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1 **Pressurized green liquid extraction of glucosinolates from *Camelina sativa* (L.) Crantz by-products:**
2 **Process optimization and biological activities of green extracts.**

3

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11

12 **Abstract**

13 The cultivation of *Camelina sativa* (L.) Crantz is rapidly increasing due to oil production resulting in a
14 substantial volume of by-products, which still have an interesting composition in secondary metabolites,
15 especially glucosinolates. Therefore, a green extraction procedure of glucosinolates by Pressurised Liquid
16 Extraction was developed and optimized using a chemometric approach. Furthermore, the glucosinolates were
17 purified by solid phase extraction, and a preliminary study on bioaccessibility and bioavailability study was
18 carried out to evaluate the resistance of the glucosinolates to the digestive process. The application of
19 pressurised liquid extraction to the recovery of glucosinolates from camelina sativa by-product, is a green,
20 automatic, and rapid method, representing a valid alternative to conventional extraction method to obtain
21 ingredients for food industries.

22 **Keywords:** Pressurized Liquid Extraction, *Camelina sativa* by-products; glucosinolates;; bioaccessibility.

23

24 Chemical compounds studied in this article.

25 Glucohirsutin (PubChem CID 44237258), Glucoarabin (PubChem CID 46173881), Glucocamelin (PubChem
26 CID 162639109)

27

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28 **1. Introduction**

29 *Camelina sativa* (L.) Crantz is an herbaceous plant in the Brassicaceae family that has received great attention
30 due to its high tolerance to different abiotic factors and its ability to grow under unfavourable conditions
31 (Blume et al., 2023), its cultivation for food and feed purpose is rapidly increasing in Europe and North
32 America. *C. sativa* seeds contain 30-40% oil rich in essential fatty acids, which can be an excellent plant-based
33 alternative to fish and flaxseed oil and high levels of bioactive molecules such as tocopherol, phytosterol, and
34 phenolic compounds (Ergönül & Özbek, 2020; Gugel & Falk, 2011; Piravi-vanak et al., 2022). Oil production
35 generates camelina pressed-cake (PC) by-product, requiring disposal. Unfortunately, this often leads to severe
36 environmental issues due to inadequate management. It is well documented that the PC has an interesting
37 chemical composition (amino acid and protein, glucosinolates, synapine, inositol phosphates, tannins, and
38 erucic acid) and could be reused in different industrial applications (Erickson et al., 2012; Zubr, 1997).
39 Glucosinolates (GLSs) are secondary metabolites particularly abundant in the Brassicaceae family; they
40 consist of a D-thioglucose group linked to a sulfonated aldoxy group and a variable side chain derived from
41 an amino acid. Nowadays, more than 130 different glucosinolates have been identified in many plants,
42 especially in the Brassicaceae (Nguyen et al., 2020; Xin et al., 2014). GLSs composition and concentration
43 vary based on species, variety, environment conditions, plant age. (Fahey et al., n.d.; Mithen et al., n.d.; Ram
44 Bhandari et al., 2015, Pagliari et al., 2022a). Recent studies have associated GLSs with several health benefits
45 such as antioxidant (direct and indirect), anti-inflammatory, antimicrobial, cholinesterase inhibitory, anti-
46 tumor, and cardiovascular disease preventive properties (Maina et al., 2020; Sanchez-Pujante et al., 2017). In
47 this context reusing the PC via extraction processes can generate potential products for the food
48 pharmaceutical, nutraceutical, and cosmetic industries.

49 Usually, the recovery of secondary metabolites from agricultural by-products requires several time-consuming
50 and potentially hazardous steps, involving the use of toxic extraction solvents. It is therefore necessary to use
51 sustainable processes with the lowest cost and environmental impact, to extract these metabolites from PC,
52 quickly, efficiently, and selectively (Belwal et al., 2018; Amran et al., 2021). Among the extraction processes
53 pressurised liquid extraction (PLE) is a highly efficient techniques compared to the traditional extraction
54 methods. PLE involves the use of liquid solvent at an elevated temperature and pressure to enhance the
55 extraction of target compounds from the matrix. The combination of pressure and temperature increase the
56 mass transfer rate by reducing the surface tension and viscosity of the solvent (Mustafa & Turner, 2011;
57 Figueroa et al., 2018; Pagano et al., 2021). This simultaneously increases the solubility of the analytes
58 facilitating the penetration of the solvent into the matrix. PLE has many advantages over conventional
59 extraction techniques (maceration, distillation, Soxhlet, etc.), such as reducing energy consumption, waste
60 management, production time, and costs, while improving consumer safety and health, regulatory compliance,
61 and preserving bioactive compounds in the matrix (Figueroa et al., 2018; Pagano et al., 2021)

62 In the present work, a PLE method was used to recover glucosinolates from camelina PC. Initially, an
63 analytical procedure based on ultra-pressure liquid chromatography (UPLC) coupled with high-resolution
64 mass spectrometry (HRMS) was developed to evaluate the chemical composition of the extract. Furthermore,
65 a response surface design was used for the identification of experimental parameters that significantly
66 influenced the extraction as well as the interaction between these parameters.

67 Under the optimized extraction conditions, the developed PLE method was compared with conventional
68 methods such as ISO and USAE (Pagliari et al., 2022a) showing better efficiency, with a reduction of time and
69 solvent consumption. Finally, the biological activities of the GLSs identified in PC, which are still poorly
70 known, were investigated using in silico and in vitro assays to identify their potential beneficial activity for
71 human health. Considering the promising chemopreventive activities and the dietary use of the species, an
72 initial bioaccessibility and bioavailability study was initiated to assess the resistance of the GLSs to the
73 digestive process and to provide information for a suitable formulation to preserve their properties.

74

75 **2. Materials and methods**

76 2.1 Standards and materials

77 MS-grade solvents used for UPLC analysis acetonitrile (MeCN) water (H_2O) and formic acid (HCOOH) were
78 provided by Romil (Cambridge, UK); analytical-grade solvents methanol (MeOH) and ethanol (EtOH) were
79 supplied by Sigma-Aldrich (Milan, Italy). H_2O was purified by using a Milli-Q system (Millipore, Bedford,
80 USA). Acetic acid (AA), ammonium hydroxide, naphthylethylene diamine dihydrochloride, phosphoric acid,
81 ascorbic acid, fluorescein sodium salt, Trizma hydrocloride (Tris-HCl) monopotassium phosphate dipotassium
82 phosphate, sodium nitroprusside dehydrate (SNP), sulphanilamide were provided by Sigma-Aldrich (Milan,
83 Italy). 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from TCI Chemicals (Tokyo,
84 Japan). Glucoarabinin potassium salt, glucocamelin potassium salt and homoglucocamelinin potassium salt
85 were purchased from Extrasynthese (Lyion, France).

