)phenolic compounds [8], both total (poly)phenolic and total anthocyanin assays were considered
321 as CMAs, i.e. the responses to be maximized and/or kept under control, directly linked with extraction
322 efficiency. These two parameters were determined through (i) the Folin-Ciocalteu assay, index of the
323 total soluble (poly)phenols (TSP) [35] and (ii) the total monomeric anthocyanins (TMA), determined by
324 the pH differential method [36].

325 Moreover, different assays were employed for assessing the antioxidant/antiradical capacity, since its
326 determination includes a wide variety of reactions, in which different scavenging mechanisms are
327 involved. The assays for the determination of antioxidant/antiradical capacity are usually split in two
328 main categories: hydrogen atom transfer (HAT) and single electron transfer (SET) [42]. HAT represents
329 the ability of an antioxidant to scavenge a free radical through the donation of a hydrogen atom. These
330 reactions are independent on both solvent and pH, they tend to be very rapid and they are also regarded
331 as the most relevant for radical scavenging evaluation, since the mechanism is the common $X^\cdot + AH \rightarrow$
332 $XH + A^\cdot$ [42]. SET is a single-electron reaction route where both the ionisation potential and
333 deprotonation of the functional group of the radical scavenging species play a major role [43, 44]. These
334 reactions are pH dependent, with electron-donating capacity increasing with pH value, and tend to be
335 slow. The SET assays measure a percentage decrease in the endpoint compound, and thus the ability of

an antioxidant to transfer an electron. Since both mechanisms tend to occur together, with balance determined by the contributions of pH and of the molecular structures of the antioxidants, and considering that in the extracts multiple antioxidants are present at the same time, in this study both HAT and SET based assays were employed and their results were chosen to represent the quality of extraction performances. Based on the considerations mentioned above, further CMAs, related to antiradical/antioxidant activity, were: (iii) DPPH assay, (iv) ABTS assay, and (v) FRAP assay. The FRAP assay, which works at low pH by reduction of ferric TPTZ to the ferrous complex, is based on the SET mechanism [42]. DPPH and ABTS are mixed-mode (HAT-SET) assays; both radicals used in these tests can be neutralised by either a SET or a HAT mechanism [45], enabling to follow both reactivities at the same time. DPPH is a solid stable free radical, which follows the $DPPH^{\cdot} + AH \rightarrow DPPH - H + A^{\cdot}$ reaction pathway [37, 46] with decolouration of the original radical as its endpoint. ABTS is an acid that after oxidation by peroxides gives an intensely coloured radical cation [38]; the decrease in colour can be directly related to the amount of free-radical scavenged by antioxidants.

3.2 Critical method parameters

The objective of a good extraction procedure is the quantitative and reproducible transfer of a series of target compounds, from a complex native matrix to a suitable medium. Analytes extraction from solid matrices is influenced by their physicochemical properties (e.g., solubility, hydrophobicity, acid-base dissociation, and partition coefficient) and molecular structures. Facing the extraction of many different compounds means dealing with a wide range of properties. To select and optimize an extraction process that efficiently encompasses such a variety, the aforementioned small group of CMPs, suitable to be equally applied to the three extraction techniques, was identified (**Table S2**).

As regards *Time*, fast extraction is always preferable, but contact time must be balanced to allow for proper penetration and diffusion of the solvent into the matrix, as well as analyte diffusion into the bulk solution. As for *OS type*, different solvents exhibit different extraction efficiencies towards target

compounds and matrices, owing to their different polarities and Van Der Waals interaction potentials. Moreover, the variation of the relative percentages of organic solvent and water is expected to exert a great influence on the extraction of polar analytes. *S/E ratio* was evaluated as the best way to control both the amount of solvent and the weight of the sample, as both are critically important variables in the extraction efficiency. Finally, *T* was selected as the specific CMP for MAE investigation, as the best way to control the power supplied to the sample matrix, avoiding potential thermal degradation of the analytes.

3.3 Screening phase

In order to obtain the maximum amount of information on all the investigated extraction techniques by performing a limited number of experiments, a screening experimental design was applied to investigate the CMPs effects on the CMAs throughout the knowledge space. During this phase, two aspects of the method development were considered, using the CMAs for evaluating extraction efficiency. The first aspect was to assess, within each extraction technique, which CMPs were pivotal in steering extraction efficiencies for the target analytes; the second was to compare the performances achieved by each technique on aliquots of the same sample to be able to select the more suitable for going on with the investigation by RSM in a proper experimental domain. The variation ranges for the CMPs were selected based on previous knowledge in phenolics extraction and analysis [7, 8]. In order to plan a homogeneous screening procedure for all the extraction methodologies investigated, the selected CMPs were studied at three levels in the same experimental domains (with the exception of *T*), as shown in **Table S2**.

Besides the aforementioned CMAs (i.e. TSP, TMA, DPPH, FRAP, and ABTS), in this screening phase, the ratio between TSP obtained in the first extraction and the sum of TSP values obtained by the first and the second extraction was also selected as a measure of the single extraction efficiency (SEE).

The effects of the different levels of the CMPs over the CMAs measured for the three techniques were evaluated through two 9-runs (MSE and UAE) and one 16-runs (MAE) symmetric screening matrices,

which allowed four (MSE and UAE) and five (MAE) factors to be studied at three levels. This set-up made it possible the estimation of a linear, no-interaction, Free-Wilson model for each extraction technique. In this type of model, each variable carries a number of coefficients equal to the number of levels minus one, leading to the estimation of the effects of each CMPs level on the CMAs. Consequently, the models used in this study present the constant term A_0 and two coefficients for each variable.

The model for both MSE and UAE is reported in Equation (5):

$$y=A_0+A_1A+A_2A+B_1B+B_2B+C_1C+C_2C+D_1D+D_2D \quad (5)$$

where A refers to *Time*, B to *OS type*, C to *OS%*, and D to *S/E ratio*.

The model for MAE is reported in Equation (6):

$$y=A_0+A_1A+A_2A+B_1B+B_2B+C_1C+C_2C+D_1D+D_2D+E_1E+E_2E \quad (6)$$

where A refers to T , B refers to *Time*, C to *OS type*, D to *OS%*, and E to *S/E ratio*.

The screening matrices, together with the measured responses of TSP, TMA, DPPH, FRAP, ABTS, and SEE, are reported in Supplementary **Tables S3, S4, and S5**, for MSE, MAE, and UAE, respectively. Each experiment of the plan was replicated twice. For each extraction technique, a graphical analysis of the effects was obtained, as shown in Supplementary **Figures S1, S2, and Figure 1** for MSE, MAE, and UAE, respectively. In these figures, plots indicated by b, d, f, h, j, and l, highlight the effect of each level of the CMP on the CMA (the longer the bar, the more the CMA is maximized), while the graphs indicated by a, c, e, g, i, and k show the statistically significance of the effect of the variation between two selected levels on each CMA. Regarding this latter type of plots, the significance of the change of CMP level is directly subject to the cut-off of the estimated experimental error, which is shown as a dotted line running across the bars. Whenever the bar length exceeds the dotted line, the level change of the CMP exerts a statistically significant effect on the CMA.

The first screened technique was MSE (Supplementary **Fig. S1**). The highest value of *Time* was the one that gave rise to the highest values of all CMAs, thus providing a clear indication for this CMP. The

analysis of the effects for *OS type* suggested acetone and methanol as equally effective for the recovery of all CMAs, with the exception of TMA for which the best results were obtained with ethanol and above all methanol. As regards *OS%*, the significance tests in level change all pointed towards the low-medium domain of this CMP (50-70% v/v), suggesting no remarkable difference between the two levels. TMA was an exception since *OS%* of 70% gave rise to a significantly higher recovery than 50%. Surprisingly, the change in *S/E ratio* fell under poor statistical significance in the investigated domain for most CMAs, with the exceptions of FRAP and SEE, which were maximized by medium-high and low-medium values, respectively.

The second technique taken into consideration was MAE. As it can be extrapolated from the plots in Supplementary **Fig. S2**, the change in *T* levels had significant effects on all CMAs, even though their maximization was obtained at different levels depending on the CMA considered (low for TMA; low-medium for TSP; medium for FRAP and high for DPPH and ABTS), suggesting the need of a more in-depth study of its influence on the MAE performances. As regards *Time*, different effects were evidenced, depending on the CMA considered. In detail, no significant effect was observed on TMA, while DPPH and ABTS were maximized at low and high values of the experimental domain. Moreover, FRAP and SEE showed higher recoveries at the central *Time* domain value. A similarity with the MSE trend emerged in the solvent selection (i.e. *OS type*), since the statistical significance across levels strongly pointed out the better performances of either methanol or acetone. Another similarity with MSE was that the change of *OS%* had a major influence on all CMAs, being the middle-low values of the experimental domain those corresponding to the highest CMA values. In contrast with MSE, *S/E ratio* was a significant parameter across most CMAs, with a strong indication towards the lower end (i.e., 0.0250) of the investigated experimental domain, in particular for maximizing TSP, FRAP and SEE.

As for UAE, the plots shown in **Figure 1** evidenced a trend for *Time* similar to that observed for MSE, pointing again towards the higher value of the domain (i.e., 15 min). Also in this case, methanol and

acetone were found the two most suitable solvents, with a strong indication of the former for TMA extraction. *OS%* and *S/E ratio* generally exerted statistically significant contributions, providing in almost all cases the highest recoveries with the medium-low range values of their experimental domain. In order to highlight which of the three extraction approaches was the best performing for the recovery of (poly)phenols, the data obtained during the screening for the different CMAs have been reported in three box plots (see Supplementary **Figure S3a-c**). The boxplots highlight the interquartile ranges of the values obtained, i.e. top of the box is the third quartile (Q3), while the bottom of the box is the first quartile (Q1). Furthermore, the plot evidenced the median and the mean values, the upper and lower whiskers corresponding to $[Q3+1.5 \cdot (Q3-Q1)]$ and $[Q1+1.5 \cdot (Q3-Q1)]$, respectively, and the outliers (i.e. observations beyond the upper or lower whisker). Based on the boxplot data, no extraction approach yielded statistically better results than the others for all investigated CMAs, using the Games-Howell comparison test at the 5% probability level. In fact, the recovery of TSPs was in the order $MSE \approx UAE > MAE$, while for TMA it was $UAE > MSE > MAE$. Moreover, the use of MAE resulted in higher DPPH values than MSE, while the three extraction systems were equivalent for FRAP and ABTS. However, considering that UAE never resulted as the extraction technique providing the worse results and that bilberry is particularly rich in anthocyanins, which were better recovered by UAE, this method was selected for the further optimization by RSM. Another point in favor of UAE implemented as ultrasonic bath is the relatively affordable cost compared to microwave and ultrasonic tip systems. In addition, the ultrasonic bath system, compared to the tip one, allows for the simultaneous extraction of a number of samples with a single device, thus increasing the analytical throughput. Within UAE, the information obtained from the screening phase made it possible to fix methanol as the organic solvent providing by far the highest recovery of TMA (**Fig. 1**), while the other parameters were investigated in detail by means of RSM. In particular, the domain for *Time* was shifted to higher levels (15-25 min),

455 while the ranges of OS % and *S/E ratio* were shifted to lower levels (40-80% v/v and 0.0250-0.0650 g
456 mL⁻¹, respectively), as reported in Supplementary **Table S2**.

457 Moreover, the results of the screening phase for SEE indicated that in general, for all the three techniques,
458 the single extraction was more efficient with respect to the second one if higher *Time* and lower *S/E ratio*
459 values (hence higher volumes of extractant) were used. Nevertheless, the TSP fraction recovered by the
460 second fraction was never negligible compared to that recovered from the first fraction, being the SEE
461 values obtained for UAE within the screening procedure in the range of 0.720-0.875 (see Supplementary
462 **Table S5**). For this reason, it was decided to perform two subsequent extractions in the analytical
463 procedure for the continuation of the study.

