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1 **Explorative investigation of the anti-glycative effect of a rapeseed by-product extract**

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15 **Keywords:** Rapeseed cake; sinapinic acid; anti-glycative activity; Advanced Glycation
16 End Products; metabolomic; HPLC-DAD; GC-MS

17

18 **Abstract**

19 Formation of Advanced Glycation End-products (AGEs) in biological systems are
20 increased during hyperglycaemia due to higher levels of circulating glucose and carbonyl
21 reactive species. AGEs are causative factors of common chronic diseases. Since synthetic
22 AGE-inhibitors exert unwanted side effects and polyphenols act as potent antiglycative
23 agents, vegetables (fruits, seeds and related by-products) are good candidates when
24 searching for natural inhibitors. The aim of this research is to explore the suitability of a
25 polyphenol-rich rapeseed cake extract (RCext) to decrease the formation of AGEs in an
26 *in vitro* model. Different phenols, amino acids, carbohydrates, organic acids and fatty
27 acids were identified in the RCext by GC-MS. Results confirm the high concentration of
28 polyphenols (73.85 ± 0.64 and 86.85 ± 2.08 mg of gallic acid equivalents g^{-1} of RCext
29 spray dried and freeze dried, respectively) which is correlated with the antioxidant
30 capacity and anti-glycative activity in a dose dependent manner. Rapeseed cake extract
31 (3.7 mg mL^{-1}) significantly reduced the formation of free fluorescent AGEs and
32 pentosidine up to 34.85%. The anti-glycative activity of the extract is likely to be due to
33 the high concentration of sinapinic acid ($0.108 \pm 0.0043 \text{ mg g}^{-1}$) in its metabolic profile,
34 and the mechanism of action is mediated by methylglyoxal trapping. Results show a
35 promising potential for using rapeseed cake extract as a food supplement to ameliorate
36 the formation of AGEs. Rapeseed cake extract should therefore be considered a potential
37 candidate for the prevention of glycation-associated complications of age-related
38 pathologies.

39

40 1. Introduction

41 Diabetes Mellitus (DM) is a chronic endocrine disorder that has a substantial influence
42 on the quality of life of citizens (1). In later stages, DM can lead to nephropathy,
43 neuropathy, cardiopathy, blindness, strokes (2) and other diabetic complications (3). The
44 number of individuals affected by DM worldwide has quadrupled between the 1980s (108
45 million) and the year 2014 (422 million) and its prevalence has doubled to 8.5% (4). The
46 absence or insufficient insulin production compromises the body's ability to convert
47 glucose to glycogen, and hence, an accumulation of high amounts of glucose in the body
48 eventually results in hyperglycaemia (5). This procedure aids the high glucose
49 accumulation through glycation and their products at the advanced stage process of the
50 Maillard Reaction (MR). The initial stage of MR involves the breakdown of Amadori
51 products to the reactive compounds deoxysones. The pH of the environment, in which
52 the reaction occurs, affects 3-deoxysones-pathway leading to the formation of very
53 reactive intermediates (6). The advanced stage of MR forms irreversible Maillard reaction
54 products (MRPs), also known as Advanced Glycation End products (AGEs), which have
55 been shown to intervene in inflammatory pathways (7-9) and result in amplified oxidative
56 stress and inflammation (10). AGEs are also strong causative factors for other common
57 chronic pathologies including cardiovascular disease, cancer, and degenerative disorders
58 such as Parkinson's and Alzheimer's. In addition, AGEs are strongly correlated with the
59 process of aging within individuals who have a high glucose diet (11).

60 Polyphenols are widely known to possess good inhibition capacity against glycation and
61 oxidation. Therefore, their potential use against AGEs is currently under intensive
62 research (12,13). Polyphenols have been identified as beneficial inhibitors of the
63 formation of AGEs and escalation of degenerative disorders. These AGEs inhibitors can
64 trap the free radicals formed in the initial stage of the MR and can protect the body from
65 the oxidation of nucleic acids, DNA, lipids and proteins (14). They achieve this through
66 attachment to the amino-carbonyl compound or by interfering with the Amadori product
67 (AP) by rearranging and discontinuing the reaction (12,15). In general, it has been
68 established that there are three key positions in the polyphenols' structure linked to their
69 anti-glycative activity. These are located at the ortho-3',4'-dihydroxy in the B ring, the
70 2,3-double bond in conjunction with the 4-oxo group in the C ring, and the presence of
71 3-OH group in C ring and 5-OH group in the A ring (13). For instance, in the case of
72 flavonoids and single phenols, such as sinapinic acid and 4-vinylsyringol, the hydroxyl

73 groups in the phenyl ring have electron-donating properties and enhance the reactivity
74 with dicarbonyl compounds since this reaction is a nucleophilic addition, thus
75 contributing to a reduction in the formation of AGEs (9,16). Synthetic inhibitors of the
76 MR have several dangerous side effects increasing the need to find natural inhibitors such
77 as polyphenols from plants and seeds (17).

78 The aim of this work is to evaluate the anti-glycative properties of the by-product of
79 *Brassica rapa* L. extracted during oil production. This by-product is mainly known as
80 rapeseed cake (RC) (18) and is used for livestock feed due to its high energy density
81 values and low selling price (19). Its macronutrients are estimated to be approximately
82 36% protein, 12% fat and 13% fibre (20). The main phenols present in rapeseed are
83 sinapinic acid derivatives with its main phenolic acid accounting for 80% in the seeds
84 (21). Currently, the protein isolated from the rapeseed cake varieties *Brassica napus* L.
85 and *Brassica rapa* L. have been accepted by the *European Food Safety Authority (EFSA)*
86 as a novel food ingredient (22). It suggests the use of rapeseed cake for human
87 consumption has been reconsidered in light of the fact that those varieties show low
88 amounts of tannins (1-3 g kg⁻¹), glucosinolates (<30 μmol g⁻¹) and erucic acid. There is
89 still not much information about its advantageous bioactive ingredients other than protein.
90 Therefore, a potential use of rapeseed cake extract in the treatment against AGEs
91 formation is investigated in this work.

