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

Marta Navarro
Bruna de Falco
Francisco J. Morales
Despoina Daliani
Alberto Fiore

This is the accepted manuscript of an article published in Food & Function 2018

The published article is available from doi: 10.1039/C8FO01408F

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

Marta Navarro[a†], Bruna de Falco[b†], Francisco J. Morales[a], Despoina Daliani[b], Alberto Fiore[b*]

[a] Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), Madrid, Spain. E-mail: fjmorales@ictan.csic.es;

[b] School of Science, Engineering & Technology, Division of Food & Drink, University of Abertay, Bell Street, DD1 1HG Dundee, Scotland, United Kingdom E-mail: b.de-falco@abertay.ac.uk, a.fiore@abertay.ac.uk

* Correspondence to: Dr Alberto Fiore

[†]Shared first authorship

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

Abstract

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

1. **Introduction**

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

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

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

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

2. Materials & methods

2.1. Materials

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

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

2.2 Extraction of rapeseed cake extract (RCext)

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

2.3 Analysis of the RCext

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

2.3.1 Total Phenolic Content (TPC) and antioxidant capacity

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

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

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

$$FRAP = p\ x\ (abs–b)/a$$

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

2.3.3 GC-MS analysis

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

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

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

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

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

$$\text{AGE Inhibition (\%)} = 1 - [[(FS - FS0) / F0] \times 100]$$

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

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

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

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

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

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

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

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

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

2.3.7 Determination of pentosidine by HPLC-fluorescence

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

Statistical analysis

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

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

Results and discussion

Total Phenolic Content and Antioxidant capacity

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

The antioxidant capacity of the samples was determined with the ABTS assay and the results are expressed as mM TEAC g^{-1}. The ABTS did not show any significant decrease of antioxidant capacity after 10 minutes roasting (Table A). However, the freeze-dried extracts and the spray-dried extracts showed the same antioxidant capacity (241.65 b ± 0.43 and 240.94 b ± 2.01 mM TEAC g^{-1}). The FRAP assay was determined on the freeze-dried sample only because, although there was not an important loss in antioxidant activity, a significant difference was found between freeze-dried and spray-dried sample for TPC. Result showed FRAP activity of 569.87 ± 7.35 Fe^{2+} eq mmol g^{-1} d.m.

GC-MS analysis

Rapeseed cake extract was analysed by using gas chromatography coupled with mass spectrometry. The detected compounds, including fatty acids, amino acids, carbohydrates, organic acids and phenols, are listed in Table B with their retention time and *m/z* values. The total ion chromatogram of one replicate is reported in Figure 1. All metabolites were detected as trimethylsilyl derivatives by using standard compounds as references. All mass spectra were characterized by a base peak at *m/z* 73 due to $[(CH_3)_3Si]$

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

Glycation

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

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

In addition, the tentative sinapinic acid content of the RCext at the IC_{50} concentration was calculated to 0.157 µg mL^{-1}. However, this value was lower than the IC_{50} obtained for the anti-glycative activity of pure sinapinic acid (89 µg mL^{-1}) in the BSA-MGO system (Table C). To gain more insight into this result, the specific MGO trapping ability of rapeseed cake extract and sinapinic acid was evaluated in the range of concentration of 0.1 to 2.5 mg mL^{-1}. However, the lowest and highest effective concentration assayed for sinapinic acid was decreased to 0.03–0.54 mg mL^{-1}, and rapeseed cake extract to 0.01 due to its high reactivity. The IC_{50} was <0.10 and 0.095 mg mL^{-1} for rapeseed cake extract and sinapinic acid for MGO trapping (Table C). This result confirms the MGO trapping capacity of both samples at simulated physiological conditions. However, the high reactivity of rapeseed cake extract against MGO does not agree with the moderate response of the extract in the BSA-MGO system as compared with sinapinic acid.

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

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

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

Conclusion

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

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

Conflict of interest

Authors declare no conflict of interest

Acknowledgements

The authors thanks Summer Harvest ltd for supplying rapeseed by-product. We thank Morag Steele, Abertay University, for the English language revision of manuscript.