464 3.4 Response surface methodology and method operable design region

465 Based on the results of the screening procedure, an RSM optimization was carried out in order to further
466 enhance UAE extraction efficiency. The new investigated experimental domain is reported in
467 Supplementary **Table S2**.

468 Using MODDE software, BBD was employed for estimating the main, interaction and quadratic effects
469 of the CMPs on the CMAs, as postulated in the following quadratic model:

$$470 \quad y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon \quad (7)$$

471 where y is the response, x_i are the independent CMPs, β_0 is the intercept, β_i are the true coefficients and
472 ε is the experimental error. BBD is a three-level design, where the experimental points are located on a
473 hypersphere and are placed at equal distance from the central point, exhibiting a high level of efficiency,
474 in terms of number of required runs with respect to the number of coefficients to be estimated [47]. The
475 experimental plan with the obtained responses is shown in **Table 1**.

476 Model refinement was carried out in a sequential way, through exclusion of the factors that were found
477 to be not significant in the coefficient plot, in order to achieve an improvement in the model predictivity
478 Q^2 [41]. The quality parameters of the calculated models are reported in Supplementary **Table S9**. All

the models resulted both valid and significant and could be used for drawing contour plots. Anyway, the quality parameters of the models were higher for TSP, TMA and DPPH, while for FRAP and ABTS a lower quality was obtained, especially in terms of model reproducibility. Thus, it was decided to explore the effects of the CMPs for all the responses using the response surfaces, while, for specifying target requirements and for building the sweet plot spot and the MODR, only TSP, TMA and DPPH were selected as CMAs.

The contour plots for TSP, TMA, DPPH, FRAP, and ABTS were reported in **Figure 2**, where the zones coloured in orange-red represent the maximization of these responses. Different trends and curvatures in the predicted responses were observed, thus highlighting the importance of using the multiresponse criteria in order to find the best compromise among the different responses. Very important quadratic effects, highlighted by pronounced curvatures in the isoresponse lines, were highlighted for OS% on TSP (**Fig. 2a**) and for *S/E ratio* on DPPH (**Fig. 2c**) and FRAP (**Fig. 2d**).

The target values for the three selected CMAs (i.e. TSP, TMA, and DPPH) were set by considering overall the results obtained in the experimental domain under study, in particular by setting the values around the mean of the results obtained, in order to find a zone that represents a good compromise for all the selected CMAs. In this study, it was not possible to fix the target values beforehand, as no certified reference materials were available for bilberry, and the responses refer to parameters that even after sample fortification with (poly)phenolic compounds cannot be determined irrespective of their native content in the pool of berries. In this latter regard, it should be noted that this problem would not have been overcome even if individual (poly)phenols would have been used as CMAs, since for these analytes reference standards that can be determined regardless of their native content (i.e. labelled standards) are not commercially available. The thresholds for the CMAs were fixed at the median level of the BBD experimental data (**Table 1**), i.e. $TSP \geq 4100$ mg CAT eq/100 g d.w; $TMA \geq 3400$ mg CYA-3-GLU eq/100

g d.w.; DPPH $\geq$ 260 μ g DPPH inhib./mg d.w. Following this approach, the same importance was given to all CMAs when calculating the MODR.

The sweet spot plots are reported in Supplementary **Figure S4** at three fixed values of *Time*. In these plots, the overlay of isoresponse surfaces led to identify the green zone as the sweet spot, namely the region where all the predicted values for the CMAs were higher than the selected thresholds. The teal-coloured zone corresponded to the zone where two CMAs reached the desired values, while the blue zone corresponded to the region where only one CMA presented a satisfactory value. In the white zone, no criterion was satisfied. At each plotted Extraction Time value, it was possible to find two green zones, at low and high values of *S/E ratio*, more or less wide.

From the application of Monte-Carlo simulations [34] to the regression models, it was possible to compute the MODR considering the uncertainty of the model parameters. In this way, method uncertainty was estimated and a level of probability that the CMAs specifications are met was calculated [33]. The risk of failure was plotted in the probability maps shown in **Figure 3**. The MODR was calculated from the set-point, which corresponded to *OS%*, 53%; *Time*, 19 min; *S/E ratio*, 0.028 g mL⁻¹, indicated by the green-coloured area, where the risk of failure of fulfilling CMAs requirements was lower than 10%. The MODR interval was the following: *OS %*, 47-59%; *Time*, 15-23 min; *S/E ratio*, 0.022-0.034 g mL⁻¹. Four experiments were run spanning the MODR for confirming that the target values were reached, finding a good agreement between the predicted and the measured values. Then, the working point was selected inside the MODR, choosing a lower value of *Time* with respect to the set-point, in order to decrease the total time of analysis. The optimum point corresponded to the following: *OS%*, 56%; *Time*, 17 min; *S/E ratio*, 0.030 g mL⁻¹. Under these experimental conditions the following results were obtained (n=3): TSP 4443 $\pm$ 176 mg (+)-catechin eq/100 g d.w., TMA 3575 $\pm$ 194 mg CYA-3-GLU eq/100 g d.w., DPPH 273 $\pm$ 54 μ g DPPH inhib./mg d.w., ABTS 1048 $\pm$ 30 μ g ABTS inhib./mg d.w., and

FRAP 183 ± 8.5 mmol Trolox eq./g d.w. It is worth noting that the experimental values obtained for TSP, TMA, and DPPH were higher than the aforementioned thresholds.

3.5 UHPLC-MS/MS analysis of the pooled sample

Since the extraction method was optimized using various macroparameters, it is interesting to investigate its effectiveness in the UHPLC-MS/MS analysis of individual (poly)phenols, in terms of both AR% and ME%. |ME%| was generally much lower than 20% (see Supplementary **Tables S7** and **S8**), which is commonly considered a threshold value below which matrix effect is negligible [48]. The only exception was MYR, which exhibited a signal enhancement of about 23%. It is remarkable that much higher suppressive/enhancing effects were determined elsewhere for MSE of bilberries using methanol/water 80/20 (v/v) [7], thus suggesting a possible role of the mechanical stirring and/or, most probably, higher percentages of methanol in the extractant mixture towards the co-extraction of matrix constituents. The evaluation of AR%, performed using LUT, SIN, and PEL-3-GLU as specified in paragraph 2.5, achieved mean recovery percentages ($n=3$) of 95 ± 12 , 91 ± 14 , and 95 ± 14 , respectively. Matrix effect was found to be very low also for the surrogate standards ($|ME\%| \leq 5\%$), thus indicating good performances of the extraction method of these reference compounds. In this regard, it is worth noting that when the extraction was carried out with MSE and methanol/water 80/20 (v/v), a stronger influence of the matrix was observed for SIN [7]. The high AR% and the low values of |ME%| highlight the very good recovery performances of the optimized extraction method.

Even though this study was not designed for (poly)phenolics profiling, it is interesting to highlight the most abundant individual (poly)phenols in the pooled sample of Ukrainian bilberries, which are here investigated for the first time regarding these analytes. To this aim, the extracts obtained under the optimized experimental conditions were analyzed by UHPLC-MS/MS following a chromatographic method elsewhere reported [7], able to determine 35 compounds among the most abundant (poly)phenols

in bilberry. The typical MRM reconstructed UHPLC-MS/MS chromatograms acquired in positive and negative ionization are shown in **Figure 4a-b**, respectively.

Mean concentrations and standard deviations of individual anthocyanins, as well as their relative percentage abundance determined in five independent replicated analyses of the pooled bilberry sample, extracted under the optimized experimental conditions, are shown in Supplementary **Figure S5**. Overall, the sum of the individual anthocyanins concentrations determined through UHPLC-MS/MS analysis (3399 mg/100 g d.w.) matched the results obtained by the TMA assay (3575 mg CYA-3-GLU eq/100 g d.w.). The concentrations of the investigated anthocyanins were distributed as follows: DEL>>MAL≈CYA>>PET≈PEO. More in detail, DEL glycosides accounted alone for 43% of the total anthocyanins determined, followed by MAL and CYA derivatives, both accounting for about 20% of the total, while PET and PEO represented a rough 10% and 5% of the total anthocyanin content, respectively. Looking at the distribution of the sugar moieties among the investigated anthocyanins, glucosides and galactosides covered 46% and 41% of the whole content, respectively, while the remaining 13% was represented by the arabinosides. This relative distribution seems to be different from that observed in bilberries harvested in Southern Europe and in the extreme North of Europe, where galactosides were lower than glucosides [7, 49] or even lower than arabinosides [50], thus suggesting a possible unique anthocyanin fingerprint for Ukrainian bilberries. However, bilberries collected in Slovenian mountains [51] showed a glycosides distribution pattern similar to that determined in this study.

An overview of the results obtained for the investigated non-anthocyanin (poly)phenols is shown in Supplementary **Figure S6**. The non-anthocyanin compounds were found to be much less abundant than anthocyanins, in agreement with findings of previous studies [7]. Moreover, the total concentration of non-anthocyanin (poly)phenols here determined (268 mg/100 g d.w.) was comparable with that reported elsewhere (252 mg/100 g d.w.) [7]. As mentioned before, the two most predominant compounds were CHL and GAL, which were found at similar concentrations and accounted together for approximately

70% of the whole non-anthocyanin group, followed by QUE-3-GAL (10%), QUE-3-GLU (4%), and COU (4%), while the remaining 12% was shared among the rest of the investigated compounds with percentages generally lower than 1%.

4 Conclusions

The development of an efficient extraction method of (poly)phenolic compounds from bilberry was approached systematically by means of an analytical QbD framework. This approach represents an innovative application in this field, which is added to the other sectors where QbD has already found its consolidated way due to its great advantages in terms of time and resources saving, while making it possible to achieve in-depth information on the phenomenon under study.

The efficiency of extraction was evaluated through several response variables, including TSP, TMA, and some antiradical/antioxidant activity related parameters. Three extraction methods, namely MSE, MAE and UAE, were explored through symmetric screening matrices. The screening procedure highlighted the UAE technique as the most suitable for extraction, especially considering anthocyanins as target analytes. Consequently, RSM was applied to this extraction approach and the contour plots were studied. In this study, the threshold values for CMAs were fixed after performing RSM, unlike what is usually done for the optimization of chromatographic separation methods, where the CMA targets are set *a priori* or after some scouting experiments. This procedure was adopted for two main reasons. (i) Since the optimization procedure must deal with several CMPs and thus with several models, it was fundamental to select the CMA models characterized by the highest statistical quality to be able to achieve a MODR, which is built by considering the model error. (ii) Since the optimization was performed on a real pooled sample with unknown (poly)phenolic composition and antioxidant/antiradical attributes, the desirable values of these variables could not be fixed *a priori* as target. Statistical quality of the models, particularly regarding predictivity, resulted higher for TSP, TMA, and DPPH; hence, target values were fixed for these CMAs and the MODR could be defined as the zone where the risk of error was lower than 10%. In

this way, it was still possible to point out a robust optimum zone inside which the optimal working point could be selected. Hence, the QbD approach has once again proved to be of great use for the optimization of analytical procedures. Since the UAE extraction approach has been selected and optimized considering TMA as a target macroparameter of great relevance, the extraction conditions optimized here for bilberry are probably suitable to be extended to the extraction of other small fruits such as raspberry or blackberry, which are very rich in anthocyanins.

Acknowledgements

The Authors wish to thank the Italian Ministry of Education, University and Research (MIUR) (“Progetto Dipartimenti di Eccellenza 2018-2022” allocated to Department of Chemistry “Ugo Schiff”, University of Florence).