92

93 **2. Materials & methods**

94 **2.1. Materials**

95 D(+)-Glucose (GLC), bovine serum albumin (BSA), methylglyoxal (MGO, 40% aqueous
96 solution), glyoxal (GO, 40% aqueous solution), aminoguanidine sulphate hydrate (AG),
97 sinapinic acid (SI), Quercetin (QE), 5-methylquinoxaline (5-MQ), nitrotetrazolium blue
98 chloride (NBT), o-phenylenediamine (OPD), heptafluorobutyric acid (HFBA), trolox (6-
99 hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,4,6-tris (2-pyridyl)-s-triazine
100 (TPTZ), 2-2'-azinobis(3-ethylbenzothiazoline- 6-sulphonic acid) diammonium salt
101 (ABTS), Folin– Ciocalteu and standards for phenolic compounds were provided by
102 Sigma-Aldrich (Dorset, UK). Iron (III) chloride was purchased from Panreac (Madrid,
103 Spain). Derivatising agents methoxyamine hydrochloride and N-methyl-N-

104 trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich (Dorset,
105 UK). Pyridine, sugars, amino acids, organic acids and polyphenols used for identification
106 and quantification were obtained from Sigma-Aldrich (Dorset, UK). Argpyrimidine
107 (ArgP) and pentosidine were obtained from PolyPeptide Laboratories (Strasbourg,
108 France). Cold pressed rapeseed cake (RC) from *Brassica napus* L var. *catana* was
109 provided by *Summer Harvest*© (Perthshire, UK) and stored at room temperature, inside
110 a sealed plastic bag.

111 **2.2 Extraction of rapeseed cake extract (RCext)**

112 The milled RC pellets were ground to a fine powder in a blender and stored at 4 °C until
113 further use. The samples were roasted at 180 °C in a Memmert (UM200) oven for 5 and
114 10 min and weighed to determine the water loss. This step was necessary in order to
115 denature the proteins and so that the material did not clog the filter in further steps. A
116 hot steam extraction was performed on roasted RC as described by Navarini *et al* (23).
117 Briefly, 15g of samples contained in a funnel-shaped filter, were extracted with the steam
118 produced by the water (75 ml) contained in an autoclave-type aluminium kettle heated by
119 an external source (on gas stove for 3 minutes), to force the same water upwards through
120 the roasted sample bed. The resulting solution was centrifuged at 1940g for 10 minutes
121 at 4°C (Joan CR231 centrifuge). Part of the supernatant was freeze-dried and used for
122 further analysis, and another part was spray-dried to evaluate the effect of spray-drying
123 on the total phenolic content and antioxidant activity. The spray-drying was performed
124 using a Büchi Mini Spray Dryer B-290 using the following conditions: inlet temperature
125 160°C, aspirator 100%, feeding pump 25%, without cleaning the nozzle and ensuring that
126 the maximum outlet temperature was always below 80 °C.

127 **2.3 Analysis of the RCext**

128 The roasted and unroasted RC and the RCext were analysed in terms of Total Phenolic
129 Content (TPC) and for their antioxidant activity (ABTS). While on the freeze-dried
130 extract FRAP assays (Ferric Reducing Ability of Plasma), metabolomic profile, anti-
131 glycative capacity, direct MGO trapping capacity, and argpyrimidine and pentosidine
132 determinations were evaluated.

133 **2.3.1 Total Phenolic Content (TPC) and antioxidant capacity**

134 The TPC was carried out using a Folin-Ciocalteu assay as previously described by
135 Singleton and Rossi (24). TPC was determined on samples at different roasting times (0,
136 5, and 10 min) and on spray dried and freeze-dried extract samples. The TPC levels are
137 expressed in gallic acid eq. g^{-1} (mg GAE g^{-1}) of dry matter (d.m.). Gallic acid was used
138 as a standard to prepare the calibration curve with a concentration range between 20 and
139 $200 \mu\text{g ml}^{-1}$. The solutions were measured at 760 nm using an UV/VIS-
140 spectrophotometer (Thermo Scientific Genesys 10S UV-Vis Spectrophotometer).

141 Antioxidant capacity was measured with an indirect method using 2,2-azino-bis-(3-
142 ethylbenzothiazoline-6-sulfonic acid (ABTS) reagent following the procedure of
143 Pellegrini *et al.* (25), with modifications as suggested in the studies of Ferracane *et al*
144 and Graziani *et al* (26,27). Results were expressed in mmol Trolox equivalent antioxidant
145 capacity (mM TEAC g^{-1}) and referred to a calibration curve ranging from 25 to $250 \mu\text{M}$.
146 All determinations were performed in triplicate.

147 The FRAP assay was carried out as previously described by Benzie *et al.* (28) with minor
148 modifications. Standards of Ferrous sulphate heptahydrate were prepared at different
149 concentrations (0.1, 0.4, 0.8 and 1 mM). The RCext was dissolved in water before
150 analysis. The FRAP reagent solution was made by mixing 100ml acetate buffer (300mM;
151 pH 3.6; stored at 4°C) with 10ml ferric chloride solution, 10ml TPTZ solution (solution
152 brought up to 10ml with HCl and stirred on heated plate at maximum 50°C before use)
153 and 12ml distilled water and incubated in a water bath before use. The FRAP solution
154 (1ml) was mixed with $30\mu\text{l}$ of each sample and incubated at room temperature for 4
155 minutes. The sample was poured inside a Fischerbrand® UV semi-micro cuvette and
156 absorbance was read at 593 nm. The FRAP reducing capacity of Fe^{2+} production was
157 calculated by the formula:

$$158 \quad FRAP = p \times (abs - b) / a$$

159 where: p = dilution factor of original sample, abs = absorbance of sample at 593 nm, a =
160 slope of the curve and b = intercept from the curve.