This work was funded by projects S2013/ABI-3028-AVANSECAL from Comunidad of Madrid and European funding from FEDER program (European Regional Development Fund). M. Navarro received grants from the JAE program (Spanish National Research Council).

Abbreviations

Bibliographic references

(1) Rasheed Z, Prasad I, Ansari NA. Advanced glycation end products (AGEs) damaged IgG, a target for circulating autoantibodies in patients with type 1 diabetes mellitus. Open Glycoscience 2009;2(1).

(2) Bourne RR, Stevens GA, White RA, Smith JL, Flaxman SR, Price H, et al. Causes of vision loss worldwide, 1990–2010: a systematic analysis. The Lancet Global Health 2013;1(6):e339-e349.

(3) Kusirisin W, Srichairatanakool S, Lerttrakarnnon P, Lailerd N, Suttajit M, Jaikang C, et al. Antioxidative activity, polyphenolic content and anti-glycation effect of some Thai medicinal plants traditionally used in diabetic patients. Medicinal Chemistry 2009;5(2):139-147.

(4) WHO. Global report on diabetes. Geneva: World Health Organization 2016:20-74.

(5) Mooyaart AL. Genetic associations in diabetic nephropathy. Clinical and experimental nephrology 2014;18(2):197-200.

(6) Van Boekel MAJS. Effect of heating on Maillard reactions in milk. Food Chem 1998;62(4):403-414.

(7) Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res 2004 Sep 1;63(4):582-592.

(8) Uribarri J, del Castillo MD, de la Maza, María Pía, Filip R, Gugliucci A, Luevano-Contreras C, et al. Dietary advanced glycation end products and their role in health and disease. Advances in nutrition 2015;6(4):461-473.

(9) Navarro M, Fiore A, Fogliano V, Morales FJ. Carbonyl trapping and antiglycative activities of olive oil mill wastewater. Food Funct 2015 Feb;6(2):574-583.

(10) Li J, Liu D, Sun L, Lu Y, Zhang Z. Advanced glycation end products and neurodegenerative diseases: mechanisms and perspective. J Neurol Sci 2012;317(1):1-5.

(11) Vlassara H, Uribarri J. Advanced glycation end products (AGE) and diabetes: cause, effect, or both? Current diabetes reports 2014;14(1):453.

(12) Mesías M, Navarro M, Gökmen V, Morales FJ. Antiglycative effect of fruit and vegetable seed extracts: inhibition of AGE formation and carbonyl- trapping abilities. J Sci Food Agric 2013;93(8):2037-2044.

(13) West BJ, Deng S, Uwaya A, Isami F, Abe Y, Yamagishi S, et al. Iridoids are natural glycation inhibitors. Glycoconj J 2016;33(4):671-681.

(14) Leccese A, Bartolini S, Viti R. Total antioxidant capacity and phenolics content in apricot fruits. International Journal of Fruit Science 2007;7(2):3-16.

(15) Navarro M, Morales FJ, Ramos S. Olive leaf extract concentrated in hydroxytyrosol attenuates protein carbonylation and the formation of advanced glycation end products in a hepatic cell line (HepG2). Food & function 2017;8(3):944-953.

(16) Shao X, Chen H, Zhu Y, Sedighi R, Ho C, Sang S. Essential structural requirements and additive effects for flavonoids to scavenge methylglyoxal. J Agric Food Chem 2014;62(14):3202-3210.

(17) Peng X, Ma J, Chen F, Wang M. Naturally occurring inhibitors against the formation of advanced glycation end-products. Food & function 2011;2(6):289-301.

(18) Bourdon D, Aumaitre A. Low-glucosinolate rapeseeds and rapeseed meals: effect of technological treatments on chemical composition, digestible energy content and feeding value for growing pigs. Anim Feed Sci Technol 1990;30(3-4):175-191.

(19) Hakeem KR, Jawaid M, Alothman OY. Agricultural biomass based potential materials. : Springer; 2015.

(20) Leming R, Lember A. Chemical composition of expeller-extracted and cold-pressed rapeseed cake. Agraarteadus 2005;16:103-109.