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1 **Quality by Design in ~~the optimizing~~ation of the extraction of (poly)phenolic compounds from**
2 ***Vaccinium myrtillus* L-berries: ~~comparison of extraction methods and application to the analysis~~**
3 **~~of Ukrainian bilberries~~**

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32 Abstract

33 Quality by Design was adopted for developing an effective extraction procedure of (poly)phenolic
34 compounds from bilberry (*Vaccinium myrtillus* L.) fruits, using a pooled sample of berries from different
35 regions of Ukraine. Mechanical solvent extraction, microwave-assisted extraction (MAE) and ultrasonic-
36 assisted extraction (UAE) were investigated by screening matrices. Extraction time (*Time*, from 5 to 15
37 min), organic solvent type (*OS type*, methanol, ethanol and acetone), organic solvent percentage (*OS%*,
38 from 50% to 90%), sample/extractant ratio (*S/E ratio*, from 0.025 to 0.1 g mL⁻¹), and, only for MAE,
39 extraction temperature (*T*, from 30 to 60 °C), were selected as critical method parameters (CMPs). The
40 spectrophotometric assays total soluble polyphenols (TSP), total monomeric anthocyanins (TMA), and
41 radical scavenging activity (evaluated by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), the 2,2'-
42 azino-di-(3-ethylbenzthiazoline sulfonic acid), and the ferric reducing antioxidant power methods) were
43 chosen as ~~responses~~critical critical method attributes (CMAs). The screening procedure allowed for
44 selecting UAE and methanol, while the other CMPs underwent further optimization through Response
45 Surface Methodology, ~~to find the coefficients of the quadratic models relating the CMPs to the responses.~~
46 Target values for TSP, TMA and DPPH were selected and the method operable design region (MODR)
47 was defined by means of Monte-Carlo simulations. The optimized conditions, with the corresponding
48 MODR intervals in bracket, were the following: (i) *Time*, 17 min (15-23 min); *OS%*, 56% (44-59%); *S/E*

49 ratio, 0.030 (0.022-0.034) g mL⁻¹. Under these experimental conditions, CMAs values of the pooled
50 sample were the following (n=3): TSP=4433±176 mg (+)-catechin eq/100 g dry weight (d.w.);
51 TMA=3575±194 mg cyanidin-3-glucoside eq/100 g d.w.; DPPH=273±5 µg DPPH inhib./mg d.w. The
52 optimized extraction method was tested for matrix effect (ME%) in the UHPLC-MS/MS analysis of 15
53 anthocyanins and 20 non-anthocyanins individual (poly)phenols commonly found in bilberries, as well
54 as for luteolin, sinapic acid, and pelargonidin-3-glucoside, absent in this fruit and therefore added to the
55 extracts as surrogate standards for evaluating apparent recovery (AR%). |ME%| was in any case ≤ 23%
56 and AR% of the surrogate standards in the range 91-95%, confirming the very good performances of the
57 optimized extraction method.

60 *Keywords:* Anthocyanins; Experimental Design; Ultrasound-Assisted extraction; Mechanical Solvent
61 Extraction; Microwave-Assisted Extraction

1 Introduction

(Poly)phenols possess important health promoting and protecting properties, which are directly related to their strong antioxidant and antiradical activities [1, 2]. Recent studies point out the potential of a (poly)phenol-rich diet to control chronic inflammation associated with various conditions, like cardiovascular diseases, diabetes and cancer [3-6].

Bilberry (*Vaccinium myrtillus* L.) fruits contain a wide variety and high concentrations of (poly)phenolic compounds, particularly anthocyanins [7, 8] and therefore they are extensively used for the formulation of supplements and drugs for the treatment of different diseases, such as minor venous circulatory disturbances, mild diarrhoea, and minor inflammations of the oral mucosa [9].

The development of effective extraction procedures for the antioxidant compounds contained in the berries' matrix has proven to be a challenge, mainly due to their thermal instability, high structural diversity, and powerful antioxidant activity. These characteristics, while beneficial in medicinal use, make them able to readily react with co-extracted matrix components, ultimately losing their ability to act as radical scavengers [10]. Mechanical solvent extraction (MSE) is an established and widely used procedure for the extraction of bioactive compounds from natural plant materials. While undoubtedly having a simple experimental setup and low energy consumption, this technique finds its main disadvantage in the time span required for extraction and in the amount of solvent employed for subsequent extractions. More recently, both microwave-assisted extraction (MAE) [10] and ultrasonic-assisted extraction (UAE) [11, 12] have been evaluated as potential alternatives to MSE. Moreover, when large-scale isolation of anthocyanins is required (e.g. for clinical trials), post extraction techniques, such as counter current chromatography is applied [13]

The mechanism and dynamics of solvent extraction are well known, with extraction efficiency rapidly reaching a natural plateau after a few subsequent extractions. Ultrasound and microwave assisted applications push extraction efficiency forward through different energy-delivery routes [14].

86 Ultrasounds work through cavitation, producing peak point-variations of pressure, which result in small
87 implosions that locally heat up and finely mince the solid structure of the plant material, enhancing the
88 solid's surface area, and favouring a more intimate and efficient contact between the solvent and the
89 extractant. Microwaves work by assisting the diffusion of target analytes from the solid matrix to the
90 solvent bulk, making molecular dipoles rotate, thus heating up the solution from the inside, and creating
91 a heating gradient that has its peak in the bulk of the solution, extending outward. The combination of
92 this heat gradient with a soaked solid matrix causes both diffusive and heat-driven motions to be aligned
93 in the same direction, ultimately aiding analytes desorption [15].

94 Several Design of Experiments driven optimizations have been carried out in different papers,
95 encompassing several fields of application for both MAE [16-18] and UAE [19-22], highlighting the
96 efficiency of these techniques in increasing the extraction yield of (poly)phenols from the investigated
97 matrices. In particular, Routray and co-workers [17] evidenced that MAE resulted in higher extraction
98 yields than the 24-h mechanical extraction procedure, whereas Xu et al. [22] proved that (poly)phenols
99 extraction yield increased by the application of a multi-frequency ultrasound procedure instead of a
100 single-frequency one. However, to our knowledge, a comprehensive Quality by Design (QbD) approach
101 to the development of these extraction procedures, particularly regarding the extraction of (poly)phenols
102 from a vegetal matrix, has not yet been investigated. QbD has been firstly introduced by the US Food
103 and Drug Administration [23] as a new and rational way to integrate quality into products and product
104 manufacturing in the pharmaceutical field. The application of this quality paradigm is also the subject of
105 the Q8(R2) Guideline by the International Council for Harmonisation (ICH) [24] and is therein defined
106 as “a systematic approach to development that begins with predefined objectives and emphasizes product
107 and process understanding and process control, based on sound science and quality risk management”.
108 While these texts focus on pharmaceutical product development, the application of QbD has also been
109 extended to different fields; notably the analytical method development in drug analysis [25-30], food

analysis [31] and environmental analysis [29, 32]. Using a risk-based and systematic strategy, QbD steers process development by building quality into the process itself, through the definition of process goals and the specification of a method operable design region (MODR), while also bringing deeper process understanding. The MODR is a multidimensional region, which ensures that critically chosen responses are included within predefined limits with an acceptable level of probability [25, 33].

In this study, a systematic evaluation of the three most used extraction alternatives, MSE, MAE and UAE, was performed employing an analytical QbD (AQbD) approach to maximize the extraction efficiency of phenolic compounds from a pooled sample of bilberries collected in different regions of Ukraine. The three extraction procedures were firstly investigated through screening matrices. ~~The critical method parameters (CMPs) were selected as: (i) “Extraction Time” (*Time*), “Organic Solvent type” (*OS type*), “Organic Solvent percentage” (*OS%*), “Sample/Extractant ratio” (*S/E ratio*, g mL⁻¹), and, only for MAE, “Extraction Temperature” (*T*). The critical method attributes (CMAs) were represented by a series of macroparameters measured by spectrophotometric assays, in order to profile the (poly)phenolic content and the antioxidant capacity of the extracts. These included total soluble polyphenols (TSP), total monomeric anthocyanins (TMA), and radical scavenging activity, the last one evaluated by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), and ferric-reducing antioxidant power (FRAP) assays.~~ On the basis of screening results, UAE was selected for further optimization by Response Surface Methodology (RSM) through Box-Behnken Design (BBD), leading to the definition of the MODR, calculated by Monte-Carlo simulations [34], and to the selection of an optimum working point. Finally, the optimized UAE extraction was applied to the UHPLC-MS/MS determination of individual (poly)phenolic compounds in the pooled sample, thus obtaining for the first time data on bilberry from this European region.

2 Materials and methods

2.1 Chemicals and reagents

Reference standards were supplied as follows: cyanidin-3-galactoside (CYA-3-GAL), cyanidin-3-glucoside (CYA-3-GLU), cyanidin-3-arabinoside (CYA-3-ARA), delphinidin-3-glucoside (DEL-3-GLU), delphinidin-3-galactoside (DEL-3-GAL), delphinidin-3-arabinoside (DEL-3-ARA), pelargonidin-3-glucoside (PEL-3-GLU), malvidin-3-glucoside (MAL-3-GLU), malvidin-3-arabinoside (MAL-3-ARA) and malvidin-3-galactoside (MAL-3-GAL) by Extrasynthese (Genay, France); peonidin-3-glucoside (PEO-3-GLU), peonidin-3-galactoside (PEO-3-GAL), peonidin-3-arabinoside (PEO-3-ARA) and petunidin-3-glucoside (PET-3-GLU) by Polyphenols Laboratories AS (Sandnes, Norway); petunidin-3-arabinoside (PET-3-ARA) and petunidin-3-galactoside (PET-3-GAL) by MEDChem Express (New Jersey, USA); gallic acid (GAC), caffeic acid (CAF), p-coumaric acid (COU), sinapic acid (SIN), ferulic acid (FER), salicylic acid (SAL), chlorogenic acid (CHL), neochlorogenic acid (NEO), cryptochlorogenic acid (CRY), esculetin (ESC), scopoletin (SCO), (+)-catechin (CAT), epicatechin (EPI), luteolin (LUT), myricetin (MYR), phloretin (PHL), phloridzin (PHZ), quercetin (QUE), quercetin-3-rhamnoside (QUE-3-RHA), quercetin-3-glucoside (QUE-3-GLU), quercetin-3-galactoside (QUE-3-GAL) and quercetin-3-rutinoside (QUE-3-RUT) by Sigma–Aldrich (St. Louis, MO, USA). LC–MS grade methanol and water were obtained from J.T. Baker (Deventer, The Netherlands). HPLC-grade methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Laboratory reagents adopted were: formic acid, sodium fluoride, Folin–Ciocalteu reagent and sodium carbonate by Merck (Darmstadt, Germany); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), ABTS, and DPPH radical by Sigma–Aldrich (St. Louis, USA); acetic acid and hydrochloric acid (36-37%) by J.T. Baker (Center Valley, PA, USA); iron (III) chloride hexahydrate by Panreac (Barcelona, Spain); potassium chloride and sodium acetate trihydrate by Carlo Erba (Milan, Italy). Nylon membranes (porosity 0.2 μ m) for the filtration of the bilberry extracts before UHPLC analysis were obtained from VWR International (Radnor, PA). Milli-Q water was taken from a Milli-Q system supplied by Millipore (Billerica, MA).

2.2 Stock solutions; ~~sample origin and extraction~~

Stock solutions were prepared for all phenolic standards at 2 mg mL⁻¹ in methanol and kept in the dark at -18°C for long-term storage. A working solution was prepared as a standard mix of all investigated compounds, at a concentration of 10 ppm in methanol, and stored under the same conditions.

2.3 Sample origin

Fully ripe *V. myrtillus* berries were harvested between July and August 2019 in different regions of Ukraine traditionally involved in the harvest of spontaneous bilberry (see Supplementary **Table S1** for full details). A sample pool of the different harvested fruits was obtained by grinding equal amounts of each individual sample by a hand blender in an ice bath.