86

87 2.2 Samples

88 The industrial PC were received after cold oil extraction from FlaNat Research srl (Milan Italy). The samples
89 It was finely blended using a knife mill, Grindomix GM-200 (Restek GmbH Germany) operated at 6000 rpm
90 (Pagliari et al., 2022b). The ground sample was sieved to obtain a powder with a homogeneous particle size
91 distribution and fraction retained on the 300-600 µm mesh was collected and stored in the dark at -80°C in
92 polyethylene bags until used in the extraction processes.

93

94 2.3 Pressurized liquid extraction (PLE)

95 The ASE300 was used for pressurized liquid extractions (Thermo Fisher, Waltham, USA). For the extraction,
96 1 g of dried material was packed into a 10 mL stainless steel extraction cell and the extraction cell's empty area
97 was filled with 4 mm solid-glass beads (Sigma-Aldrich, Milan, Italy), and a paper filter (Whatman n°1) was
98 placed at the bottom of the extraction cell.

99 Preliminary tests were performed with EtOH 80%, 3 cycles, and static of 4 minutes to identify the extraction
100 temperature range to be used in the experimental design. The extracts were collected in a glass vial (60 mL)
101 and the solvent was evaporated using a rotary evaporator (G3, heiVAP core, Heidolph Germany) to calculate
102 the extraction yield.

103

104 2.4 Optimization of PLE condition by an Experimental Design

105 Chemometric approach was used to find the best PLE extraction condition using Statgraphic Centurion XVI
106 16.1 version (Rockville, USA.). A response surface was employed to investigate the effects of four independent
107 variables (extraction temperature, number of cycles, solvent composition, static time) on the dependent
108 variable (g/gDM of GLS9, GLS10, GLS11). A Box-Behnken design 2-factor interaction with 3 canter point,
109 an error of 12 degree of freedom, for a total of 27 randomized run was used (Table 1). The four independent
110 variables were examined at three levels (low, medium, and high) using this design: extraction temperature
111 (Temp) at 70, 110, and 130 °C, number of cycles 2, 4, and 6, composition of solvent modifier (EtOH%) at 60,
112 80, and 100%, and static time 2, 4, and 6 minutes.

113 To get the parameters of the statistical models, data from the CCD were submitted to regression analysis using
114 least square regression methods. ANOVA was used to determine the statistical significance of independent
115 variable (A, B, C, and D) contributions and their first order interaction. The answers obtained from the
116 statistical analysis were fitted to a second-degree model capable of taking into account the individual parameter
117 interactions together with their quadratic relationships (eq 1).

118 γ=β_0+∑_(j=1)^k β_j x_j+∑_(j=1)^k β_jj x_j^2+∑_(j=1)^k.∑_(j=1)^k β_ij x_i x_j (eq1)

119

120 2.5 Purification of glucosinolates by solid phase extraction

121 To obtain a GLSs rich extract the solid-phase extraction (SPE) was carried out based on our previous study
122 (Pagliari et al., 2022a) with slight modification. Briefly, a strong anion exchange (SAX) Mega Bond Elute
123 NH_2 cartridges (1 g) were activated with 10 mL of MeOH and equilibrated with 10 mL of H_2O 1% AA. The
124 PLE liquid extract was loaded onto the NH_3^+ cartridge, washed with 5 mL of MeOH 1% AA; finally, the
125 glucosinolate fraction was eluted with 10 mL of freshly prepared H_2O 2% NH_4OH solution. The purified
126 extract was evaporated to dryness in a vacuum evaporator at 40° C, dissolved in water at a concentration of 1
127 mg mL^{-1} and filtered with 0.22 µm PES filter before in vitro assays.

128

129 2.6 Comparative analysis of extraction techniques

130 An extraction technique previously developed was used as to compare the performance of the developed PLE
131 method. The ultrasound-assisted extraction (USAE) was developed and optimised in our previous study
132 (Pagliari et al., 2022a). Briefly 1 g of ground samples was extracted 2 time with 5 mL of 65% EtOH in in the
133 ultrasonic bath (Sonorex TK 52; Bandelin electronic, Berlin, Germany) operating at 35 kHz and power, 100%.
134 All the extraction were performed in triplicate, centrifuged (ALC centrifuge PK 120, Thermo Electro
135 Corporation, San Jose, CA, USA) for 3 minutes at 13000 rpm (19.8 g). The supernatants were filtered with
136 paper filter (Whatman No. 1 filter) and stored at -20 °C until the analysis by UPLC-HRMS.

137

138 2.7 Qualitative and quantitative analysis by HRMS/MS analysis

139 Qualitative and quantitative analyses of extracts and were carried out using an acquity UPLC system coupled
140 with a Xevo G2-XS QTof mass spectrometer (Waters Corp., Milford, MA, United States). The mass
141 spectrometer equipped with an electrospray ion source (ESI), was used in negative and positive ionization
142 modes to acquire full-scan MS, and spectra were recorded in the range of 50-1000 m/z. The ESI parameters
143 were as follows: electrospray capillary voltage 2.0 kV, source temperature 150 °C and desolvation temperature
144 600 °C. The cone and desolvation gas flow were 20 and 900 L h^{-1}, respectively, and a scan time of 0.3 s was
145 employed. Cone voltage was set at 70 V, and source offset at 20. The mass spectrometer was calibrated with
146 0.5 M sodium formate, and 100 pg μL^{-1} of standard leucine-enkkephaline at m/z 554.2615 was infused with
147 the flow of column at 5 µL min^{-1} as the lock mass and acquired for 1 s each 30 s. The total ion current (TIC)
148 used for qualitative analysis was acquired, and MS/MS spectrum of each compound at different collision
149 energy was acquired and compared to reference standards from which the GLSs identification was performed.
150 A quantitative analysis was performed using multiple reaction monitoring (MRM) data acquisition mode by
151 monitoring three characteristic fragments for each target compounds of [M+H]- ion of glucoarabinin (506.1523
152 > 442.14, 248.11, 96.96) glucocamelinin (520.1684 > 456.16, 262.12. 96.96) and homoglucocamelinin
153 (534.1819 > 470.18, 276.14, 96.96) and ramping collision energy from 25 to 30 V to produce abundant product
154 ions before the detection. In order to quantify the GLS compounds in the extracts, an external standard
155 calibration was conducted six points between 0.01 and 10 µg mL^{-1}. Each level was acquired in triplicate. The
156 analysis of variance (ANOVA) was carried out to test the regression curves, and the linear model was found
157 appropriate over the concentration range (R^2 values > 0.9992). Precision and intraday repeatability were also
158 estimated in all the concentration levels with a coefficient of variation lower than 5%. The results of the
159 quantitative analysis for each analyte were expressed as µg g^{-1} of dry matter (DM). The Mass Lynx software
160 (version 4.2) was used for instrument control, data acquisition, and processing.