161 **2.3.3 GC-MS analysis**

162 Rapeseed cake extracts were derivatized prior to GC-MS analysis in order to obtain
163 volatile and stable compounds. The analysis was carried out according to de Falco *et al.*

164 (29). For this purpose, an aliquot of diluted sample was evaporated to dryness in a vacuum
165 centrifuge (Eppendorf Concentrator 5301) and oxymated with 50 μl of methoxyamine
166 hydrochloride (20 mg mL^{-1}) in pyridine at 60°C for 45 min. The active hydrogens, such as
167 OH, SH and NH, were silylated with MSTFA at 60°C for 45 min. Analyses were
168 performed in triplicate to reduce the possible variability of samples due to extraction,
169 derivatization and injection. Each derivatized sample was injected in a pulsed splitless
170 mode into an Agilent-7820A GC system with 5977E MSD operating in EI mode at 70
171 eV. The system was equipped with a 30 m x 0.25 mm id fused-silica capillary column
172 with 0.25 μm HP-5MS stationary phase (Agilent technologies, UK). One μl of each
173 sample was injected and the temperature was set at 270°C. Helium was used as carrier
174 gas at a constant flow rate of 1 mL min^{-1} . Separation of metabolites was achieved under
175 the following temperature program: 2 min of isothermal heating at 70°C, followed by a
176 10°C min^{-1} oven temperature ramp to 320°C, and a final 2 min heating at 320°C. Spectra
177 were recorded in the mass range 50 to 800 m/z . MassHunter Qualitative Analysis B.07.00
178 software was used to analyse both chromatograms and mass spectra. The identification
179 was achieved by comparison with standard compounds and with spectra in National
180 Institute of Standard and Technologies library NIST MS Search 2.2. Data were processed
181 with the AMDIS software to deconvolute co-eluting peaks. Artefact peaks, such as peaks
182 due to derivatising agents, were not considered in the final analysis. The quantification of
183 separated metabolites was calculated from Total Ion Chromatography (TIC) by the
184 computerized integrator and with standard compounds.

185 **2.3.4 Anti-Glycation Activity of Polyphenols by *in vitro* glycation assay with bovine** 186 **serum albumin (BSA) induced by methylglyoxal (MGO) and glucose (GLC).**

187 The glycation models of BSA with MGO (BSA-MGO assay), and GLC (BSA-GLC
188 assay) were carried out as described by Mesías *et al.* (30) with slight modifications. The
189 range of concentrations of RCext and pure sinapinic acid were from 0.14 to 3.57, and 0.04
190 to 0.77 mg mL^{-1} , respectively. Aminoguanidine (0.07 to 0.14 mg mL^{-1}) was used as
191 positive control of the inhibition for the formation of fluorescent AGEs, and quercetin
192 (0.07 to 0.14 mg mL^{-1}) as the polyphenol of reference. The concentrations of the reactants
193 were 0.23 mg mL^{-1} for MGO, 100 mg mL^{-1} for GLC and 10 mg mL^{-1} for BSA. Briefly,
194 the mixtures (blank, control, or sample) were incubated at 37 °C for 14 days (BSA-MGO
195 assay) or 21 days (BSA-GLC assay) in a PBS buffer (100 mM, pH 7.4). An aliquot was
196 taken just before incubation and stored in at -80 °C as a control to establish the increase

197 in fluorescence. Fluorescence (n = 4) was measured in a microplate reader (Synergy™
198 HT-multimode Biotek, VT, USA) with temperature control. In parallel, samples of RCext
199 and sinapinic acid were incubated at 37 °C for 14 days or 21 days in order to establish
200 their intrinsic fluorescence, if any. The formation of AGEs was monitored by
201 fluorescence with excitation/emission at 340/420 nm for BSA-MGO or BSA-GO and
202 360/420 nm for BSA-GLC. Results were expressed as a percentage of inhibition of AGE
203 formation, and it was calculated according to the following equation:

$$204 \quad \text{AGE Inhibition (\%)} = 1 - [(FS - FS_0) / F_0] \times 100$$

205 where: FS = fluorescence of the system with sample, FS₀ = intrinsic fluorescence of
206 sample, F₀ = fluorescence of the system without sample.

207 The concentration required to inhibit glycation by 50% (IC₅₀) was calculated from the
208 dose–response curve using the Microsoft-Excel computer software package.

209 **2.3.5 Evaluation of direct MGO trapping capacity by HPLC-DAD.**

210 Direct MGO trapping capacity was determined as described by Mesias et al. (30) after
211 MGO conversion into the respective quinoxaline derivative (2-MQ). The range of
212 concentrations of RCext and sinapinic acid was 0.1–2.5 mg mL⁻¹ and 0.03–0.54 mg mL⁻¹,
213 respectively. The incubation was carried out at 37 °C for 168 h in PBS (100 mM, pH 7.4).
214 Remaining MGO as its quinoxaline derivative was determined by HPLC-DAD.
215 Chromatograms were recorded at 315 nm with the retention times of 2-MQ, and 5-MQ,
216 4.1, and 5.6 min, respectively. The amount of unreacted MGO was calculated from the
217 ratio of 2-MQ and 5-MQ (internal standard) as compared with the control. The percentage
218 of inhibition of MGO was calculated with the following formula:

$$219 \quad \text{MGO inhibition (\%)} = [(CTL - S) / CTL] \times 100$$

220 where: CTL = amount of MGO in control, S = amount of MGO in sample with the target
221 compound for evaluation,

222 The concentration required to trap MGO by 50% (IC₅₀) was obtained from the dose–
223 response curves using the Microsoft-Excel computer software package.