2.4 Design of the experiments and extraction conditions

In the screening step, the pool was extracted with ~~the three methods listed below~~ MSE, MAE, and UAE, ~~varying the selected~~ investigating the following critical method parameters (CMPs): ~~values (i)~~ “Extraction Time” (Time), (ii) “Organic Solvent type” (OS type), (iii) “Organic Solvent percentage” (OS%), (iv) “Sample/Extractant ratio” (S/E ratio, g mL⁻¹), and, only for MAE, (v) “Extraction Temperature” (T). For all extraction methods, 0.250 g aliquots of dry weight (d.w.) material were extracted using mixtures of water and organic solvent (i.e. methanol, ethanol or acetone) under the following range of experimental conditions (see Supplementary **Table S2**): organic solvent percentage 50-90%, extraction time 5-15 min, extractant volume 2.5-10 mL, temperature (for MAE only) 30-60 °C. In all cases, 10 mM NaF was added to the extractant to inactivate polyphenol oxidase. After extraction, the obtained mixture was centrifuged at 8500xg for 3 min and the supernatant recovered. The procedure was repeated twice, and the extracts were analysed separately. ~~(Supplementary Table S2) and For each applying the extraction approach, a specific number of experiments was performed as conditions as described in the corresponding experimental matrices reported in the Supplementary Material (Table S3 for MSE, Table S4 for MAE, and Table S5 for MSE,~~

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MAE, and UAE, respectively}. MSE was carried out by magnetic stirring at room temperature in flat bottom glass vials of proper capacity. MAE was performed through an Ethos 1 Microwave Digester supplied by Milestone s.r.l. (Sorisole, Italy), while UAE was performed using a Bandelin Sonorex Digitech Type DT512H (Bandelin electronic, Berlin, Germany) ultrasonic bath.

~~For all extraction methods, 0.250 g aliquots of dry weight (d.w.) material were extracted using mixtures of water and organic solvent (i.e. methanol, ethanol or acetone) under the following range of experimental conditions: organic solvent percentage 50-90%, extraction time 5-15 min, extractant volume 2.5-10 mL. For MAE, temperature values in the range 30-60 °C were adopted. In all cases, 10 mM NaF was added to the extractant to inactivate polyphenol oxidase. After extraction, the obtained mixture was centrifuged at 9000xg for 3 min and the supernatant recovered. The procedure was repeated twice, and the extracts were analysed separately.~~

In the final optimized conditions, UAE was employed for the extraction of 0.250 g aliquots of d.w. material for 17 min with 8.3 mL of methanol 56%, corresponding to a Sample/Extractant ratio of 0.030 g mL⁻¹. The MODR corresponded to the following interval: Extraction Time, 15-23 min; Organic Solvent percentage, 44-59%; Sample/Extractant ratio, 0.022-0.034 g mL⁻¹.

The critical method attributes (CMAs) used as tools for comparing the extraction efficiency of the three methods were represented by a series of macroparameters measured by spectrophotometric assays, in order to profile the (poly)phenolic content and the antioxidant capacity of the extracts. These included total soluble polyphenols (TSP), total monomeric anthocyanins (TMA), and radical scavenging activity, the last one evaluated by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays.

2.3.2.5 Antioxidant capacity Spectrophotometric assays

2.3.2.5.1 Analysis of total soluble polyphenols

TSP were spectrophotometrically determined [35] as follows: 25-200 µL of the extract (depending on the estimated concentration of (poly)phenols in the extract) were mixed with Milli-Q water and 200 µL of Folin-Ciocalteu reagent to achieve in any case a final volume of 9.4 mL. After 3 min of incubation in the dark, 400 µL of an aqueous solution saturated with sodium carbonate were added and the mixture obtained was dark incubated for 1 h; afterwards the absorbance was measured at 740 nm and (poly)phenol concentration calculated on the basis of a CAT calibration curve; accordingly, the results were expressed as mg of CAT eq/100 g of d.w. berries.

2.3.22.5.2 Analysis of total monomeric anthocyanins

TMA were measured by the following procedure [36]. Aliquots of 50-500 µL of bilberry extract were diluted in solutions at pH=1.0 (KCl 1.86 g + 6 mL HCl 37%; final volume 1 L Milli-Q water) and pH=4.5 (anhydrous CH₃COONa 32.81 g + 20 mL HCl 37%; final volume 1 L Milli-Q water), to obtain a final volume of 10 mL. The absorbance (*Abs*) of both solutions were measured at 520 and 700 nm and the quantity ΔAbs was calculated according to Equation (1).

$$\Delta Abs = (Abs_{pH=1.0}^{520\text{ nm}} - Abs_{pH=1.0}^{700\text{ nm}}) - (Abs_{pH=4.5}^{520\text{ nm}} - Abs_{pH=4.5}^{700\text{ nm}}) \quad (1)$$

Similarly, ΔAbs values were also calculated for different concentrations of CYA-3-GLU, used as reference standard, and plotted as a function of CYA-3-GLU concentrations. The best equation fitting the experimental points was calculated by the least square method, thus obtaining a linear calibration curve. TMA in the extracts were finally calculated using this calibration curve as mg of CYA-3-GLU eq/100 g of d.w. berries.

2.3.32.5.3 DPPH radical scavenging activity assay

Radical scavenging activity (RSA) was spectrophotometrically measured [37] using the free radical DPPH, according to the following procedure. Five different volumes of each extract (5-100 µL and 10-200 µL, for the first and second extraction aliquot respectively) were added to 5 mL of a 0.1 mM DPPH solution in methanol. For each extract, a blank was performed by replacing the extract with Milli-Q

water. The decrease in absorbance at 517 nm was monitored every 30 min up until 360 min of the incubation in the dark when the reaction reached a plateau. For each concentration tested, the reaction kinetics was plotted. The percentage of DPPH neutralized at the steady state (%DPPH) was determined by using Equation (2):

$$\%DPPH = 100 \times \frac{(Abs_0^E - Abs_{ss}^E) - (Abs_0^B - Abs_{ss}^B)}{Abs_0^E} \quad (2)$$

where Abs_0^E , Abs_{ss}^E , Abs_0^B , Abs_{ss}^B are the absorbance values of the extract (E) and of the blank (B) at time zero (0) and at the steady state (ss).

The DPPH inhibition percentages obtained were plotted as a function of the corresponding amount of bilberry (d.w.) in the five samples analysed and the best equation fitting the experimental points was calculated by the least square method. IC50 was defined as the concentration of the sample (mg of fruit, d.w.) in 1 mL of reaction mixture, necessary to decrease the initial DPPH concentration (which was kept constant in all the experiments) by 50%; therefore, higher IC50 values correspond to lower RSA values. The RSA of samples (RSA_{sample}) can be derived from IC50 values ($\mu\text{g d.w.}/\mu\text{mol DPPH solution}$) determined on bilberry extracts ($IC50_{sample}$) by using Equation (3), allowing for obtaining a parameter that increases with the rise in antiradical activity and that is expressed as $\mu\text{g DPPH inhibited by 1 mg of d.w. berry sample}$.

$$RSA_{sample} = \frac{MW_{DPPH}}{IC50_{sample}} \quad (3)$$

2.3.42.5.4 ABTS radical scavenging activity assay

RSA was spectrophotometrically measured using the free radical ABTS [38], according to a procedure similar to that described for DPPH. ABTS was converted to its radical cation by addition of 175 μL of 140 mM potassium persulfate to 9.825 mL of 7 mM ABTS (both solutions in Milli-Q water), followed by incubation in the dark for 12-16 h. The obtained solution was diluted 1/100 (v/v) in buffer solution at pH=4.5 (anhydrous CH_3COONa 32.81 g + 20 mL HCl 37%; final volume 1 L MilliQ water) and used

for the analyses. As the method is very sensitive, ABTS-RSA was performed using 5-25 μ L or 20-100 μ L of diluted extracts (dilution ratio 5-10 times). The absorbance was measured at 734 nm. All measurements and calculations were carried out as described for DPPH.

2.3.5.2.5.5 FRAP antioxidant activity assay

FRAP reagent [39] was prepared with 10 volumes of 300 mM acetate buffer pH=3.6 (anhydrous CH_3COONa 1.9 g + CH_3COOH 99% 16 mL; final volume 1 L Milli-Q water), 1 volume of 10 mM TPTZ in 40 mM HCl and 1 volume of 20 mM FeCl_3 . Aliquots of 25-500 μ L of a properly diluted extract (to obtain an absorbance value included in the calibration line) were added to 5 mL of FRAP reagent pre-warmed at 37°C. After 4 hours of incubation in the dark at 37°C, absorbance was read at 593 nm. Results are expressed as mmol of Trolox equivalents per g of d.w. berries.

2.4.2.6 UHPLC-MS/MS instrumentation and analysis

Pooled sample extracts were filtered before UHPLC-MS/MS analysis on 0.22 μ m nylon membranes, and further diluted 1/1000 (v/v). Analysis of the individual compounds was performed through an UHPLC system, hyphenated with an electrospray ionization (ESI) source and a tandem (MS/MS) mass spectrometer, working as triple quadrupole. The UHPLC system (Shimadzu, Kyoto, Japan) consisted of a low-pressure gradient quaternary pump Nexera X2 LC-30AD, a CTO/20AC thermostatted column compartment, a SIL-30AC autoinjector, a DGU-20A 5R degassing unit and a CBM-20A module controller. The detector was a 5500 QTrapTM hybrid triple quadrupole-linear ion trap mass spectrometer (Sciex, Ontario, Canada) through a Turbo VTM interface equipped with an ESI probe.

The chromatographic separation was performed on a Phenomenex Kinetex XB-C18 chromatographic column (150 $\times$ 2.1 mm i.d., particle size 2.6 μ m) equipped with a guard column using the same stationary phase. Chromatographic analysis of the non-anthocyanin (poly)phenols was performed at 50 °C, eluting the injected volumes with water/formic acid 99.9/0.1 (v/v) (eluent A) and methanol/formic acid 99.9/0.1 (v/v) (eluent B), according to the following gradient: 0-3 min isocratic 2% (eluent B), 3-4 min linear

gradient 2-15% (eluent B), 4-27 min linear gradient 15-60% (eluent B), 27-29 min linear gradient 60-95% (eluent B), 29-33 min isocratic 95% (eluent B). Chromatographic analysis of the anthocyanins was performed at 50 °C using water/formic acid 95/5 (v/v) (eluent A) and methanol/formic acid 95/5 (v/v) (eluent B), according to the following gradient: 0.1-10 min linear gradient 7-17% (eluent B), 10-20 min linear gradient 17-22% (eluent B), 20-22 min linear gradient up to 95% (eluent B), 22-27 min isocratic 95% (eluent B). Flow rate and injection volume were set at 0.3 mL min⁻¹ and 5 µL respectively, for both chromatographic runs.

Target compound MS/MS analysis was carried out using the Multiple Reaction Monitoring mode (MRM) using the ESI source in negative or positive ionization mode, depending on the analyte considered. Source dependent parameters were optimized through flow injection analysis, at optimal LC flow and mobile phase composition. For the negative ionization mode, ESI (-), optimal source parameters were set as follows: curtain gas, 30; CAD gas, medium; temperature, 600 °C; gas 1, 50; gas 2, 65; interface heater on; ion spray voltage, -4500 V. For the positive ionization mode, ESI (+), optimal source parameters were: curtain gas, 30; CAD gas, medium; temperature, 600 °C; gas 1, 40; gas 2, 65; interface heater on; ion spray voltage, 5500 V. For each investigated compound, the most intense transition current was used for its quantification and the second most intense one for confirming its identification [40]. Compound dependent parameters were optimized by direct infusion of properly diluted target analyte standard solutions and are reported in Supplementary **Table S6**.