161

162 2.8 In vitro antioxidant activities

163 2.8.1 ABTS assay

164 ABTS assay was used to evaluate the antioxidant activity of all sample extracts. The experimental conditions
165 were reported by Pagano et al. (Pagano, Sánchez-Camargo, et al., 2018). Briefly, 5 mL of PBS (control),

166 Trolox (0.25-1 mg mL-1) and extracts 1 mgmL-1 were mixed with 500 mL of ABTS standard solution at a
167 concentration of 1mM. 300 mL of each mixture were transferred into a 96 well plate and were incubated,
168 protected from light, and after 60 min the absorbance was read at 734 nm using a Multiskan Go
169 spectrophotometer (Thermo Fischer Scientific). Results of ABTS assay were expressed as Trolox equivalent
170 TEAC mmol/mg, and they were employed to quantify the antioxidant activity of the tested solution expressed
171 as standard deviation (SD) of three measurements.

172

173 2.8.2 Oxygen radical absorbance capacity (ORAC)

174 The scavenging capacity of extracts against oxygen radicals was evaluated using the ORAC assay. The
175 experiment is performed in black 96-well plates according to the protocol reported by Sánchez-Martínez et al.
176 (Sánchez-Martínez et al., 2022). Each well is filled with 100 µL of extract at different concentrations in water,
177 100 µL of AAPH (590mM) in 30mM phosphate buffer saline (PBS) at pH=7.5, 25 µL of fluorescein in PBS
178 and 100 µL of PBS to make up the volume. Fluorescence was measured with λ excitation= 485 nm and λ
179 emission= 530 nm every 5 minutes for 1 hour at 37°C. Ascorbic acid was used as a positive control. The
180 peroxyl radical scavenging ability of the extract was expressed as IC50 (ug of extract able to inhibit 50% of
181 the AAPH radical).

182

183 2.8.3 RNS scavenging capacity

184 RNS radical scavenging capacity was measured by a point spectrophotometric assay capable of measuring the
185 ability of the sample to neutralize nitric oxide (NO) radicals. The assay was prepared according to Sánchez-
186 Martínez et al. (Sánchez-Martínez et al., 2022). The assay was performed in transparent 96-well plates. Each
187 well was filled with 100 µL of the extract at different concentrations in water and 50 µL of SNP (5mM)
188 solubilized in 30mM PBS at pH= 7.5. After incubation for 2 h under the white light of a lamp at room
189 temperature, 100 µL of Griess reagent (prepared by mixing 500 mg sulfanilamide with 50 mg naphthyl-
190 ethylenediamine dihydrochloride and 1.25 mL phosphoric acid in 48.5 mL water) is added. After incubation
191 for 5 minutes, the absorbance at 734 nm is recorded to measure the concentration of nitrite radicals. Ascorbic
192 acid is used as a reference standard. The results are expressed as µg ascorbic acid equivalent/mg extract.

193

194 2.9 In vitro gastrointestinal digestion simulation

195 Gastrointestinal digestion was carried out following the INFOGEST protocol described by Minekus (Minekus
196 et al., 2014a), with light modification by Pagliari (Pagliari et al., 2023). Briefly, the oral, gastric, and intestinal
197 phases were simulated by mimicking the salt and enzyme composition, pH, time, and temperature conditions
198 of each phase of the digestive process. Specifically, in the oral phase, 2 mL of PC extract was added to 1.4 mL
199 of oral saline (SSF), 390 µL of water, 200 µL of amylase 75 U/mL and 10 µL of $CaCl_2$ 0.3 M. After 2 minutes
200 at 37°C, 3 mL of gastric saline (SGF), 640 µL of pepsin 2000 U/mL, 258 µL of water and 2 µL of $CaCl_2$ 0.3
201 M and 160 µL of HCl 4M were added to the 4 mL of oral phase to achieve a pH between 2-3. After 2 hours at
202 37°C, the final intestinal phase was started by adding 4.4 mL intestinal salt phase (SIF), 2 mL porcine
203 pancreatin to obtain trypsin at 100 U/mL, 14 mg porcine bile, 1.58 mL water and 16 µL $CaCl_2$ 0.3M. The pH
204 was maintained at 8 during the intestinal phase (2 h at 37°C). At the end of the process, the digested extracts
205 were analysed by UPLC-HRMS to quantify the GLSs compounds. The Bioaccessibility and bioavailability
206 were calculated according to Eq. (1) and Eq. (2) respectively end expressed as percentage as reported by Canas
207 (Canas et al., 2022):

208 $$\%\, bioaccessibility = \frac{digested\ fraction}{non{-}digested\ fraction} * 100 \qquad \text{Eq. (1)}$$

209 Where non-digested fraction and digested fractions are the concentration of compound before and after
210 digestion simulation, respectively.

211 $$\% \, bioavailaility = \frac{digested\ fraction * absorption}{non-digested\ fraction} * 100 \qquad \text{Eq. (2)}$$

212

213 The absorption was estimated in silico using pkCSM-pharmacokinetics (http://biosig.unimelb.edu.au/pkcsm/,
214 consulted on 5 September 2023) e ADMETlab (https://admet.scbdd.com/, consulted on 5 September 2023).

215

216 2.10 Statistical Analysis

217 All data were performed in triplicate and results were presented as average ± standard deviation. Analysis of
218 variance (ANOVA) was used to compare the means while Turkey's test was used to assess the statistically
219 significant difference among extraction conditions using JMP 14 software. A p-value of □ 0.05 was considered
220 significant.

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222 **3 Results and discussion**

223 3.1 Analysis of PLE extract

224 Although the characterization of the PC extract obtained by USAE had already been reported in our previously
225 work (Pagliari et al., 2022a), we carried out further analysis using UHPL-HRMS/MS on the PLE extract to
226 verify the stability of its chemical composition under the combined influence of temperature and pressure.
227 Indeed, the temperatures and pressures employed in PLE can modify the chemical composition of extract and
228 may lead to the degradation of molecules when compared to USAE. For this reason, an extraction under mild
229 conditions (70°C, two extraction cycles and 65% EtOH) was performed to investigate the preliminary
230 chemical composition of the PLE extract. Identification of compounds were assigned by using all chemical
231 information; retention times (Tr), UV/vis signals, accurate mass, molecular formula, MS/MS spectra, reference
232 standards whenever available, combined with chemo-taxonomic databases. The detailed characterisation of
233 the phytochemical compounds selected condition was performed using UPLC-HRMS and the obtained results
234 were in agreement with those reported in our previous study (Pagliari et al., 2022a). In detail as reported in
235 Figure S1, the untargeted analysis in negative ion mode reveals the presence of 11 main metabolites, including
236 phenols (epicatechin-O-glucoside isomer (1 and 2), rutin-2-O-apioside (4), rutin (6), Kaempferol-3-O-
237 gentiobioside-7-O-rhamnoside (7), Kaempferol-3-O-neohesperidoside (8), Isorhamnetin-3-O-β-rutinoside (9),
238 Tamarixetin-7-O-rutinoside(11) and three glucosinolates, glucoarabin (3) (GLS9), glucocamelinin (5)
239 (GLS10) and homoglucocamelinin (10) (GLS11).