(21) Khattab R, Eskin M, Aliani M, Thiyam U. Determination of sinapic acid derivatives in canola extracts using high-performance liquid chromatography. J Am Oil Chem Soc 2010;87(2):147-155.

(22) EFSA. Scientific Opinion on the safety of " rapeseed protein isolate" as a Novel Food ingredient. EFSA Journal 2013;11(10):n/a-n/a.

(23) Navarini L, Nobile E, Pinto F, Scheri A, Suggi-Liverani F. Experimental investigation of steam pressure coffee extraction in a stove-top coffee maker. Appl Therm Eng 2009;29(5):998-1004.

(24) Singleton V, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 1965;16(3):144-158.

(25) Pellegrini N, Serafini M, Colombi B, Del Rio D, Salvatore S, Bianchi M, et al. Total Antioxidant Capacity of Plant Foods, Beverages and Oils Consumed in Italy Assessed by Three Different In Vitro Assays. J Nutr 2003;133(9):2812-2819.

(26) Ferracane R, Graziani G, Gallo M, Fogliano V, Ritieni A. Metabolic profile of the bioactive compounds of burdock (Arctium lappa) seeds, roots and leaves. J Pharm Biomed Anal 2010;51(2):399-404.

(27) Graziani G, Pernice R, Lanzuise S, Vitaglione P, Anese M, Fogliano V. Effect of peeling and heating on carotenoid content and antioxidant activity of tomato and tomato- virgin olive oil systems. Eur Food Res Technol 2003;216(2):116-121.

(28) Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239(1):70-76.

(29) de Falco B, Fiore A, Rossi R, Amato M, Lanzotti V. Metabolomics driven analysis by UAEGC-MS and antioxidant activity of Chia (Salvia hispanica L.) commercial and mutant seeds. Food Chem 2018.

(30) Mesías M, Navarro M, Gökmen V, Morales FJ. Antiglycative effect of fruit and vegetable seed extracts: inhibition of AGE formation and carbonyl-trapping abilities. J Sci Food Agric 2013;93(8):2037-2044.

(31) Navarro M, Morales FJ. In vitro investigation on the antiglycative and carbonyl trapping activities of hydroxytyrosol. European Food Research and Technology 2016;242(7):1101-1110.

(32) Wilker SC, Chellan P, Arnold BM, Nagaraj RH. Chromatographic quantification of argpyrimidine, a methylglyoxal-derived product in tissue proteins: comparison with pentosidine. Anal Biochem 2001;290(2):353-358.

(33) Almuziny M, Decker C, Wang D, Gerard P, Tharayil N. Nutrient Supply and Simulated Herbivory Differentially Alter the Metabolite Pools and the Efficacy of the Glucosinolate-Based Defense System in Brassica Species. J Chem Ecol 2017:1-14.

(34) Wu C, Huang S, Lin J, Yen G. Inhibition of advanced glycation endproduct formation by foodstuffs. Food & function 2011;2(5):224-234.

(35) Henle T, Schwarzenbolz U, Klostermeyer H. Detection and quantification of pentosidine in foods. Zeitschrift für Lebensmitteluntersuchung und-Forschung A 1997;204(2):95-98.

(36) Shipanova IN, Glomb MA, Nagaraj RH. Protein modification by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct. Arch Biochem Biophys 1997;344(1):29-36.

(37) Donner TW. The metabolic effects of dietary supplementation with D-tagatose in patients with type 2 diabetes. Diabetes 2006;55:A110.

(38) Lu Y, Levin G, Donner T. Tagatose, a new antidiabetic and obesity control drug. Diabetes, obesity and metabolism 2008;10(2):109-134.

(39) Oku T, Murata-Takenoshita Y, Yamazaki Y, Shimura F, Nakamura S. D-sorbose inhibits disaccharidase activity and demonstrates suppressive action on postprandial blood levels of glucose and insulin in the rat. Nutr Res 2014;34(11):961-967.