2.5.2.7 UHPLC-MS/MS method

The analytical performance of the UHPLC-MS/MS method here adopted for the quantification of the individual (poly)phenols was checked in terms of: (i) instrumental detection limits (LODs), (ii) instrumental limit of quantification (LOQ), (iii) instrumental repeatability, and (iv) linearity range. These data are shown in Supplementary **Tables S7** and **S8** for positively and negatively ionisable compounds, respectively.

~~LODs~~ DLs were considered as the concentration corresponding to a signal-to-noise ratio of three and were evaluated through successive injections of standard solutions at increasing dilutions. The linearity of the method was tested measuring standard solutions at seven different concentration levels (LOQ – 5 – 25 – 50 – 100 – 250 – 500 ppb) in triplicate. Intraday repeatability was measured as RSD of five different injections of standards at 25 ppb and 250 ppb concentration levels in LC-MS water. Spiked standards for apparent recovery (AR%) evaluation were LUT and SIN for negative ionization, and PEL-3-GLU for positive ionization. The AR% were evaluated to account for the goodness of the whole extraction and analysis procedure, by spiking aliquots of dried bilberry powder with these standards and subjecting them to the optimised extraction procedure, as to obtain a theoretical extract concentration of 100 ppb for each standard. Matrix effect (ME%) was investigated by spiking the extracts with different concentrations of target analytes (5, 25, 100, 250 and 500 ppb) and analysing them by UHPLC-MS/MS, together with the non-spiked ones. For each compound, the chromatographic areas found in the spiked extracts were subtracted from those of the non-spiked ones and the resulting differences were plotted as a function of the spiked concentrations. The best curve fitting the experimental points was calculated by least square method and its slope (s_{matrix}) was compared with the one of the corresponding calibration curve in water ($s_{solvent}$). ME% values were calculated according to the following equation (4) [7].

$$ME\% = \left(\frac{s_{matrix}}{s_{solvent}} \cdot 100 \right) - 100 \quad (4)$$

2.62.8 Softwares

Chromatograms were elaborated using Sciex Analyst software (version 1.6.2). Peak attribution and quantitative determination were performed using MultiQuant software version 3.0.2 (Sciex). NemrodW software (NemrodW, LPRAI sarl, Marseille, France) was used to set up the screening matrices used during the screening phase and for the related data elaboration. MODDE 13 software (Sartorius Stedim Data Analytics AB-Umetrics, Umeå, Sweden) was employed to plan BBD selected for RSM, to

perform the relative data treatment and to define the MODR by means of Monte-Carlo simulations. The target for defect per million opportunities (DPMO) [41] was fixed at 10,000, corresponding to a risk of error of 10%.

3 Results and discussion

An AQbD procedure was followed for the optimization of the extraction conditions of (poly)phenolic compounds. This procedure consisted in (i) the definition of the Analytical Target Profile (ATP), (ii) the selection of ~~Critical Method Attributes (CMAs)~~, (iii) the identification of CMPs, (iv) the screening phase, (v) RSM optimization and MODR definition. The selected knowledge space was investigated separately for MSE, MAE, and UAE through three distinct screening experimental designs. The most performing extraction technique was then selected for RSM optimization and MODR definition, and a convenient working point was finally defined for the extraction of the target compounds from the fruit matrix.

3.1 Analytical target profile and critical method attributes

The establishment of the ATP represents the first step of an AQbD process. Delineating an ATP consists in selecting a set of criteria that encompasses which performance characteristics the developed procedure should exhibit, as well as the means to measure such characteristics [24, 33]. The purpose of optimizing the extraction procedure of (poly)phenols from bilberries was to achieve the best possible extraction efficiency within the limits of a reasonable extraction time, with low solvent consumption, ease of handling and cost efficiency.

A series of readily available and widely employed assays was selected for evaluating performance variations during each extraction procedure by tracing the (poly)phenolic contents, as well as the antiradical/antioxidant activity of the final extracts.

Since bilberry is a fruit well known for its large content of anthocyanins, but also of non-anthocyanin (poly)phenolic compounds [8], both total (poly)phenolic and total anthocyanin assays were considered as CMAs, i.e. the responses to be maximized and/or kept under control, directly linked with extraction

347 efficiency. These two parameters were determined through (i) the Folin-Ciocalteu assay, index of the
348 total soluble (poly)phenols (TSP) [35] and (ii) the total monomeric anthocyanins (TMA), determined by
349 the pH differential method [36].

350 Moreover, different assays were employed for assessing the antioxidant/antiradical capacity, since its
351 determination includes a wide variety of reactions, in which different scavenging mechanisms are
352 involved. The assays for the determination of antioxidant/antiradical capacity are usually split in two
353 main categories: hydrogen atom transfer (HAT) and single electron transfer (SET) [42]. HAT represents
354 the ability of an antioxidant to scavenge a free radical through the donation of a hydrogen atom. These
355 reactions are independent on both solvent and pH, they tend to be very rapid and they are also regarded
356 as the most relevant for radical scavenging evaluation, since the mechanism is the common $X^{\cdot} + AH \rightarrow$
357 $XH + A^{\cdot}$ [42]. SET is a single-electron reaction route where both the ionisation potential and
358 deprotonation of the functional group of the radical scavenging species play a major role [43, 44]. These
359 reactions are pH dependent, with electron-donating capacity increasing with pH value, and tend to be
360 slow. The SET assays measure a percentage decrease in the endpoint compound, and thus the ability of
361 an antioxidant to transfer an electron. Since both mechanisms tend to occur together, with balance
362 determined by the contributions of pH and of the molecular structures of the antioxidants, and
363 considering that in the extracts multiple antioxidants are present at the same time, in this study both HAT
364 and SET based assays were employed and their results were chosen to represent the quality of extraction
365 performances. Based on the considerations mentioned above, further CMAs, related to
366 antiradical/antioxidant activity, were: (iii) DPPH assay, (iv) ABTS assay, and (v) FRAP assay. The
367 FRAP assay, which works at low pH by reduction of ferric TPTZ to the ferrous complex, is based on the
368 SET mechanism [42]. DPPH and ABTS are mixed-mode (HAT-SET) assays; both radicals used in these
369 tests can be neutralised by either a SET or a HAT mechanism [45], enabling to follow both reactivities
370 at the same time. DPPH is a solid stable free radical, which follows the $DPPH^{\cdot} + AH \rightarrow DPPH - H +$

371 A' reaction pathway [37, 46] with decolouration of the original radical as its endpoint. ABTS is an acid
372 that after oxidation by peroxides gives an intensely coloured radical cation [38]; the decrease in colour
373 can be directly related to the amount of free-radical scavenged by antioxidants.

374 3.2 Critical method parameters

375 The objective of a good extraction procedure is the quantitative and reproducible transfer of a series of
376 target compounds, from a complex native matrix to a suitable medium. Analytes extraction from solid
377 matrices is influenced by their physicochemical properties (e.g., solubility, hydrophobicity, acid-base
378 dissociation, and partition coefficient) and molecular structures. Facing the extraction of many different
379 compounds means dealing with a wide range of properties. To select and optimize an extraction process

380 that efficiently encompasses such a variety, the aforementioned a small group of CMPs, suitable to be
381 equally applied to the three extraction techniques, was identified (Table S2), as reported in
382 Supplementary Table S2. These CMPs were: (i) Extraction Time (*Time*), (ii) Organic Solvent type (*OS*
383 *type*), (iii) Organic Solvent percentage (*OS%*) and (iv) Sample/Extractant ratio (*S/E ratio*); (v) Extraction
384 temperature (*T*) was selected as further CMP only for MAE.

385 As regards *Time*, -fast extraction is always preferable, but contact time must be balanced to allow for
386 proper penetration and diffusion of the solvent into the matrix, as well as analyte diffusion into the bulk
387 solution. As for *OS type*, different solvents exhibit different extraction efficiencies towards target
388 compounds and matrices, owing to their different polarities and Van Der Waals interaction potentials.
389 Moreover, the variation of the relative percentages of organic solvent and water is expected to exert a
390 great influence on the extraction of polar analytes. *S/E ratio* was evaluated as the best way to control
391 both the amount of solvent and the weight of the sample, as both are critically important variables in the
392 extraction efficiency. Finally, *T* was selected as the specific CMP for MAE investigation, as the best way
393 to control the power supplied to the sample matrix, avoiding potential thermal degradation of the
394 analytes.

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395 3.3 Screening phase

396 In order to obtain the maximum amount of information on all the investigated extraction techniques by
397 performing a limited number of experiments, a screening experimental design was applied to investigate
398 the CMPs effects on the CMAs throughout the knowledge space. During this phase, two aspects of the
399 method development were considered, using the CMAs for evaluating extraction efficiency. The first
400 aspect was to assess, within each extraction technique, which CMPs were pivotal in steering extraction
401 efficiencies for the target analytes; the second was to compare the performances achieved by each
402 technique on aliquots of the same sample to be able to select the more suitable for going on with the
403 investigation by RSM in a proper experimental domain. The variation ranges for the CMPs were selected
404 based on previous knowledge in phenolics extraction and analysis [7, 8]. In order to plan a homogeneous
405 screening procedure for all the extraction methodologies investigated, the selected CMPs were studied
406 at three levels in the same experimental domains (with the exception of T), as shown in [Supplementary](#)
407 **Table S2**.

408 Besides the aforementioned CMAs (i.e. TSP, TMA, DPPH, FRAP, and ABTS), in this screening phase,
409 the ratio between TSP obtained in the first extraction and the sum of TSP values obtained by the first and
410 the second extraction was also selected as a measure of the single extraction efficiency (SEE).

411 The effects of the different levels of the CMPs over the CMAs measured for the three techniques were
412 evaluated through two 9-runs (MSE and UAE) and one 16-runs (MAE) symmetric screening matrices,
413 which allowed four (MSE and UAE) and five (MAE) factors to be studied at three levels. This set-up
414 made it possible the estimation of a linear, no-interaction, Free-Wilson model for each extraction
415 technique. In this type of model, each variable carries a number of coefficients equal to the number of
416 levels minus one, leading to the estimation of the effects of each CMPs level on the CMAs. Consequently,
417 the models used in this study present the constant term A_0 and two coefficients for each variable.

418 The model for both MSE and UAE is reported in Equation (5):

$$y=A_0+A_1A+A_2A+B_1B+B_2B+C_1C+C_2C+D_1D+D_2D \quad (5)$$

where *A* refers to *Time*, *B* to *OS type*, *C* to *OS%*, and *D* to *S/E ratio*.

The model for MAE is reported in Equation (6):

$$y=A_0+A_1A+A_2A+B_1B+B_2B+C_1C+C_2C+D_1D+D_2D+E_1E+E_2E \quad (6)$$

where *A* refers to *T*, *B* refers to *Time*, *C* to *OS type*, *D* to *OS%*, and *E* to *S/E ratio*.

The screening matrices, together with the measured responses of TSP, TMA, DPPH, FRAP, ABTS, and SEE, are reported in Supplementary **Tables S3, S4, and S5**, for MSE, MAE, and UAE, respectively.

Each experiment of the plan was replicated twice. For each extraction technique, a graphical analysis of the effects was obtained, as shown in Supplementary **Figures S1, S2, and Figure 1** for MSE, MAE, and UAE, respectively. In these figures, plots indicated by b, d, f, h, j, and l, highlight the effect of each level of the CMP on the CMA (the longer the bar, the more the CMA is maximized), while the graphs indicated by a, c, e, g, i, and k show the statistical significance of the effect of the variation between two selected levels on each CMA. Regarding this latter type of plots, the significance of the change of CMP level is directly subject to the cut-off of the estimated experimental error, which is shown as a dotted line running across the bars. Whenever the bar length exceeds the dotted line, the level change of the CMP exerts a statistically significant effect on the CMA.