240 3.2 Optimization of glucosinolates extraction by PLE

241 3.2.1 Preliminary selection of solvent composition and temperature

242 Once the presence of GLSs in PLE extract has been confirmed through qualitative analysis, the extraction
243 conditions were optimised using chemometric approach. As generally reported in the literature, the parameters
244 that most influence the PLE process are temperature and solvent composition (Pagano, Sánchez-Camargo, et
245 al., 2018). Preliminary experiments were carried out to determine the ranges of temperature and solvent
246 composition to be used before chemometric optimization. The effect of PLE temperature on extraction
247 efficiency of GLSs was showed in Figure 1. The results show a direct correlation between the rise of PLE
248 temperature and the extraction yield of GLSs up to a temperature of 110°C, beyond which the recovery of
249 glucosinolates starts to decrease, probably because of the degradation of these compounds. Indeed, it is known
250 that GLSs are thermosensitive molecules and most of them could be degraded over at 100°C (Hanschen, Bauer,
251 et al., 2012; Oerlemans et al., 2006). Based on these results, the temperature range of 70-130 °C was used in
252 the chemometric optimization. Regarding the solvent composition, EtOH was chosen as Generally Recognized
253 as Safe (GRAS) solvent for safe food use and for its efficiency in the recovery of GLSs, as previously
254 demonstrated in USAE (Pagliari et al., 2022a). As demonstrated for the USAE extraction, waters produce a
255 swelling of matrix due to the presence of saponins and mucilage in the camelina seeds, causing the clogging
256 of the PLE system preventing the emptying of extraction cell, for this reason the minimum EtOH content to
257 ensure the proper functioning of the PLE is EtOH 60%. Following these results, the solvent composition range
258 was set between 60% and 100% EtOH.

259

260 3.2.2 Response surface design of PLE process

261 After conducting preliminary experiments, to determine the temperature and solvent composition ranges, a
262 Box-Behnken two-factor interaction design was employed to investigate the effect of four independent
263 variables (temperature, number of cycles, static time and percentage of ethanol) on the dependent variable (the
264 extraction efficiency of the three glucosinolates). Table 1 shows the experimental conditions for each run and
265 the experimental values of the responses (GLS9, GLS10 and GLS11) to the different experimental conditions.

266 The statistical analysis reported in Table 2 allows us to identify the significant factors and their impact on the
267 response variables. Based on the results, the model showed a high correlation (R2 ≅ 77-79%), indicating a low
268 variance of the data and a good prediction of the model with respect to all the response variables considered.

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269 The obtained results demonstrate that only the percentage of ethanol and its combination with the number of
270 cycles have a significant influence on the efficiency of the GLS9, GLS10, and GLS11 recovery (p-value <
271 0.05). The regression model equations that best describe the recovery of GLS as a function of process variables
272 are shown in Table S1. As shown in the response surface (Figure 2 a-c), the percentage of EtOH has a linear
273 effect on the recovery of the three GLSs in the range from 60 to 65-70%, above 65-70% the desired effect
274 decreases. This confirms the ability of PLE to use the combination of high temperature and pressure to
275 significantly modify the chemical properties of the extraction solvent, improving the extraction efficiency of
276 the water and reducing the necessity to use a high concentration of organic solvent. The number of cycles
277 linearly increases the extraction efficiency when the EtOH percentage was low, whereas at high EtOH
278 concentration increasing the number of cycles has a negative effect by reducing the recovery of the three GLSs
279 (figure 2 d-f). The temperature follows a parabolic profile, indicating a peak after which recovery may decrease
280 due to GLS structure degradation at high temperatures (MacLeod et al., 1981), while the extraction process
281 was not affected by the static time. Temperature and static time were found to be statistically insignificant
282 (Table 2). The optimized conditions extrapolated from DOE to maximize GLSs recovery were as follow 65%
283 EtOH, 6 cycles, 118°C and 2 minutes static time (Opt 1), resulting in a desirability of 97.84%. However, as
284 the extraction temperature did not show statistical significance (p-value > 0.05) in the regression model, as the
285 concentration of GLS9, GLS10, and GLS11 did not change significantly by varying the temperature, as shown
286 in the response surface (Figure 2 g-i), alternative extraction conditions were investigated. These conditions
287 (Opt 2) were the same as adopted in Opt 1 but the extraction was conducted at 70°C representing the minimum
288 value tested in response surface) was tested. The results of conditions Opt1 and Opt2 indicate that there is no
289 statistically significant difference (p-value > 0.05) between the two tested conditions. Therefore, Opt 2 was
290 selected as a better solution to minimize energy consumption and to avoid the possible thermal degradation of
291 GLSs, with an extraction yield of 148.08 ± 0.02 mg/g DM. Furthermore, the actual values, reported in table 3,
292 were in good agreement with the predicted values for the concentration of the three main glucosinolates by
293 PLE, indicating the suitability of the established regression models for the prediction of response values.

294 Once the optimal PLE conditions for recovering GLS from PC were identified, we decided to conduct a
295 comparison between PLE and our previously optimized method based on USAE (Pagliari et al., 2022a). As
296 expected, the pressurized liquid extraction proved to be the most efficient techniques for glucosinolates
297 recovery, in fact, using this method, the recovery of the three GLSs increased by approximately 690% (GLS9),
298 643% (GLS10), and 801% (GSL11) compared to the USAE procedure. However, the use of PLE requires
299 highly qualified personnel and a high initial equipment cost. The results of the recovery of GLSs from the PC
300 showed a high extraction efficiency of green techniques compared to the ISO technique.

301

302 3.3 Purification of the GLSs by solid phase extraction.

303 Considering the good content of GLSs in PC it was decided to purify the extract by SPE to test the potential
304 biological activity of an extract rich in GLSs.