224 **2.3.6 Determination of argpyrimidine (ArgP) by HPLC-fluorescence.**

225 The determination of ArgP formation was carried out as described by Navarro & Morales
226 (31) after acid hydrolysis (6 M HCl for 20 h at 110°C) of the sample treated in the BSA-
227 MGO glycation assay. Samples (10µL) were eluted onto a Mediterranean-Sea-ODS2
228 column (250 mm × 4 mm, 5 µm; Teknokroma, Barcelona, Spain) at a flow rate of 0.8 mL
229 min⁻¹. The mobile phase consisted of HFBA (1 mL L⁻¹) (solvent A) and Acetonitrile
230 (ACN) (500 mL L⁻¹) containing HFBA (1 mL L⁻¹) (solvent B) using the following
231 gradient elution: 0 min, 20% B; 25 min, 100% B; 26 min, 100% B; 27–37 min, 20% B.
232 ArgP was detected at 335 nm and 385 nm as the excitation and emission wavelengths,
233 respectively, and eluted at 15.1 min. Calibration was carried out in the range 0.01–0.5 µg
234 mL⁻¹ with a pure standard. The analysis was carried out on a Prominence HPLC system
235 with an RF-20A XS fluorescence detector (Shimadzu Corporation, Kyoto, Japan).

236 **2.3.7 Determination of pentosidine by HPLC-fluorescence**

237 Pentosidine was determined in samples from the BSA-Glucose glycation assay following
238 the HPLC method described by Wilker, Chellan, Arnold, and Nagaraj (32) with minor
239 modifications. Samples were incubated with 1.5 mL of sodium borate (0.2 M, pH 9.2)
240 and 1 mL of sodium borohydride (1 M in 0.1 M NaOH) for 4 h at room temperature.
241 Then, samples were hydrolysed with 5 mL of HCl (final concentration of 6 M/20 h/110°C)
242 and 500 µL was dried in a Speed-Vac concentrator (ThermoFisher Scientific,
243 Courtaboeuf, France). The residue was reconstituted in 1 mL of formic acid (1 mL L⁻¹)
244 and the pentosidine content of the samples was measured by HPLC-fluorescence.
245 Samples (10 µL) were eluted onto a Mediterranean-Sea-ODS2 column (250 mm x 4 mm,
246 5 µm; Teknokroma, Barcelona, Spain) at a flow rate of 0.8 mL min⁻¹ under gradient
247 elution. The mobile phase consisted of HFBA (1 mL L⁻¹) (solvent A) and acetonitrile
248 (500 mL L⁻¹) containing HFBA (1 mL L⁻¹) as solvent B and the applied gradient program
249 was as follows: 0 min, 20% B; 25 min, 100% B; 26 min, 100% B; and 27–37 min, 20%
250 B. Pentosidine was detected at excitation and emission wavelengths of 335 and 385 nm
251 respectively, and eluted at 15.9 min. Calibration was carried out in the range 0.0005–0.05
252 µg mL⁻¹ with a pure standard.

253 **Statistical analysis**

254 Data were analysed by ANOVA using XLStat (version 2014.5.03, Addinsoft, NY). All
255 the results were expressed as mean ± SD. Significant differences between the samples

256 with a confidence interval of 95% were performed by using the Tukey test. The average
257 was calculated using the results of the thermal treatment replicates and the technical
258 replicates (at least four observations per sample).

259 **Results and discussion**

260 **Total Phenolic Content and Antioxidant capacity**

261 The content of total phenols in RC unroasted and roasted, and in RCext freeze-dried and
262 spray-dried samples is shown in Table A. Results indicated no significant differences
263 between the 5 and 10 minutes of roasting time compared to the unroasted sample.
264 However, 5 minutes of roasting were needed in order to guarantee a mild protein
265 degradation for the further extraction. A significant difference was observed between
266 freeze-dried extracts and spray-dried extracts; with the freeze-dried extract showing a
267 higher concentration of polyphenol equivalent content. This could be explained by the
268 thermal treatment that the product undergoes during the spray drying process. For this
269 reason, the freeze-dried extract was selected for the anti-glycative activity and FRAP
270 measurements.

271 The antioxidant capacity of the samples was determined with the ABTS assay and the
272 results are expressed as mM TEAC g⁻¹. The ABTS did not show any significant decrease
273 of antioxidant capacity after 10 minutes roasting (Table A). However, the freeze-dried
274 extracts and the spray-dried extracts showed the same antioxidant capacity (241.65 b ±
275 0.43 and 240.94 b ± 2.01 mM TEAC g⁻¹). The FRAP assay was determined on the freeze-
276 dried sample only because, although there was not an important loss in antioxidant
277 activity, a significant difference was found between freeze-dried and spray-dried sample
278 for TPC. Result showed FRAP activity of 569.87 ± 7.35 Fe²⁺ eq mmol g⁻¹ d.m.

279 **GC-MS analysis**

280 Rapeseed cake extract was analysed by using gas chromatography coupled with mass
281 spectrometry. The detected compounds, including fatty acids, amino acids,
282 carbohydrates, organic acids and phenols, are listed in Table B with their retention time
283 and *m/z* values. The total ion chromatogram of one replicate is reported in Figure 1. All
284 metabolites were detected as trimethylsilyl derivatives by using standard compounds as
285 references. All mass spectra were characterized by a base peak at *m/z* 73 due to [(CH₃)₃Si]

286 group. According to other reports, sugars are present as main metabolites (Figure 2) and
287 sucrose (Sucr) is the most abundant one (33). Other sugars identified were sorbose (Sorb),
288 tagatose (Tag) and galactose (Gal), present in very similar amount in the rapeseed extracts
289 (0.091; 0.090; 0.088 mg g⁻¹ respectively), while mannitol (Man) was present in higher
290 amounts (0.389 mg g⁻¹). Lactic acid is the major organic acid identified, followed by
291 benzoic acid. Sinapinic acid was identified in the metabolic profile of RCext as sinapinic
292 acid 2 trimethylsilyl derivative (2TMS), and the quantitative analysis showed high
293 amount of this compound (0.108 mg g⁻¹). The main fatty acids detected were palmitic
294 (PA) and stearic acid (ST), with a base peak at m/z 313 and 341 respectively (Table B)
295 due to the loss of a methyl group from the TMS ester group.