The first screened technique was MSE (Supplementary **Fig. S1**). The highest value of *Time* was the one that gave rise to the highest values of all CMAs, thus providing a clear indication for this CMP. The analysis of the effects for *OS type* suggested acetone and methanol as equally effective for the recovery of all CMAs, with the exception of TMA for which the best results were obtained with ethanol and above all methanol. As regards *OS%*, the significance tests in level change all pointed towards the low-medium domain of this CMP (50-70% v/v), suggesting no remarkable difference between the two levels. TMA was an exception since *OS%* of 70% gave rise to a significantly higher recovery than 50%. Surprisingly, the change in *S/E ratio* fell under poor statistical significance in the investigated domain for most CMAs,

with the exceptions of FRAP and SEE, which were maximized by medium-high and low-medium values, respectively.

The second technique taken into consideration was MAE. As it can be extrapolated from the plots in Supplementary **Fig. S2**, the change in *T* levels had significant effects on all CMAs, even though their maximization was obtained at different levels depending on the CMA considered (low for TMA; low-medium for TSP; medium for FRAP and high for DPPH and ABTS), suggesting the need of a more in-depth study of its influence on the MAE performances. As regards *Time*, different effects were evidenced, depending on the CMA considered. In detail, no significant effect was observed on TMA, while DPPH and ABTS were maximized at low and high values of the experimental domain. Moreover, FRAP and SEE showed higher recoveries at the central *Time* domain value. A similarity with the MSE trend emerged in the solvent selection (i.e. *OS type*), since the statistical significance across levels strongly pointed out the better performances of either methanol or acetone. Another similarity with MSE was that the change of *OS%* had a major influence on all CMAs, being the middle-low values of the experimental domain those corresponding to the highest CMA values. In contrast with MSE, *S/E ratio* was a significant parameter across most CMAs, with a strong indication towards the lower end (i.e., 0.0250) of the investigated experimental domain, in particular for maximizing TSP, FRAP and SEE.

As for UAE, the plots shown in **Figure 1** evidenced a trend for *Time* similar to that observed for MSE, pointing again towards the higher value of the domain (i.e., 15 min). Also in this case, methanol and acetone were found the two most suitable solvents, with a strong indication of the former for TMA extraction. *OS%* and *S/E ratio* generally exerted statistically significant contributions, providing in almost all cases the highest recoveries with the medium-low range values of their experimental domain. In order to highlight which of the three extraction approaches was the best performing for the recovery of (poly)phenols, the data obtained during the screening for the different CMAs have been reported in three box plots (see Supplementary **Figure S3a-c**). The boxplots highlight the interquartile ranges of the

values obtained, i.e. top of the box is the third quartile (Q3), while the bottom of the box is the first quartile (Q1). Furthermore, the plot evidenced the median and the mean values, the upper and lower whiskers corresponding to $[Q3+1.5\cdot(Q3-Q1)]$ and $[Q1+1.5\cdot(Q3-Q1)]$, respectively, and the outliers (i.e. observations beyond the upper or lower whisker). Based on the boxplot data, no extraction approach yielded ~~significantly~~ statistically better results than the others for all investigated CMAs, using the Games-Howell comparison test at the 5% probability level. In fact, the recovery of TSPs was in the order $MSE \approx UAE > MAE$, while for TMA it was $UAE > MSE > MAE$. Moreover, the use of MAE resulted in higher DPPH values than MSE, while the three extraction systems were equivalent for FRAP and ABTS. However, considering that UAE never resulted as the extraction technique providing the worse results and that bilberry is particularly rich in anthocyanins, which were better recovered by UAE, this method was selected for the further optimization by RSM. Another point in favor of UAE implemented as ultrasonic bath is the relatively affordable cost compared to microwave and ultrasonic tip systems. In addition, the ultrasonic bath system, compared to the tip one, allows for the simultaneous extraction of a number of samples with a single device, thus increasing the analytical throughput. Within UAE, the information obtained from the screening phase made it possible to fix methanol as the organic solvent providing by far the highest recovery of TMA (**Fig. 1**), while the other parameters were investigated in ~~greater~~ detail by means of RSM. In ~~detail~~ particular, the domain for *Time* was shifted to higher levels (15-25 min), while the ranges of *OS %* and *S/E ratio* were shifted to lower levels (40-80% v/v and 0.0250-0.0650 g mL⁻¹, respectively), as reported in Supplementary **Table S2**. Moreover, the results of the screening phase for SEE indicated that in general, for all the three techniques, the single extraction was more efficient with respect to the second one if higher *Time* and lower *S/E ratio* values (hence higher volumes of extractant) were used. Nevertheless, the TSP fraction recovered by the second fraction was never negligible compared to that recovered from the first fraction, being the SEE values obtained for UAE within the screening procedure in the range of 0.720-0.875 (see Supplementary

491 **Table S5**). For this reason, it was decided to perform two subsequent extractions in the analytical
492 procedure for the continuation of the study.

493 3.4 Response surface methodology and method operable design region

494 Based on the results of the screening procedure, an RSM optimization was carried out in order to further
495 enhance UAE extraction efficiency. The new investigated experimental domain is reported in
496 Supplementary **Table S2**.

497 Using MODDE software, BBD was employed for estimating the main, interaction and quadratic effects
498 of the CMPs on the CMAs, as postulated in the following quadratic model:

$$499 y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon \quad (7)$$

500 where y is the response, x_i are the independent CMPs, β_0 is the intercept, β_i are the true coefficients and
501 ε is the experimental error. BBD is a three-level design, where the experimental points are located on a
502 hypersphere and are placed at equal distance from the central point, exhibiting a high level of efficiency,
503 in terms of number of required runs with respect to the number of coefficients to be estimated [47]. The
504 experimental plan with the obtained responses is shown in **Table 1**.

505 Model refinement was carried out in a sequential way, through exclusion of the factors that were found
506 to be not significant in the coefficient plot, in order to achieve an improvement in the model predictivity
507 Q^2 [41]. The quality parameters of the calculated models are reported in Supplementary **Table S9**. All
508 the models resulted both valid and significant and could be used for drawing contour plots. Anyway, the
509 quality parameters of the models were higher for TSP, TMA and DPPH, while for FRAP and ABTS a
510 lower quality was obtained, especially in terms of model reproducibility. Thus, it was decided to explore
511 the effects of the CMPs for all the responses using the response surfaces, while, for specifying target
512 requirements and for building the sweet plot spot and the MODR, only TSP, TMA and DPPH were
513 selected as CMAs.

514 The contour plots for TSP, TMA, DPPH, FRAP, and ABTS were reported in **Figure 2**, where the zones
515 coloured in orange-red represent the maximization of these responses. Different trends and curvatures in
516 the predicted responses were observed, thus highlighting the importance of using the multiresponse
517 criteria in order to find the best compromise among the different responses. Very important quadratic
518 effects, highlighted by pronounced curvatures in the isoresponse lines, were highlighted for *OS%* on TSP
519 (**Fig. 2a**) and for *S/E ratio* on DPPH (**Fig. 2c**) and FRAP (**Fig. 2d**).

520 The target values for the three selected CMAs (i.e. TSP, TMA, and DPPH) were set by considering
521 overall the results obtained in the experimental domain under study, in particular by setting the values
522 around the mean of the results obtained, in order to find a zone that represents a good compromise for
523 all the selected CMAs. In this study, it was not possible to fix the target values beforehand, as no certified
524 reference materials were available for bilberry, and the responses refer to parameters that even after
525 sample fortification with (poly)phenolic compounds cannot be determined irrespective of their native
526 content in the pool of berries. In this latter regard, it should be noted that this problem would not have
527 been overcome even if individual (poly)phenols would have been used as CMAs, since for these analytes
528 reference standards that can be determined regardless of their native content (i.e. labelled standards) are
529 not commercially available. The thresholds for the CMAs were fixed at the median level of the BBD
530 experimental data (**Table 1**), i.e. $TSP \geq 4100$ mg CAT eq/100 g d.w; $TMA \geq 3400$ mg CYA-3-GLU eq/100
531 g d.w.; $DPPH \geq 260$ μ g DPPH inhib./mg d.w. Following this approach, the same importance was given
532 to all CMAs when calculating the MODR.

533 The sweet spot plots are reported in Supplementary **Figure S4** at three fixed values of *Time*. In these
534 plots, the overlay of isoresponse surfaces led to identify the green zone as the sweet spot, namely the
535 region where all the predicted values for the CMAs were higher than the selected thresholds. The teal-
536 coloured zone corresponded to the zone where two CMAs reached the desired values, while the blue
537 zone corresponded to the region where only one CMA presented a satisfactory value. In the white zone,

no criterion was satisfied. At each plotted Extraction Time value, it was possible to find two green zones, at low and high values of *S/E ratio*, more or less wide.

From the application of Monte-Carlo simulations [34] to the regression models, it was possible to compute the MODR considering the uncertainty of the model parameters. In this way, method uncertainty was estimated and a level of probability that the CMAs specifications are met was calculated [33]. The risk of failure was plotted in the probability maps shown in **Figure 3**. The MODR was calculated from the set-point, which corresponded to *OS%*, 53%; *Time*, 19 min; *S/E ratio*, 0.028 g mL⁻¹, indicated by the green-coloured area, where the risk of failure of fulfilling CMAs requirements was lower than 10%. The MODR interval was the following: *OS %*, 47-59%; *Time*, 15-23 min; *S/E ratio*, 0.022-0.034 g mL⁻¹. Four experiments were run spanning the MODR for confirming that the target values were reached, finding a good agreement between the predicted and the measured values. Then, the working point was selected inside the MODR, choosing a lower value of *Time* with respect to the set-point, in order to decrease the total time of analysis. The optimum point corresponded to the following: *OS%*, 56%; *Time*, 17 min; *S/E ratio*, 0.030 g mL⁻¹. Under these experimental conditions the following results were obtained (n=3): TSP 4443±176 mg (+)-catechin eq/100 g d.w., TMA 3575±194 mg CYA-3-GLU eq/100 g d.w., DPPH 273±54 µg DPPH inhib./mg d.w., ABTS 1048±30 µg ABTS inhib./mg d.w., and FRAP 183±8.5 mmol Trolox eq./g d.w. It is worth noting that the experimental values obtained for TSP, TMA, and DPPH were higher than the aforementioned thresholds.

3.5 UHPLC-MS/MS analysis of the pooled sample

Since the extraction method was optimized using various macroparameters, it is interesting to investigate its effectiveness in the UHPLC-MS/MS analysis of individual (poly)phenols, in terms of both AR% and ME%. |ME%| was generally much lower than 20% (see Supplementary **Tables S7** and **S8**), which is commonly considered a threshold value below which matrix effect is negligible [48]. The only exception was MYR, which exhibited a ME% signal enhancement of about 23%. It is remarkable that much higher

562 suppressive/enhancing effects were determined elsewhere for MSE of bilberries using methanol/water
563 80/20 (v/v) [7], thus suggesting a possible role of the mechanical stirring and/or, most probably, higher
564 percentages of methanol in the extractant mixture towards the co-extraction of matrix constituents. The
565 evaluation of AR%, performed using LUT, SIN, and PEL-3-GLU as specified in paragraph 2.5, achieved
566 mean recovery percentages (n=3) of 95±12, 91±14, and 95±14, respectively. Matrix effect was found to
567 be very low also for the surrogate standards ($|ME\%| \leq 5\%$), thus indicating good performances of the
568 extraction method of these reference compounds. In this regard, it is worth noting that when the extraction
569 was carried out with MSE and methanol/water 80/20 (v/v), a stronger influence of the matrix was
570 observed for SIN [7]. The high AR% and the low values of $|ME\%|$ highlight the very good recovery
571 performances of the optimized extraction method.