Table A. Results of total polyphenol content and antioxidant capacity on unroasted 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	$14.48^{a} \pm 0.39$	$95.10^{a} \pm 12.42$	
5' roasting	$14.01^{a} \pm 1.22$	$89.98^{a} \pm 8.52$	
10' roasting	$14.20^{a} \pm 1.05$	$78.74^{a} \pm 5.43$	
RCext Freeze dried	$86.85^{c} \pm 2.08$	$241.65^{b} \pm 0.43$	569.87 ± 7.35
RCext Spray dried	$73.85^{b} \pm 0.64$	$240.94^{b} \pm 2.01$	

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

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**	***m/z***	**mg/g**
1	Lactic Acid, 2TMS	LA	7.25	$C_9H_{22}O_3Si_2$	219, 191, 147, 133, 117, 73, 45	0.139±0.001
2	Benzoic Acid, TMS	BE	10.02	$C_{10}H_{14}O_2Si$	194, 179, 135, 105, 77	0.046±0.0007
3	Glycerol, 3TMS	GLYC	10.46	$C_{12}H_{32}O_3Si_3$	218, 205, 147, 117, 89, 73, 45	0.028±0.0006
4	Levoglucosano, 3TMS	Levo	16.37	$C_{15}H_{34}O_5Si_3$	333, 217, 204, 147, 129, 103, 73	0.047±0.001
5	Shikimic acid	Sh	17.48	$C_{19}H_{42}O_5Si_4$	462, 255, 204, 147, 73	0.023±0.0008
6	Tagatose, MEOX	Tag	18.38	$C_{22}H_{55}NO_6Si_5$	307, 217, 147, 103, 73	0.090±0.0007
7	Sorbose, MEOX	Sorb	18.47	$C_{22}H_{55}NO_6Si_5$	307, 217, 147, 103, 73	0.091±0.0007
8	Galactose, MEOX	Gal	18.68	$C_{22}H_{55}NO_6Si_5$	319, 205, 147, 103, 73	0.088±0.0009
9	Mannitol, 6TMS	Man	19.12	$C_{24}H_{62}O_6Si_6$	421, 345, 319, 205, 147, 103, 73	0.389±0.0045
10	Palmitic Acid, TMS	PA	19.87	$C_{19}H_{40}O_2Si$	328, 313, 145, 117, 73	0.372±0.0007
11	Myo-Inositol, 6TMS	Myo	20.70	$C_{24}H_{60}O_6Si_6$	305, 217, 147, 129, 73	0.261±0.007
12	Stearic acid, TMS	ST	21.79	$C_{21}H_{44}O_2Si$	356, 341, 132, 117, 73	0.521±0.0067
13	Sinapinic acid, 2TMS	SI	21.90	$C_{17}H_{28}O_5Si_2$	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

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

Anti-glycative activity	**IC_{50} (mg mL^{-1})**	**range (mg mL^{-1})**
BSA-GLC glycation assay		
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	
BSA-MGO glycation assay		
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	
MGO trapping		
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 rapeseed cake extract (RCext), sinapinic acid, and controls (aminoguanidine, quercetin).

	Inhibition (%)	**Conc. (mg mL^{-1})**
Argpyrimidine		
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
Pentosidine		
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 ($p < 0.05$). Sinapinic acid content of 0.154 and 0.386 µg mL^{-1} in the RCext of 1.43 and 3.57 mg mL^{-1}, respectively.

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

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

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	$14.48^{a} \pm 0.39$	$95.10^{a} \pm 12.42$	
5' roasting	$14.01^{a} \pm 1.22$	$89.98^{a} \pm 8.52$	
10' roasting	$14.20^{a} \pm 1.05$	$78.74^{a} \pm 5.43$	
RCext Freeze dried	$86.85^{c} \pm 2.08$	$241.65^{b} \pm 0.43$	569.87 ± 7.35
RCext Spray dried	$73.85^{b} \pm 0.64$	$240.94^{b} \pm 2.01$	

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	$C_9H_{22}O_3Si_2$	219, 191, 147, 133, 117, 73, 45	0.139±0.001
2	Benzoic Acid, TMS	BE	10.02	$C_{10}H_{14}O_2Si$	194, 179, 135, 105, 77	0.046±0.0007
3	Glycerol, 3TMS	GLYC	10.46	$C_{12}H_{32}O_3Si_3$	218, 205, 147, 117, 89, 73, 45	0.028±0.0006
4	Levoglucosano, 3TMS	Levo	16.37	$C_{15}H_{34}O_5Si_3$	333, 217, 204, 147, 129, 103, 73	0.047±0.001
5	Shikimic acid	Sh	17.48	$C_{19}H_{42}O_5Si_4$	462, 255, 204, 147, 73	0.023±0.0008
6	Tagatose, MEOX	Tag	18.38	$C_{22}H_{55}NO_6Si_5$	307, 217, 147, 103, 73	0.090±0.0007
7	Sorbose, MEOX	Sorb	18.47	$C_{22}H_{55}NO_6Si_5$	307, 217, 147, 103, 73	0.091±0.0007
8	Galactose, MEOX	Gal	18.68	$C_{22}H_{55}NO_6Si_5$	319, 205, 147, 103, 73	0.088±0.0009
9	Mannitol, 6TMS	Man	19.12	$C_{24}H_{62}O_6Si_6$	421, 345, 319, 205, 147, 103, 73	0.389±0.0045
10	Palmitic Acid, TMS	PA	19.87	$C_{19}H_{40}O_2Si$	328, 313, 145, 117, 73	0.372±0.0007
11	Myo-Inositol, 6TMS	Myo	20.70	$C_{24}H_{60}O_6Si_6$	305, 217, 147, 129, 73	0.261±0.007
12	Stearic acid, TMS	ST	21.79	$C_{21}H_{44}O_2Si$	356, 341, 132, 117, 73	0.521±0.0067
13	Sinapinic acid, 2TMS	SI	21.90	$C_{17}H_{28}O_5Si_2$	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 37ºC on rapeseed cake extracts and controls

Anti-glycative activity	**IC_{50} (mg mL^{-1})**	**range (mg mL^{-1})**
BSA-GLC glycation assay		
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	
BSA-MGO glycation assay		
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	
MGO trapping		
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})**
Argpyrimidine		
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
Pentosidine		
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 ($p < 0.05$).

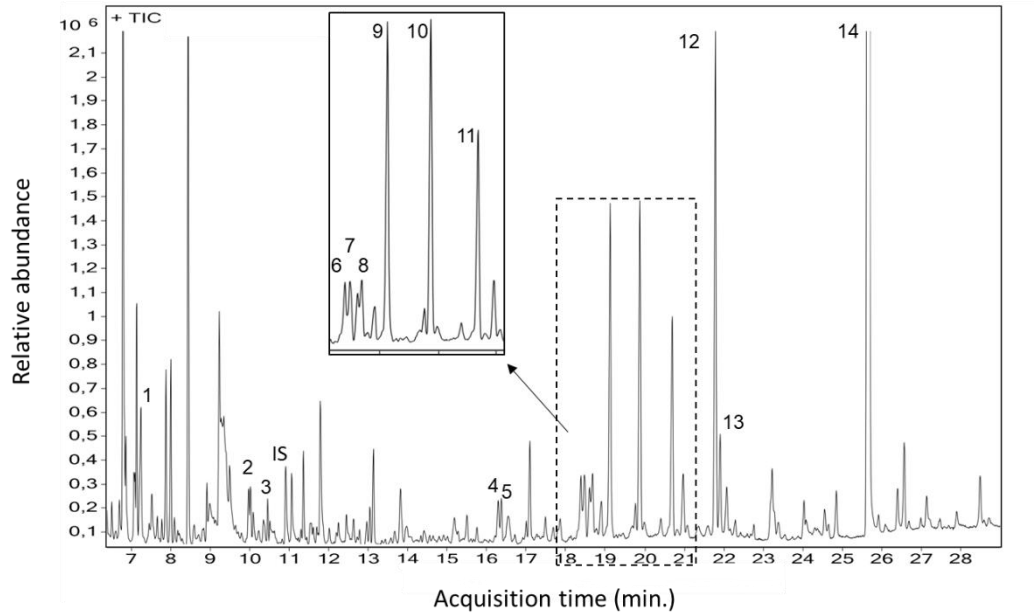

Figure 1. Total Ion Chromatogram (TIC) of rapeseed cake extract with detected metabolites.