305 Both wash and elution fractions were collected, and each was analysed by UPLC-HRMS-DAD to detect the
306 presence of GLSs and to verify the purity of the SPE extracts. Figure 3 shows the HRMS chromatographic
307 profiling of the elution fractions and crude extract. Chromatographic analysis shows the selective interaction
308 of the negatively charged GLSs in the basic state with the NH_4^+ cartridges compared to the neutral phenolic
309 compounds, which can not interact with the positive charges of the phase. Due to the concentration capacity
310 of SPE procedure, the presence of new minority peaks in the purified PLE extract has been detected. For these
311 reasons, a data dependent HRMS analysis was performed to tentatively identify the compounds detected.
312 Among all these compounds, the peak at a retention time of 3.80 min with an accurate mass of 492.1036 m/z
313 showed the specific fragmentation pattern of aliphatic glucosinolates according to MS/MS analysis (Fabre et
314 al., 2007; Glauser et al., 2012). Specifically, the 477.0805 m/z production corresponds to the loss of a methyl
315 (-15 Da), while the 234.0807 m/z ion corresponds to the fragment remaining after the loss of thioglucose and
316 methyl sulfoxide and the presence of the 96.9596 m/z fragment corresponding to the sulphate group (Figure
317 4). Based on this fragmentation pattern the glucohirsutin (GLS8) compound was tentatively assigned. To the
318 best of our knowledge, this is the first time that GLS8 has been identified in the camelina sativa species.

319

320 3.4 Predictive Bioactivities of the purified extract

321 The evaluation of the general biological potential of the four specific GLSs identified in C. sativa extract was
322 initially investigated *in silico* using Prediction of activity spectra for substance (PASS) on-line software. The
323 analysis was obtained by entering the simplified molecular input line entry Simplified Molecular Input Line
324 Entry System (SMILES) strings of each molecule into the software and obtaining in response a table containing
325 the probable activity (PA) and probable inactivity (PI) values with a related biological activity variability
326 between 0.000 and 1.000. Biological predictions with PA > 0.700 show the greater potential biological activity.

327 The results reported in Table S2 show probable chemotherapeutic activity, in particular apoptosis agonist,
328 chemopreventive and antineoplastic effects with Pa > 0.9 are predicted depending on their structure, indicating
329 a very high probability results being reliable. These results are in agreement with the few data reported in
330 literature regarding the activity of GLSs (Cabello-Hurtado et al., 2012). Hence, to investigate the biological
331 activity of GLSs in purified PLE extract which was predicted using in silico analysis, spectrophotometric
332 assays and in vitro cell analysis were conducted.

333

334 3.5 Scavenging effect of purified glucosinolates extract

335 Oxidative stress is one of the primary mechanisms involved in the onset and progression of non-communicable
336 diseases such as cancer (Seyedsadjadi & Grant, 2021; Hariri & Ghiasvand, 2016). Oxidative stress is caused
337 by the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In physiological
338 conditions, the production of radicals is minimal and is counteracted by endogenous cellular mechanisms.
339 However, exogenous or endogenous factors can increase oxidative stress and impair the activity of cellular
340 oxidoreductive processes (Borsoi et al., 2023). This disruption in the balance between radical production and
341 endogenous mechanisms may contribute to the development and progression of various diseases including
342 cancer. In this context, the antioxidant activity of natural molecules consumed through the diet play a crucial
343 role in the prevention of pathological conditions. The predicted anticancer and chemopreventive activities of
344 GLSs may be attributed to their potential antioxidant activity. Several studies have been reported the direct
345 and/or indirect antioxidant capacity of GLSs, although their properties exhibit significant variations depending
346 on their specific chemical structure. Hence, it was decided to evaluate the ability of the main GLSs identified
347 in camelina sativa expeller to directly neutralise ROS and RNS. Scavenging activity was studied using three
348 spectrophotometric assays (ABTS, ORAC and RNS). The ABTS assay showed a TE value of 69.77±2.78
349 μg/mL EXT for the purified camelina extract, indicating a relatively low of capacity to neutralise ROS. This
350 low capacity to neutralise ROS was also confirmed by the ORAC assay, which showed an IC50 value of
351 percentage inactivation of 7.75±0.22 μg/mL EXT, which was higher than the IC50 of ascorbic acid used as a
352 positive reference (1.06±0.07 μg/mL EXT). Regarding the activity against nitric radicals, a better neutralising
353 effect was observed with an IC50 of 902.07±19.35 μg/mL EXT much closer to that of ascorbic acid
354 842.18±10.25. This suggests a better capacity of the purified extract to neutralise RNS. Based on the obtained
355 result, the GLSs-enriched extract indicates a reduced direct antioxidant capacity, with enhanced efficiency
356 against RNS radicals. These results agree with our previous study, which observed an increase in oxidative
357 stress following its administration in tumour cells (Pagliari et al., 2022a). These results, combined with
358 literature data, suggest an indirect antioxidant capacity, capable of stimulating the production of antioxidant
359 enzymes in healthy cells, but not directly neutralise radical species.

360 3.6 Bioaccessibility and bioavailability of GLSs

361 To evaluate the potential application of GLSs-enriched extract as a health-promoting ingredient for dietary
362 supplements, it was decided to verify the stability of GLSs following the gastrointestinal digestion simulation.
363 INFOGEST is widely used for bioaccessibility studies of phytochemical compounds such as polyphenols,
364 methylxanthines and numerous secondary metabolites, as well as for observing the digestive fate of proteins,
365 lipids and carbohydrates (Brodkorb et al., 2019; Minekus et al., 2014a). The results obtained showed an
366 influence of the digestive process on the degradation of the main GLSs. This finding is in line with the

367 literature, which shows that GLSs are molecules with reduced stability, sensitive to the action of pH and
368 enzymes. Specifically, after the intestinal digestion step, the concentrations of GLS9 GLS10 and GLS11
369 remained at 32.24±2.15, 50.03±3.67 and 11.53±0.99 µg/mg EXT, respectively, with a bioaccessibility of 28%
370 (GLS9), 26.80% (GLS10) and 15.70% (GLS11). However, in addition to the bioaccessibility of the molecules,
371 it is also important to assess their bioavailability, or the percentage of the compound that is passively absorbed
372 across the gastrointestinal barrier. Specifically, a bioavailability of 0%-3.45% has been calculated, indicating
373 an inability to cross the intestinal barrier. This may be due to the structure of the molecule, which has a high
374 molecular weight resulting in low permeability through the cell membrane. Further in-depth studies, perhaps
375 using in vitro cellular systems to simulate the gastrointestinal barrier and evaluate its possible active absorption
376 via transporters rather than the development of a suitable formulation to facilitate its passage, will therefore be
377 required.

378 **4. Conclusions**

379 This study evaluated the efficient extraction of glucosinolates from camelina sativa by-product using
380 pressurized liquid extraction. Through systematic optimization using an experimental design we have
381 successfully identified the key parameters that maximize the yield of bioactive compounds, reducing the
382 environmental impact. In detail, the optimized conditions extrapolated by Box-Behnken design were: EtOH
383 65%, temperature 70°C, 6 cycles, and static duration 2 minutes. After the identification of optimal extraction
384 conditions for GLSs recovery, a comparative analysis of various extraction techniques (ISO and USAE) reveals
385 that the optimized PLE extract has the highest GLSs recovery by reducing the time and organic solvent used.
386 This result not only highlights the efficacy of the PLE method but also underscores its advantages in terms of
387 sustainability, align with the principles of green chemistry. Following the optimization of extraction
388 conditions, a selective purification procedure employing SPE have been used to selectively concentrate the
389 GLSs and concurrently eliminate the interfering compounds. By employing this purification procedure which
390 produced an GLS-rich extract the glucohirsutin, a previously unidentified glucosinolate in camelina sativa was
391 discovered. Finally, the antioxidant activities and simulated digestion of the GLS-rich extract were
392 investigated. The obtained extract exhibited significant potential for application in the food and pharmaceutical
393 industries contributing to the growing demand for natural bioactive compounds with health-promoting
394 benefits.

395

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396 **Declaration of competing interest**

397 The authors declare that they have no know competing financial interest or personal relationship that could
398 have appeared to influence the work reported in this paper.

399

400 **CRediT authorship contribution statement**

401

402 **Stefania Pagliari:** Data Curation, Writing, Investigation. **Gloria Domínguez-Rodríguez:** formal analysis,

403 **Alejandro Cifuentes:** Supervision, writing review & editing. **Elena Ibáñez:** Supervision, writing review &
404 editing. **Massimo Labra:** Supervision**,** Funding acquisition, conceptualization. **Luca Campone:** Supervision**,**
405 Funding acquisition, Conceptualization Supervision, writing review & editing.

406

407 All authors have read and agreed to the published version of the manuscript.

408

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417

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420 **Data Availability**

421 Data will be made available on request.

422

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543 • The Camelina sativa by-products as source of glucosinolates was explored.
544 • A higher bioactive content can be obtained by unconventional developed method.
545 • Optimization of extraction parameter by response surface design.
546 • Glucohirsutin were identified by UPLC-UV-HRMS/MS for the first time in Camelina sativa.

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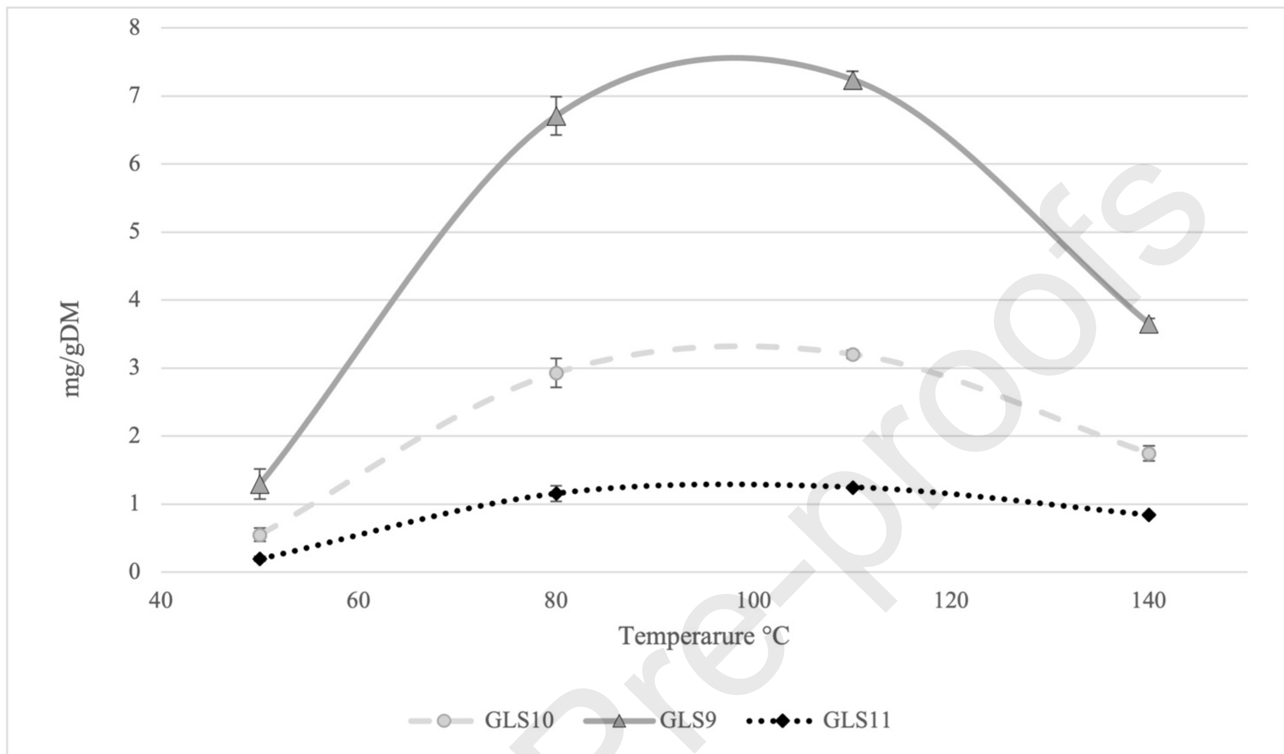

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556 **Figure 1** Effect of the temperature on of the three GLSs yield (mg/g DM) in PLE extracts.

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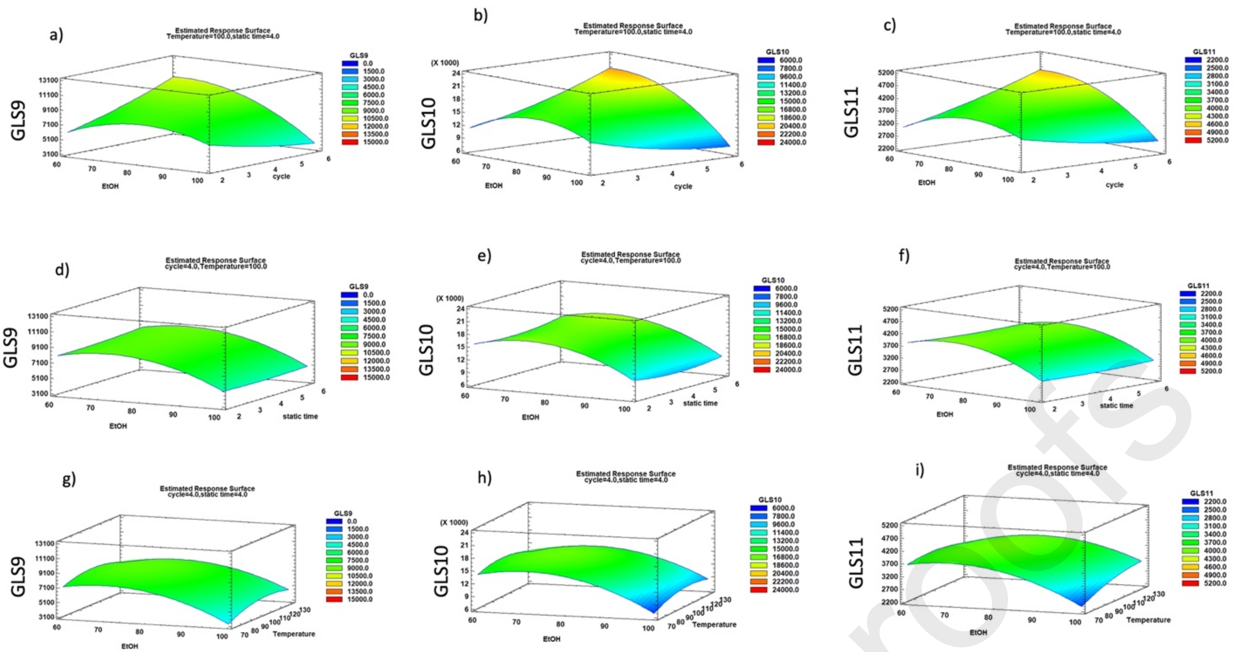

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561 **Figure 2** Response surface plots showing the effects of combination of %EtOH vs number of cycles (a-c);
562 %EtOH vs Static time (d-f); %EtOH vs Temperature (g-i), on the recovery of glucosinolates.

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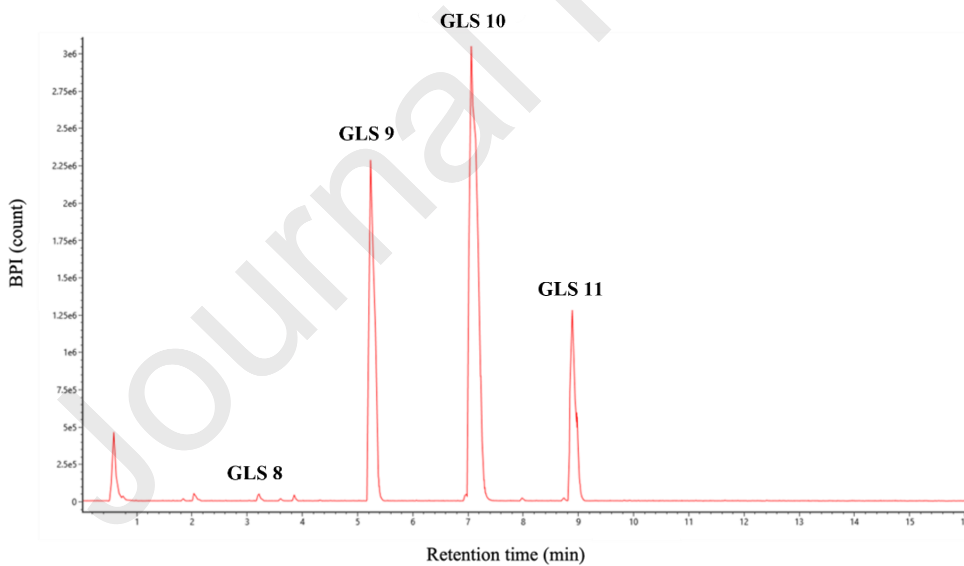

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565 **Figure 3** UPLC full MS chromatograms of pressurized liquid extraction after purification by solid phase
566 extraction.

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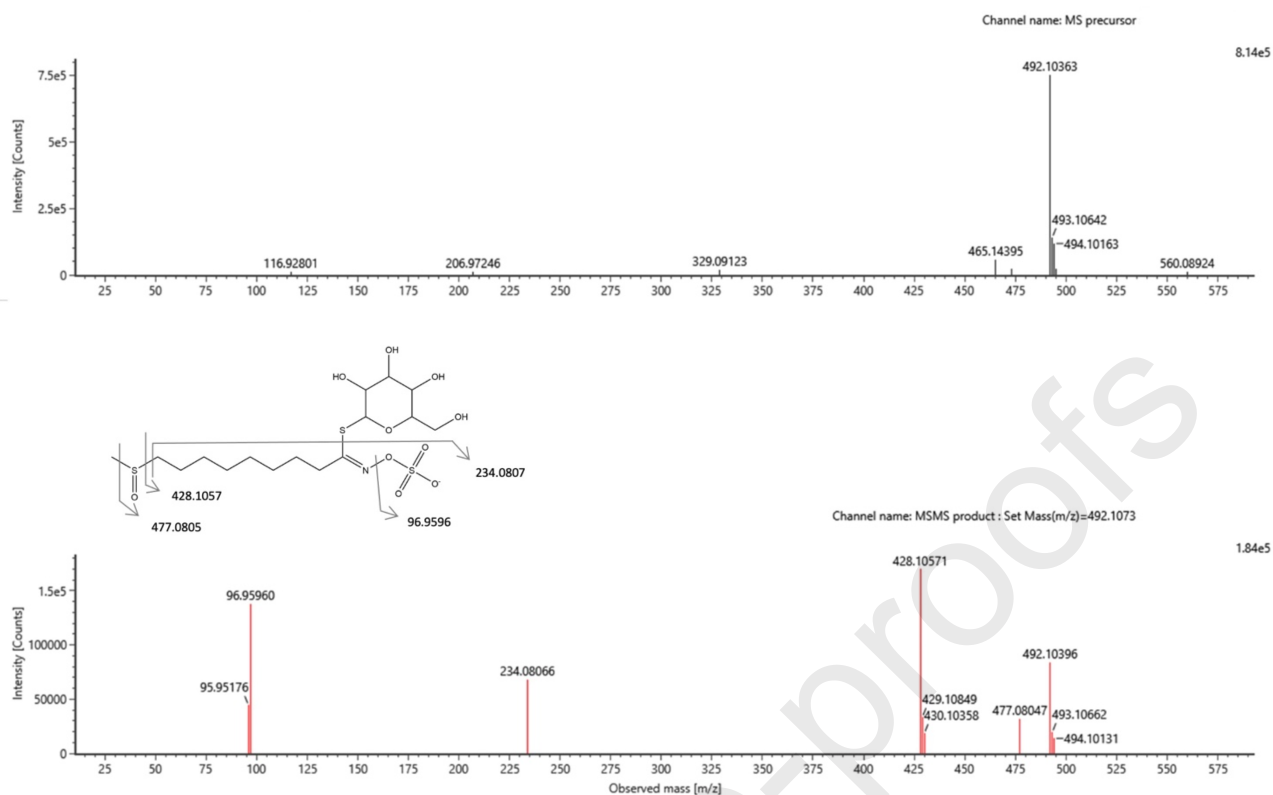

569

570 **Figure 4** full mass, MS/MS with fragmentation pathway of glucohirsutin (GLS8)

571 in negative ion mode

572

573

574 **Table 1** Experimental condition of the response surface design and experimental quantitative value of the
575 response variable (GLS9, GLS10 and GLS11.

576

Run	EtOH (%)	Cycles (n°)	Temp (°C)	S.time (min)	GLS 9 (mg/gDM)	GLS 10 (mg/gDM)	GLS 11 (mg/gDM)
1	100	6	100	4	3,9	7,8	2,7
2	80	4	130	2	7,5	15,3	4,0
3	80	4	100	4	8,9	17,5	4,1
4	100	2	100	4	5,1	10,8	3,1

5	80	6	130	4	6,6	12,8	3,9
6	100	4	100	2	5,0	10,7	3,0
7	80	4	70	6	8,7	18,3	4,0
8	60	4	130	4	8,6	16,2	4,1
9	80	6	100	2	10,3	21,8	4,7
10	60	4	70	4	6,4	13,5	3,7
11	100	4	130	4	6,7	11,9	3,6
12	60	4	100	6	7,5	14,2	3,4
13	80	2	70	4	7,9	15,7	4,0
14	80	2	130	4	7,6	16,3	3,4
15	60	4	100	2	7,7	15,2	3,7
16	80	2	100	2	8,2	15,2	3,9
17	100	4	100	6	4,4	8,8	2,8
18	60	6	100	4	11,6	22,1	4,7
19	80	4	70	2	6,7	14,4	3,4
20	80	6	70	4	7,0	14,2	3,5
21	80	4	100	4	7,4	14,8	3,6
22	60	2	100	4	5,5	10,4	2,7
23	80	2	100	6	9,4	19,1	4,4

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24	80	4	130	6	6,5	12,7	3,5
25	80	6	100	6	10,1	21,9	4,4
26	80	4	100	4	8,7	16,0	4,0
27	100	4	70	4	3,1	6,4	2,2

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578

579 **Table 2** Analysis of variance of the regression model

	Sum of squares			**Mean squares**		
	GLS9	**GLS10**	**GLS11**	**GLS9**	**GLS10**	**GLS11**
A:EtOH	3.01023E7	1.03447E8	2.07917E6	3.01023E7	1.03447E8	2.07917E6
B:cycle	2.86163E6	1.47054E7	468470	2.86163E6	1.47054E7	468470
C:Temperature	1.02726E6	617894	249697	1.02726E6	617894.	249697
D:static time	122614	520833	2610.75	122614	520833.	2610.75
A^2	1.88627E7	8.3087E7	1.94837E6	1.88627E7.	8.3087E7	1.94837E6
B^2	534674	5.83389E6	54225.9	534674	5.83389E6	54225.9
C^2	4.5387E6	1.2012E7	133001	4.5387E6	1.2012E7	133001
D^2	107163	5.09212E6	18934.3	107163.	5.09212E6	18934.3
AB	1.32169E7	5.40666E7	1.35956E6	1.32169E7	5.40666E7	1.35956E6
AC	463080	1.90026E6	213444	463080	1.90026E6	213444
AD	44944.0	182329.	2862.25	44944.0	182329.	2862.25
BC	576.0	1.02414E6	245025	576.0	1.02414E6	245025

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BD	475410	3.51938E6	160400	475410	3.51938E6	160400
CD	2.26804E6	1.06994E7	301950	2.26804E6	1.06994E7	301950
Total error	2.3288E7	8.9001E7	2.11423E6	1.94067E6	7.41675E6	176186
Total (corr.)	1.02605E8	4.23083E8	9.94614E6	-	-	-
R^2	77.3032	78.9637	78.7432	-	-	-
Adj R^2	50.8237	54.4214	53.9437	-	-	-
		F-value			**P-value**	
	GLS9	**GLS10**	**GLS11**	**GLS9**	**GLS10**	**GLS11**
A:EtOH	15.51	13.95	11.80	**0.0020**[a]	**0.0028**[a]	**0.0049**[a]
B:cycle	1.47	1.98	2.66	0.2480	0.1845	0.1284
C:Temperature	0.53	0.08	1.42	0.4808	0.7778	0.2569
D:static time	0.06	0.07	0.01	0.8058	0.7955	0.9051
A^2	9.72	11.20	11.06	**0.0089**[a]	**0.0058**[a]	**0.0060**[a]
B^2	0.28	0.79	0.31	0.6092	0.3926	0.5892
C^2	2.34	1.62	0.75	0.1521	0.2273	0.4020
D^2	0.06	0.69	0.11	0.8182	0.4235	0.7487
AB	6.81	7.29	7.72	**0.0228**[a]	**0.0193**[a]	**0.0167**[a]
AC	0.24	0.26	1.21	0.6340	0.6219	0.2926
AD	0.02	0.02	0.02	0.8816	0.8780	0.9007
BC	0.00	0.14	1.39	0.9865	0.7167	0.2611
BD	0.24	0.47	0.91	0.6296	0.5040	0.3588

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CD	1.17	1.44	1.71	0.3009	0.2529	0.2150

580 R^2 = Quadratic correlation coefficient. [a] Significant ($p < 0.05$).

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583 **Table 3** Quantitative analysis of GLS9, GLS10 and GLS11 between the optimal design condition suggested
584 by software (Optimized 1) and the hypothetic optimal condition with the lowest energy consumption
585 (Optimized 2).

GLSs	Predicted Optimized 1 mg g^{-1} DM	Optimized 1 mg g^{-1} DM	Predicted Optimized 2 mg g^{-1} DM	Optimized 2 mg g^{-1} DM
GLS 9	11.84^{a}	12.4 ± 1.25^{a}	9.25^{a}	11.6 ± 1.15^{a}
GLS 10	22.49 ^{b}	22.98 ± 1.16^{b}	20.21^{b}	21.37 ± 1.36^{b}
GLS 11	5.16 ^{c}	5.22 ± 0.41^{c}	4.26^{c}	5.01 ± 0.25^{c}

586 *a,b,c – means with the same letter are not significantly different

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