572 Even though this study was not designed for (poly)phenolics profiling, it is interesting to highlight the
573 most abundant individual (poly)phenols in the pooled sample of Ukrainian bilberries, which are here
574 investigated for the first time regarding these analytes. To this aim, the extracts obtained under the
575 optimized experimental conditions were analyzed by UHPLC-MS/MS following a chromatographic
576 method elsewhere reported [7], able to determine 35 compounds among the most abundant (poly)phenols
577 in bilberry. The typical MRM reconstructed UHPLC-MS/MS chromatograms acquired in positive and
578 negative ionization are shown in **Figure 4a-b**, respectively.

579 Mean concentrations and standard deviations of individual anthocyanins, as well as their relative
580 percentage abundance determined in five independent replicated analyses of the pooled bilberry sample,
581 extracted under the optimized experimental conditions, are shown in Supplementary **Figure S5**. Overall,
582 the sum of the individual anthocyanins concentrations determined through UHPLC-MS/MS analysis
583 (3399 mg/100 g d.w.) matched the results obtained by the TMA assay (3575 mg CYA-3-GLU eq/100 g
584 d.w.). The concentrations of the investigated anthocyanins were distributed as follows:
585 DEL>>MAL≈CYA>>PET≈PEO. More in detail, DEL glycosides accounted alone for 43% of the total

anthocyanins determined, followed by MAL and CYA derivatives, both accounting for about 20% of the total, while PET and PEO represented a rough 10% and 5% of the total anthocyanin content, respectively. Looking at the distribution of the sugar moieties among the investigated anthocyanins, glucosides and galactosides covered 46% and 41% of the whole content, respectively, while the remaining 13% was represented by the arabinosides. This relative distribution seems to be different from that observed in bilberries harvested in Southern Europe and in the extreme North of Europe, where galactosides were lower than glucosides [7, 49] or even lower than arabinosides [50], thus suggesting a possible unique anthocyanin fingerprint for Ukrainian bilberries. However, bilberries collected in Slovenian mountains [51] showed a glycosides distribution pattern similar to that determined in this study.

An overview of the results obtained for the investigated non-anthocyanin (poly)phenols is shown in Supplementary **Figure S6**. The non-anthocyanin compounds were found to be much less abundant than anthocyanins, in agreement with findings of previous studies [7]. Moreover, the total concentration of non-anthocyanin (poly)phenols here determined (268 mg/100 g d.w.) was comparable with that reported elsewhere (252 mg/100 g d.w.) [7]. As mentioned before, the two most predominant compounds were CHL and GAL, which were found at similar concentrations and accounted together for approximately 70% of the whole non-anthocyanin group, followed by QUE-3-GAL (10%), QUE-3-GLU (4%), and COU (4%), while the remaining 12% was shared among the rest of the investigated compounds with percentages generally lower than 1%.

4 Conclusions

The development of an efficient extraction method of (poly)phenolic compounds from bilberry was approached systematically by means of an analytical QbD framework. This approach represents an innovative application in this field, which is added to the other sectors where QbD has already found its consolidated way due to its great advantages in terms of time and resources saving, while making it possible to achieve in-depth information on the phenomenon under study.

The efficiency of extraction was evaluated through several response variables, including TSP, TMA, and some antiradical/antioxidant activity related parameters. Three extraction methods, namely MSE, MAE and UAE, were explored through symmetric screening matrices. The screening procedure highlighted the UAE technique as the most suitable for extraction, especially considering anthocyanins as target analytes. Consequently, RSM was applied to this extraction approach and the contour plots were studied. In this study, the threshold values for CMAs were fixed after performing RSM, unlike what is usually done for the optimization of chromatographic separation methods, where the CMA targets are set *a priori* or after some scouting experiments. This procedure was adopted for two main reasons. (i) Since the optimization procedure must deal with several CMPs and thus with several models, it was fundamental to select the CMA models characterized by the highest statistical quality to be able to achieve a MODR, which is built by considering the model error. (ii) Since the optimization was performed on a real pooled sample with unknown (poly)phenolic composition and antioxidant/antiradical attributes, the desirable values of these variables could not be fixed *a priori* as target. Statistical quality of the models, particularly regarding predictivity, resulted higher for TSP, TMA, and DPPH; hence, target values were fixed for these CMAs and the MODR could be defined as the zone where the risk of error was lower than 10%. In this way, it was still possible to point out a robust optimum zone inside which the optimal working point could be selected. Hence, the QbD approach has once again proved to be of great use for the optimization of analytical procedures. Since the UAE extraction approach has been selected and optimized considering TMA as a target macroparameter of great relevance, the extraction conditions optimized here for bilberry are probably suitable to be extended to the extraction of other small fruits such as raspberry or blackberry, which are very rich in anthocyanins.

Acknowledgements

The Authors wish to thank the Italian Ministry of Education, University and Research (MIUR) (“Progetto Dipartimenti di Eccellenza 2018-2022” allocated to Department of Chemistry “Ugo Schiff”, University of Florence).

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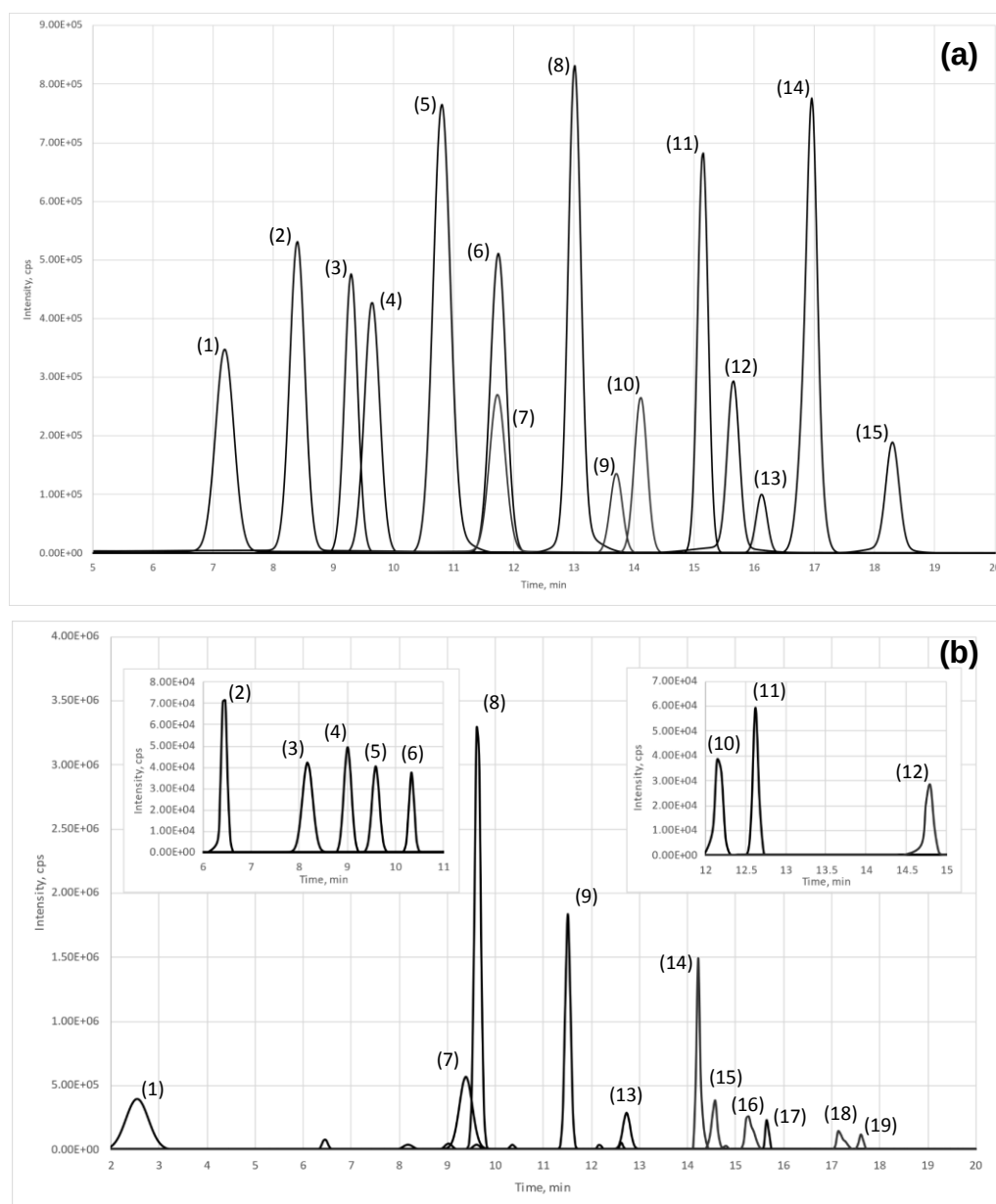


Figure 4 – Typical MRM reconstructed LC–MS/MS chromatograms of the Ukrainian bilberry pooled sample. (a) ESI (+) compounds, identified as follows: (1) DEL-3-GAL, (2) DEL-3-GLU, (3) CYA-3-GAL, (4) DEL-3-ARA, (5) CYA-3-GLU, (6) CYA-3-ARA, (7) PET-3-GAL, (8) PET-3-GLU, (9) PEO-3-GAL, (10) PET-3-ARA, (11) PEO-3-GLU, (12) MAL-3-GAL, (13) PEO-3-ARA, (14) MAL-3-GLU, (15) MAL-3-ARA. (b) ESI (-) compounds, identified as follows: (1) GAC, (2) NEO, (3) CAT, (4) ESC, (5) CRY, (6) EPI, (7) CAF, (8) CHL, (9) COU, (10) SCO, (11) FER, (12) PHZ, (13) SAL, (14) QUE-3-GAL, (15) QUE-3-GLU, (16) MYR, (17) QUE-3-RHA, (18) QUE, (19) PHL.