296 **Glycation**

297 The anti-glycative activity of the rapeseed cake extract and standards (sinapinic acid,
298 aminoguanidine, and quercetin) is summarized in Table C. Accumulation of AGE-
299 specific fluorescence applied in this investigation is a general measure to assess the
300 protein glycation damage (34). The reaction was carried out at moderate conditions for
301 14 or 21 days of incubation at 37 °C for BSA–MGO and BSA–GLC systems,
302 respectively, in order to avoid any potential formation of artefacts when the reaction is
303 speeded at higher temperature and shortest time. The reaction proceeded slower in the
304 BSA-GLC since the conversion of glucose is necessary for its open-chain form and the
305 parallel reaction of autoxidation to generate reactive dicarbonyls. In addition, the intrinsic
306 fluorescence of samples was residual, accounting for <5 % of the total fluorescence in the
307 glycation models. The anti-glycative capacity of samples was initially assayed in a range
308 from 0.5 to 5 mg mL⁻¹. However, due to the limited solubility of the RCext, the maximum
309 effective concentration was set at 3.57 mg mL⁻¹, and the minimum effective
310 concentration of sinapinic acid was set at 0.04 mg mL⁻¹ due to its high activity. Rapeseed
311 cake extract and sinapinic acid exerted a dose dependent anti-glycation activity in BSA–
312 MGO and BSA–GLC systems in the concentration range studied (Table C). The
313 concentration required to inhibit glycation by 50% (IC₅₀) in the BSA-GLC was 0.36 and
314 0.16 mg mL⁻¹ for rapeseed cake extract and sinapinic acid, respectively. The anti-
315 glycative activity for the BSA-GLC system was nearly double for the sinapinic acid as
316 compared with the rapeseed cake extract, but it was in the same order of magnitude.
317 However, the anti-glycative activity of sinapinic acid in the BSA-MGO system was
318 nearly sixteen times more effective than the RCext with values of 0.09 and 1.45 mg mL⁻¹,

319 respectively. The anti-glycative activity of sinapinic acid occurs mainly through the
320 trapping of MGO which significantly reduces the formation of fluorescent AGEs in the
321 BSA-MGO system. However, the rapeseed cake extract did not show a similar response
322 against MGO. In both glycation models, the activity of sinapinic acid was similar to that
323 of aminoguanidine which is used as reference, being 0.15 and 0.11 mg mL⁻¹ for the BSA-
324 GLC and BSA-MGO system, respectively.

325 In addition, the tentative sinapinic acid content of the RCext at the IC₅₀ concentration was
326 calculated to 0.157 µg mL⁻¹. However, this value was lower than the IC₅₀ obtained for
327 the anti-glycative activity of pure sinapinic acid (89 µg mL⁻¹) in the BSA-MGO system
328 (Table C). To gain more insight into this result, the specific MGO trapping ability of
329 rapeseed cake extract and sinapinic acid was evaluated in the range of concentration of
330 0.1 to 2.5 mg mL⁻¹. However, the lowest and highest effective concentration assayed for
331 sinapinic acid was decreased to 0.03–0.54 mg mL⁻¹, and rapeseed cake extract to 0.01
332 due to its high reactivity. The IC₅₀ was <0.10 and 0.095 mg mL⁻¹ for rapeseed cake extract
333 and sinapinic acid for MGO trapping (Table C). This result confirms the MGO trapping
334 capacity of both samples at simulated physiological conditions. However, the high
335 reactivity of rapeseed cake extract against MGO does not agree with the moderate
336 response of the extract in the BSA-MGO system as compared with sinapinic acid.

337 Pentosidine is a protein-crosslinking fluorescent AGE that is formed in reactions of
338 pentose with lysine and arginine, but also formed from glucose, fructose, 3-
339 deoxyglycosone or Amadori compounds, through an oxidative route (35). Rapeseed cake
340 extract reduced up to 34.85% (at the concentration of 3.57 mg mL⁻¹) the formation of
341 pentosidine. Similar results were obtained for sinapinic acid, in that it was able to mitigate
342 up to 47.09% the formation of pentosidine at the concentration of 0.77 mg mL⁻¹ (Table
343 D). These results are in line with those obtained for the BSA-GLC system, where both
344 the extract and sinapinic acid exerted anti-glycation activity. However, the investigation
345 on ArgP reveals the specific effectivity of sinapinic acid for trapping MGO. ArgP is a
346 fluorescent AGE produced from the reaction between two molecules of MGO and
347 arginine (36). ArgP was measured in the BSA-MGO system incubated with rapeseed cake
348 extract and sinapinic acid in the range of 0.14-3.57 and 0.39-0.77 mg mL⁻¹, respectively.
349 Inhibition of ArgP (<5%) by the rapeseed cake extract was not detected, but sinapinic
350 acid (0.77 mg mL⁻¹) already reached an inhibition of 18.3% (Table D). These results

351 confirm the preference of sinapinic acid to MGO that could explain its anti-glycative
352 activity.

353 In contrast, the anti-glycative activity exerted by rapeseed cake extract is not only due to
354 the MGO-trapping capacity but other variables are should be further considered. The
355 coexistence of anti- and pro-glycative compounds in the extract was observed from the
356 GC-MS analysis. Sorbose and tagatose are reducing monosaccharides which differ in the
357 position of hydroxyl groups, could participate in the glycation as promoters. However,
358 they have had a great deal of attention in recent years due to their health benefits and
359 similar properties to sucrose. In particular, the sweetness of tagatose is 92% that of
360 sucrose but it has no glycaemic effect (37). Later on, Lu *et al.* (38) also demonstrated that
361 the increase of blood glucose after the ingestion of sucrose is reduced by tagatose. In
362 addition, a more recent study showed that sorbose could be used as a sweetener to
363 suppress the postprandial elevation of blood levels of glucose and insulin and for the
364 prevention of lifestyle-related diseases, such as type 2 diabetes mellitus (39).

365 **Conclusion**

366 The research investigated the anti-glycative effect of a freeze-dried rapeseed cake extract
367 compared to sinapinic acid, the major phenolic compound in the extract. The use of spray-
368 drying as alternative processing to freeze-dried was also considered, but results did not
369 show a significant difference in the antioxidant capacity, except in the TPC. The content
370 of sinapinic acid in the rapeseed cake obtained by freeze-drying was higher than those
371 obtained by spray-drying. The sinapinic acid exerted a positive effect for trapping of
372 MGO in both BSA–MGO and BSA–GLC glycation systems. Interestingly, the sinapinic
373 acid showed a specific activity against protein crosslinking by the inhibition of the
374 formation of pentosidine. However, the results for the anti-glycative activity of the RCext
375 were contradictory. Surprisingly, the RCext showed an excellent MGO trapping capacity
376 in the BSA-GLC glycation system, but the MGO trapping activity was moderate in the
377 BSA-MGO glycation model. The extract showed better results for the BSA-GLC
378 glycation model than BSA-MGO where the RCext did not display an activity directly
379 proportional to the excellent MGO trapping capacity. The RCext and sinapinic acid
380 exerted a promising inhibitory activity against the formation of pentosidine, but only at
381 the highest concentrations tested. In conclusion, this product could have a potential
382 application as a food supplement in the mitigation of AGEs, but further investigation will

383 be necessary on the removal of the pro-glycative compounds as highlighted from the GC-
384 MS analysis.

385

386 **Conflict of interest**

387 Authors declare no conflict of interest

388

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396

397 **Abbreviations**

398

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500 blood levels of glucose and insulin in the rat. Nutr Res 2014;34(11):961-967.

501

502 **Table A.** Results of total polyphenol content and antioxidant capacity on unroasted
503 rapeseed cake and on freeze-dried and spray-dried extract.

Samples	TPC mg GAE g⁻¹	ABTS mM TEAC g⁻¹	FRAP Fe²⁺eq mmol g⁻¹
Unroasted	14.48 ^a ± 0.39	95.10 ^a ± 12.42	
5' roasting	14.01 ^a ± 1.22	89.98 ^a ± 8.52	
10' roasting	14.20 ^a ± 1.05	78.74 ^a ± 5.43	
RCext Freeze dried	86.85 ^c ± 2.08	241.65 ^b ± 0.43	569.87 ± 7.35
RCext Spray dried	73.85 ^b ± 0.64	240.94 ^b ± 2.01	

504 Significant differences were determined by ANOVA analysis and Tukey test ($p \leq 0.05$). Different letters
505 indicate significant differences.

506

507 **Table B.** Metabolites detected by GC-MS in the rapeseed cake extract with their,
 508 abbreviation (Abb.) retention time (RT), empirical formula, mass-to-charge (*m/z*), and
 509 concentration (mg/g).

510

	Metabolite	Abb.	RT	Formula	<i>m/z</i>	mg/g
1	Lactic Acid, 2TMS	LA	7.25	C ₉ H ₂₂ O ₃ Si ₂	219, 191, 147, 133, 117, 73, 45	0.139±0.001
2	Benzoic Acid, TMS	BE	10.02	C ₁₀ H ₁₄ O ₂ Si	194, 179, 135, 105, 77	0.046±0.0007
3	Glycerol, 3TMS	GLY C	10.46	C ₁₂ H ₃₂ O ₃ Si ₃	218, 205, 147, 117, 89, 73, 45	0.028±0.0006
4	Levoglucoano, 3TMS	Levo	16.37	C ₁₅ H ₃₄ O ₅ Si ₃	333, 217, 204, 147, 129, 103, 73	0.047±0.001
5	Shikimic acid	Sh	17.48	C ₁₉ H ₄₂ O ₅ Si ₄	462, 255, 204, 147, 73	0.023±0.0008
6	Tagatose, MEOX	Tag	18.38	C ₂₂ H ₅₅ NO ₆ Si ₅	307, 217, 147, 103, 73	0.090±0.0007
7	Sorbose, MEOX	Sorb	18.47	C ₂₂ H ₅₅ NO ₆ Si ₅	307, 217, 147, 103, 73	0.091±0.0007
8	Galactose, MEOX	Gal	18.68	C ₂₂ H ₅₅ NO ₆ Si ₅	319, 205, 147, 103, 73	0.088±0.0009
9	Mannitol, 6TMS	Man	19.12	C ₂₄ H ₆₂ O ₆ Si ₆	421, 345, 319, 205, 147, 103, 73	0.389±0.0045
10	Palmitic Acid, TMS	PA	19.87	C ₁₉ H ₄₀ O ₂ Si	328, 313, 145, 117, 73	0.372±0.0007
11	Myo-Inositol, 6TMS	Myo	20.70	C ₂₄ H ₆₀ O ₆ Si ₆	305, 217, 147, 129, 73	0.261±0.007
12	Stearic acid, TMS	ST	21.79	C ₂₁ H ₄₄ O ₂ Si	356, 341, 132, 117, 73	0.521±0.0067
13	Sinapinic acid, 2TMS	SI	21.90	C ₁₇ H ₂₈ O ₅ Si ₂	368, 353, 338, 323, 279, 249, 73	0.108±0.0043
14	Sucrose, 8TMS	Sucr	2.56	C ₃₆ H ₈₆ O ₁₁ Si ₈	437, 361, 319, 271, 217, 147, 103, 73	9.176±0.149

511 *TMS trimethylsilyl derivative

512 **MEOX methyloxime

513

514

515 **Table C.** Summary of the antiglycative activity of rapeseed cake extracts (RCext),
 516 sinapinic acid, controls (aminoguanidine and quercetin) in the glycation assays (BSA-
 517 Glucose, and BSA-Methylglyoxal), and the trapping for MGO at a fixed time reaction of
 518 168h at 37°C.

Anti-glycative activity	IC₅₀ (mg mL⁻¹)	range (mg mL⁻¹)
<i>BSA-GLC glycation assay</i>		
RCext	0.36 ^c ±0.053	0.14 – 3.57
sinapinic acid	0.156 ^b ±0.006	0.04 – 7.71
aminoguanidine	0.149 ^b ±0.030	
quercetin	0.054 ^a ±0.001	
<i>BSA-MGO glycation assay</i>		
RCext	1.454 ^B ±0.269	0.14 – 3.57
sinapinic acid	0.089 ^A ±0.006	0.04 – 7.71
aminoguanidine	0.106 ^A ±0.007	
quercetin	0.058 ^A ±0.001	
<i>MGO trapping</i>		
RCext	< 0.1*	0.1 – 2.5
sinapinic acid	0.095±0.001	0.027 – 0.54

519 * Lower concentration saturates the system

520 Different lower case letters in the results of BSA-GLC glycation assay and capital letters in the results of
 521 BSA-MGO glycation assay denote significant differences (p < 0.05).

522

523

524 **Table D.** Summary of determination of argpyrimidine and determination of pentosidine
 525 on rapeseed cake extract (RCext), sinapinic acid, and controls (aminoguanidine,
 526 quercetin).

	Inhibition (%)	Conc. (mg mL⁻¹)
<i>Argpyrimidine</i>		
RCext	*	1.43
	*	3.57
sinapinic acid	5.90 ^a ±4.27	0.39
	18.30 ^b ±6.06	0.77
aminoguanidine	41.70 ^c ±0.22	0.14
quercetin	24.60 ^b ±2.79	0.14
<i>Pentosidine</i>		
RCext	6.26 ^A ±5.26	1.43
	34.85 ^{BC} ±8.27	3.57
sinapinic acid	22.43 ^{AB} ±3.54	0.39
	47.09 ^C ±3.43	0.77
aminoguanidine	10.00 ^A ±0.64	0.14
quercetin	5.15 ^A ±1.08	0.14

527 * not observed. Inhibition < 5%

528 Different lower case letters in the results of inhibition of Argpyrimidine formation and capital letters in the
 529 results of inhibition of Pentosidine formation denote significant differences (p < 0.05). Sinapinic acid
 530 content of 0.154 and 0.386 µg mL⁻¹ in the RCext of 1.43 and 3.57 mg mL⁻¹, respectively.

531

532 **Figure 1.** Total Ion Chromatogram (TIC) of rapeseed cake extract with detected
533 metabolites. Numbers of metabolites as described in table B.

534

535 ~~**Figure 2.** Quantitative determination of detected metabolites expressed as mg g^{-1} of~~
536 ~~rapeseed extract. The abbreviation of each compounds is reported in Table B.~~

537

Table A. Results of total polyphenol content and antioxidant capacity on crude rapeseed cake and on freeze-dried and spray dried extract

Samples	TPC mg GAE g⁻¹	ABTS mM TEAC g⁻¹	FRAP Fe²⁺eq mmol g⁻¹
Unroasted	14.48 ^a ± 0.39	95.10 ^a ± 12.42	
5' roasting	14.01 ^a ± 1.22	89.98 ^a ± 8.52	
10' roasting	14.20 ^a ± 1.05	78.74 ^a ± 5.43	
RCext Freeze dried	86.85 ^c ± 2.08	241.65 ^b ± 0.43	569.87 ± 7.35
RCext Spray dried	73.85 ^b ± 0.64	240.94 ^b ± 2.01	

Significant differences were determined by ANOVA analysis and Tukey test ($p \leq 0.05$). Different letters indicate significant differences.

Table B. Metabolites detected by GC-MS in the rapeseed cake extract with their retention time.

Metabolite	Abbreviation	RT	Formula	m/z	mg/g
1 Lactic Acid, 2TMS	LA	7.25	C ₉ H ₂₂ O ₃ Si ₂	219, 191, 147, 133, 117, 73, 45	0.139±0.001
2 Benzoic Acid, TMS	BE	10.02	C ₁₀ H ₁₄ O ₂ Si	194, 179, 135, 105, 77	0.046±0.0007
3 Glycerol, 3TMS	GLYC	10.46	C ₁₂ H ₃₂ O ₃ Si ₃	218, 205, 147, 117, 89, 73, 45	0.028±0.0006
4 Levoglucosano, 3TMS	Levo	16.37	C ₁₅ H ₃₄ O ₅ Si ₃	333, 217, 204, 147, 129, 103, 73	0.047±0.001
5 Shikimic acid	Sh	17.48	C ₁₉ H ₄₂ O ₅ Si ₄	462, 255, 204, 147, 73	0.023±0.0008
6 Tagatose, MEOX	Tag	18.38	C ₂₂ H ₅₅ NO ₆ Si ₅	307, 217, 147, 103, 73	0.090±0.0007
7 Sorbose, MEOX	Sorb	18.47	C ₂₂ H ₅₅ NO ₆ Si ₅	307, 217, 147, 103, 73	0.091±0.0007
8 Galactose, MEOX	Gal	18.68	C ₂₂ H ₅₅ NO ₆ Si ₅	319, 205, 147, 103, 73	0.088±0.0009
9 Mannitol, 6TMS	Man	19.12	C ₂₄ H ₆₂ O ₆ Si ₆	421, 345, 319, 205, 147, 103, 73	0.389±0.0045
10 Palmitic Acid, TMS	PA	19.87	C ₁₉ H ₄₀ O ₂ Si	328, 313, 145, 117, 73	0.372±0.0007
11 Myo-Inositol, 6TMS	Myo	20.70	C ₂₄ H ₆₀ O ₆ Si ₆	305, 217, 147, 129, 73	0.261±0.007
12 Stearic acid, TMS	ST	21.79	C ₂₁ H ₄₄ O ₂ Si	356, 341, 132, 117, 73	0.521±0.0067
13 Sinapinic acid, 2TMS	SI	21.90	C ₁₇ H ₂₈ O ₅ Si ₂	368, 353, 338, 323, 279, 249, 73	0.108±0.0043
14 Sucrose, 8TMS	Sucr	2.56	C ₃₆ H ₈₆ O ₁₁ Si ₈	437, 361, 319, 271, 217, 147, 103, 73	9.176±0.149

*TMS trimethylsilyl derivative

**MEOX methyloxime

Table C. Summary of Glycation (BSA-GLUCOSE, and BSA-Methylglyoxal), trapping for MGO at a fixed time reaction of 168h at 37°C on rapeseed cake extracts and controls

Anti-glycative activity	IC₅₀ (mg mL⁻¹)	range (mg mL⁻¹)
<i>BSA-GLC glycation assay</i>		
RCext	0.36 ^c ±0.053	0.14 – 3.57
sinapinic acid	0.156 ^b ±0.006	0.04 – 7.71
aminoguanidine	0.149 ^b ±0.030	
quercetin	0.054 ^a ±0.001	
<i>BSA-MGO glycation assay</i>		
RCext	1.454 ^B ±0.269	0.14 – 3.57
sinapinic acid	0.089 ^A ±0.006	0.04 – 7.71
aminoguanidine	0.106 ^A ±0.007	
quercetin	0.058 ^A ±0.001	
<i>MGO trapping</i>		
RCext	< 0.1*	0.1 – 2.5
sinapinic acid	0.095±0.001	0.027 – 0.54

* Lower concentration saturates the system

Different lower case letters in the results of BSA-GLC glycation assay and capital letters in the results of BSA-MGO glycation assay denote significant differences ($p < 0.05$).

Table D. Summary of determination of argpyrimidine and determination of pentosidine on RCext and controls

	Inhibition (%)	Conc. (mg mL⁻¹)
<i>Argpyrimidine</i>		
RCext	*	1.43
	*	3.57
sinapinic acid	5.90 ^a ±4.27	0.39
	18.30 ^b ±6.06	0.77
aminoguanidine	41.70 ^c ±0.22	0.14
quercetin	24.60 ^b ±2.79	0.14
<i>Pentosidine</i>		
RCext	6.26 ^A ±5.26	1.43
	34.85 ^{BC} ±8.27	3.57
sinapinic acid	22.43 ^{AB} ±3.54	0.39
	47.09 ^C ±3.43	0.77
aminoguanidine	10.00 ^A ±0.64	0.14
quercetin	5.15 ^A ±1.08	0.14

* not observed. Inhibition < 5%

Different lower case letters in the results of inhibition of Argpyrimidine formation and capital letters in the results of inhibition of Pentosidine formation denote significant differences ($p < 0.05$).

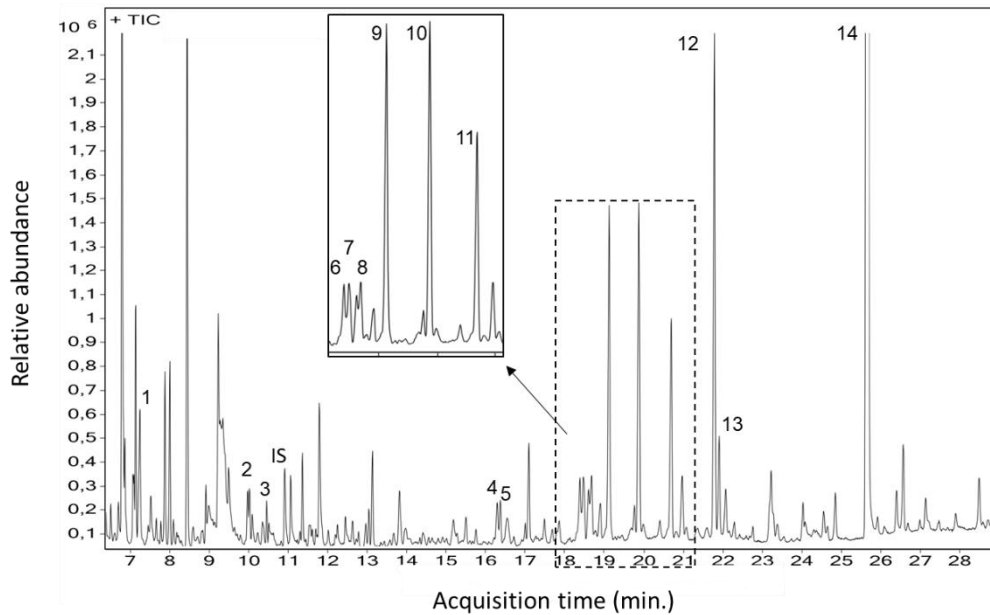


Figure 1. Total Ion Chromatogram (TIC) of rapeseed cake extract with detected metabolites.