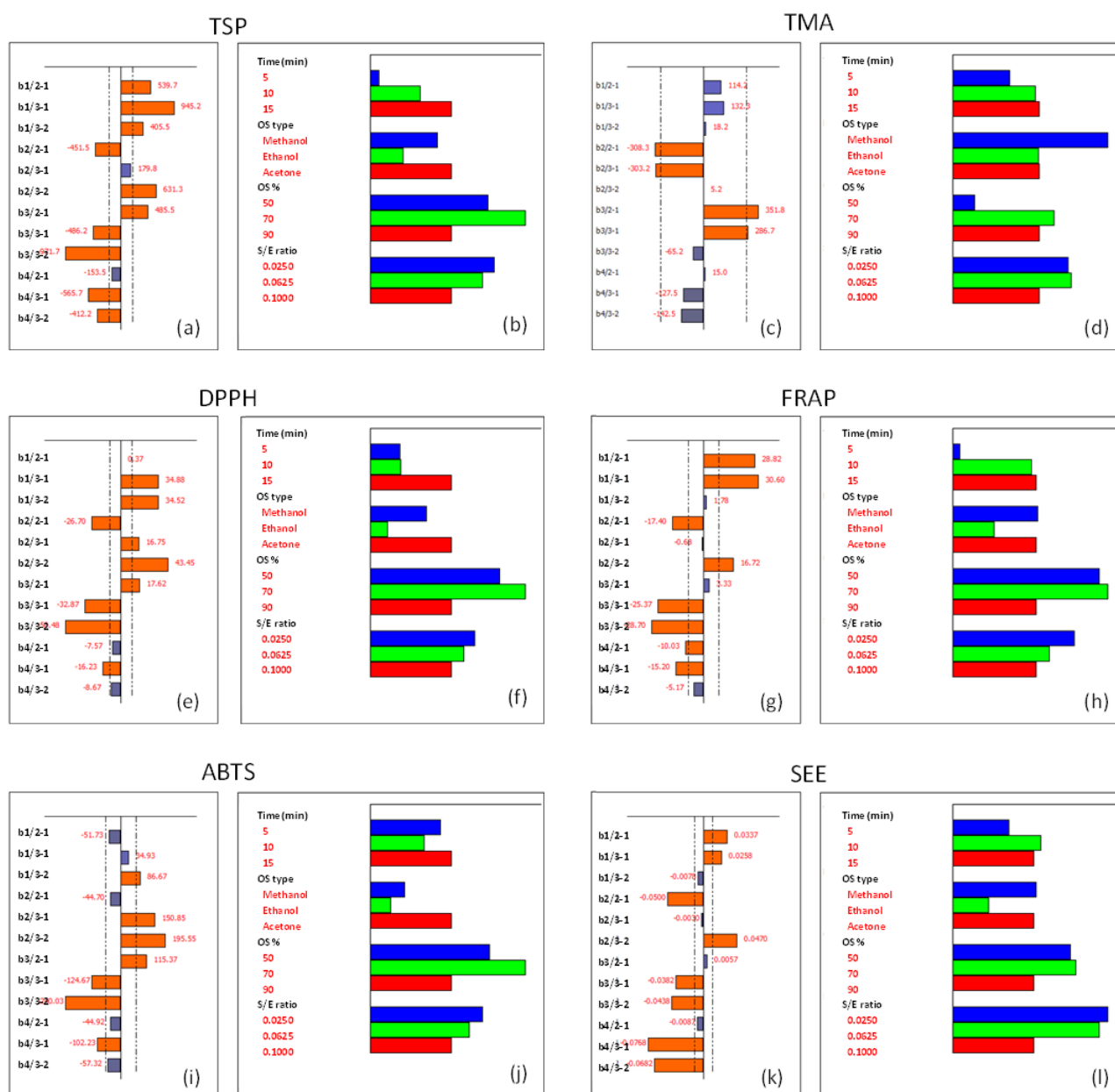


Figure 1 – Graphical analysis of effects resulting from the UAE screening phase. Critical method attributes: (a,b) TSP; (c,d) TMA; (e,f) DPPH; (g,h) FRAP; (i,j) ABTS; (k,l), SEE. In the plots identified with the letters a, c, e, g, i, and k, the length of the bars is related to the difference of effect between two selected levels of each CMP on the response, while the orange colour points out the statistically significant differences. In the plots identified with the letters b, d, f, h, j, and l, the bars correspond to the effects of the single levels of each CMP on the response.

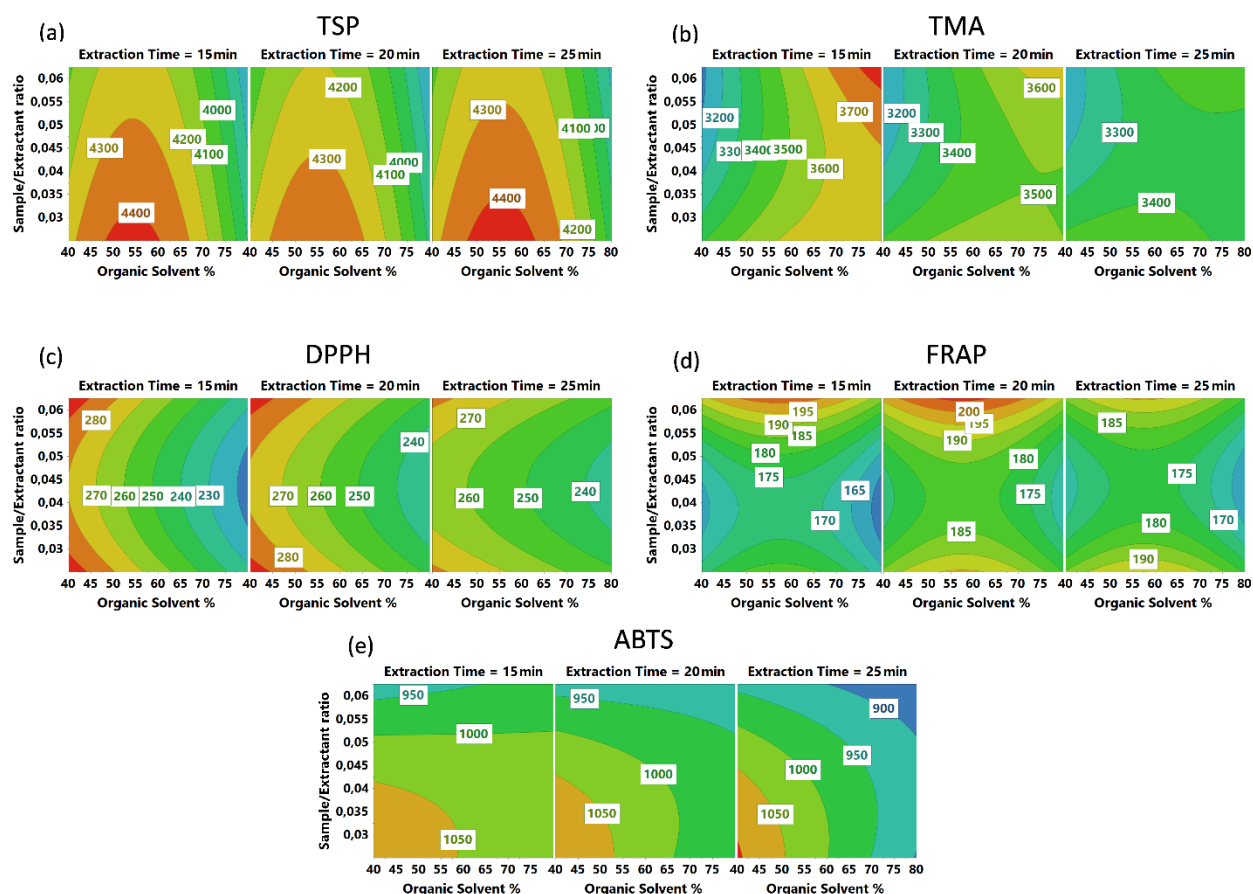


Figure 2 – 2-D contour plots obtained plotting Sample/Extractant ratio vs. Organic Solvent percentage at three levels of Extraction Time (15, 20, and 25 min). (a) TSP; (b) TMA; (c) DPPH; (d) FRAP; (e) ABTS.

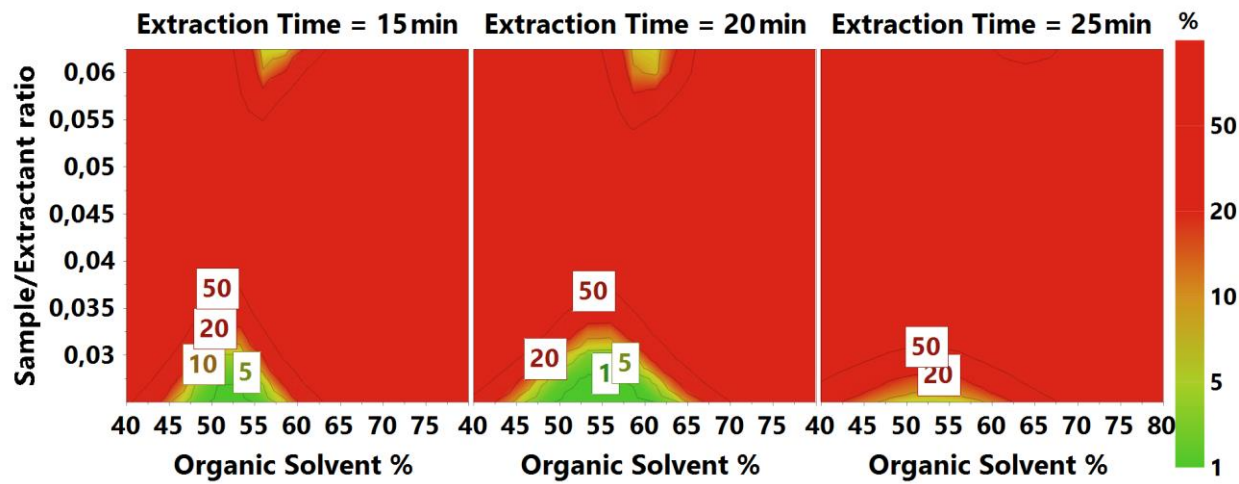


Figure 3 – 2-D probability plots (risk of error %) obtained by plotting Sample/Extractant ratio vs. Organic Solvent percentage at three levels of Extraction Time (15, 20 and 25 min). The MODR is represented by the green zone included in the 10% isoprobability line.

Table 1 – UAE: RSM Box-Behnken Design. Experimental plan with two replicates.

<i>Exp No</i>	<i>Time</i>	<i>OS %</i>	<i>S/E ratio</i>	<i>TSP</i>	<i>TMA</i>	<i>DPPH</i>	<i>FRAP</i>	<i>ABTS</i>
1	15	40	0.04375	4250	3305	266.6	154.8	1060.2
2	25	40	0.04375	4189	3182	261.6	159.8	1013.6
3	15	80	0.04375	3804	3765	221.6	150.8	1039.2
4	25	80	0.04375	3853	3248	225.1	160.4	879.2
5	15	60	0.02500	4324	3518	249.3	168.9	1015
6	25	60	0.02500	4470	3615	268.0	188.3	1010.2
7	15	60	0.06250	4225	3495	267.4	207.9	873.0
8	25	60	0.06250	4179	3325	276.2	193.2	959.0
9	20	40	0.02500	4329	3388	303.0	194.3	1105.8
10	20	80	0.02500	3802	3525	239.2	175.6	926.7
11	20	40	0.06250	4096	3085	291.7	185.0	952.7
12	20	80	0.06250	3611	3630	248.6	185.2	860.3
13	20	60	0.04375	4240	3353	250.7	182.3	969.3
14	15	40	0.04375	4117	3053	288.2	177.2	1025.2
15	25	40	0.04375	4067	3125	253.1	166.7	1007.2
16	15	80	0.04375	3887	3690	222.2	168.8	1068.0
17	25	80	0.04375	3872	3345	244.8	164.8	933.1
18	15	60	0.02500	4467	3593	264.6	189.2	1063.8
19	25	60	0.02500	4500	3453	274.1	200.2	1030.1
20	15	60	0.06250	4124	3593	273.3	198.2	934.7
21	25	60	0.06250	4275	3475	272.4	204.3	885.8
22	20	40	0.02500	4059	3455	306.2	190.1	1119.4
23	20	80	0.02500	3874	3479	247.5	180.6	920.6
24	20	40	0.06250	4043	3141	292.0	201.6	965.6
25	20	80	0.06250	3793	3735	237.6	194.9	952.5
26	20	60	0.04375	4343	3553	271.2	182.3	1050.9

CMPs units of measurements: *Time*, min; *OS%*, %v/v; *S/E ratio*, g mL⁻¹.

CMAs units of measurements: *TSP*, mg CAT eq/100 g d.w.; *TMA*, mg CYA-3-GLU eq/100 g d.w.; *DPPH*, inhib. DPPH µg/mg d.w.; *FRAP*, mg Trolox/g d.w.; *ABTS*, inhib. ABTS µg/mg d.w.

CRedit Author Statement

Luca Marzullo: Investigation, Writing - Original Draft. **Oleksander Ochkur:** Investigation. **Serena Orlandini:** Software, Writing - Review & Editing. **Lapo Renai:** Writing - Original Draft. **Roberto Gotti:** Software, Visualization. **Oleh Koshovyi:** Conceptualization, Resources. **Sandra Furlanetto:** Formal analysis, Visualization **Massimo Del Bubba:** Conceptualization, Formal analysis, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: