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Corresponding Author	Family Name	**Hidalgo**
	Particle	
	Given Name	**Alyssa**
	Suffix	
	Division	Department of Food, Environmental and Nutritional Sciences (DeFENS)
	Organization	University of Milan
	Address	Via Celoria 2, 20133, Milan, Italy
	Phone	
	Fax	
	Email	alyssa.hidalgovidal@unimi.it
	URL	
	ORCID	
Author	Family Name	**Olivos**
	Particle	
	Given Name	**Juan Edgar Santa Cruz**
	Suffix	
	Division	Departamento de Tecnología de Alimentos y Productos Agropecuarios, Facultad de Industrias Alimentarias
	Organization	Universidad Nacional Agraria la Molina
	Address	Av. La Molina s/n, Lima 12, Peru
	Phone	
	Fax	
	Email	
	URL	
	ORCID	http://orcid.org/0000-0002-3311-814X
Author	Family Name	**Noni**
	Particle	**De**
	Given Name	**Ivano**
	Suffix	
	Division	Department of Food, Environmental and Nutritional Sciences (DeFENS)
	Organization	University of Milan
	Address	Via Celoria 2, 20133, Milan, Italy
	Phone	
	Fax	
	Email	
	URL	

	ORCID	http://orcid.org/0000-0003-1281-7053
Author	Family Name	**Brandolini**
	Particle	
	Given Name	**Andrea**
	Suffix	
	Division	Consiglio Per la Ricerca in Agricoltura e l'analisi Dell'economia Agraria
	Organization	Unità di Ricerca Per la Selezione dei Cereali e la Valorizzazione Delle Varietà Vegetali (CRA-SCV)
	Address	Via Forlani 3, 26866, S. Angelo Lodigiano (LO), Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	http://orcid.org/0000-0002-4552-4081
Author	Family Name	**Yilmaz**
	Particle	
	Given Name	**Volkan Arif**
	Suffix	
	Division	Department of Food Engineering, Faculty of Engineering
	Organization	Ondokuz Mayis University
	Address	Kurupelit, 55139, Samsun, Turkey
	Phone	
	Fax	
	Email	
	URL	
	ORCID	http://orcid.org/0000-0001-5039-4026
Author	Family Name	**Cattaneo**
	Particle	
	Given Name	**Stefano**
	Suffix	
	Division	Department of Food, Environmental and Nutritional Sciences (DeFENS)
	Organization	University of Milan
	Address	Via Celoria 2, 20133, Milan, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	http://orcid.org/0000-0003-1188-3526
Author	Family Name	**Ragg**
	Particle	
	Given Name	**Enzio M.**
	Suffix	
	Division	Department of Food, Environmental and Nutritional Sciences (DeFENS)
	Organization	University of Milan
	Address	Via Celoria 2, 20133, Milan, Italy

Phone	
Fax	
Email	
URL	
ORCID	http://orcid.org/0000-0002-2757-8369

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Abstract

Aim of this research was to study the evolution of heat damage, phenolic acid content and in vitro antioxidant capacity of whole meal einkorn water biscuits baked at 205 °C for increasing times (10 min steps) from 25 to 75 min. The heat damage was gauged by determining furosine, hydroxymethylfurfural (HMF), furfural and glucosylisomaltol (GLI) contents. Furosine increased up to 50 min baking, when HMF started to form; furfural augmented only after 65 min treatment, whereas GLI did not change. An unknown compound, apparently related to the severity of the heat load, aroses through the aldolic condensation of HMF with the acetone used for the extraction of phenolic acids; hence the use of acetone-based solvents in thermally processed cereal products should be avoided. The conjugated phenolic acids ferulic, vanillic, syringic, *p*-coumaric, *p*-hydroxybenzoic and syringaldehyde and the bound phenolic acids ferulic, *p*-coumaric, syringic, and *p*-hydroxybenzoic were identified in water biscuits. The stronger heating treatments led to an increase of the soluble conjugated compounds, but did not influence the bound fraction. The in vitro antioxidant capacity of water biscuits augmented significantly as baking time increased, likely for the formation of antioxidant compounds as a consequence of heat damage.

Keywords (separated by '-'): ABTS - FRAP - Heat damage - Insoluble-bound phenolics - Soluble conjugated phenolics - *Triticum monococcum*

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1 ORIGINAL PAPER

2 Phenolic acid content and in vitro antioxidant capacity of einkorn 3 water biscuits as affected by baking time

4 Juan Edgar Santa Cruz Olivos[1] · Ivano De Noni[2] · Alyssa Hidalgo[2] · Andrea Brandolini[3] · Volkan Arif Yilmaz[4] ·
5 Stefano Cattaneo[2] · Enzio M. Ragg[2]

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8 Abstract

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20 **Keywords** ABTS · FRAP · Heat damage · Insoluble-bound phenolics · Soluble conjugated phenolics · *Triticum*
21 *monococcum*

22 Introduction

23 Phenols are secondary metabolites synthesized by plants in
24 response to pests, diseases and stresses. They are excellent
25 oxygen radical scavengers, and as such can exert a beneficial

A1 ✉ Alyssa Hidalgo
A2 alyssa.hidalgovidal@unimi.it

A3–A6 [1] Departamento de Tecnología de Alimentos y Productos Agropecuarios, Facultad de Industrias Alimentarias, Universidad Nacional Agraria la Molina, Av. La Molina s/n, Lima 12, Peru

A7–A9 [2] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via Celoria 2, 20133 Milan, Italy

A10–A14 [3] Consiglio Per la Ricerca in Agricoltura e l'analisi Dell'economia Agraria, Unità di Ricerca Per la Selezione dei Cereali e la Valorizzazione Delle Varietà Vegetali (CRA-SCV), Via Forlani 3, 26866 S. Angelo Lodigiano (LO), Italy

A15–A16 [4] Department of Food Engineering, Faculty of Engineering, Ondokuz Mayis University, Kurupelit 55139 Samsun, Turkey

impact on human health; in particular, they show anti- 26
inflammatory, anti-microbial, anti-thrombotic, anti-athero- 27
genic, vasodilatatory and cardio-protective effects [1]. Phe- 28
nolic acids, the main phenols present in wheat kernels [2], 29
are often scarce in commercial wheat flours, because they 30
are mainly present in the aleuronic and hyaline layers, in the 31
germ and in the seed coat [3, 4], which are usually elimi- 32
nated during milling. Phenolic acids are present as soluble 33
free, soluble conjugated and insoluble-bound [5]. The free 34
soluble fraction represents about 1% of total phenols. The 35
soluble conjugated phenolic acids, esterified to sugars and 36
other low molecular weight components, and the insoluble- 37
bound phenolic acids, linked to cell wall constituents, repre- 38
sent about 22% and 77% of the total phenols [2]. However, 39
the free soluble phenols easily cross the human intestinal 40
barrier, whereas bound and conjugated forms are not readily 41
absorbable. A partial conversion from bound to conjugated 42
during in vitro intestinal digestion has been observed [6]; 43
additionally, bound phenolic acids can be partly degraded 44
in the colon by bacterial enzymes [7, 8]. Although the bio- 45
availability of phenolic compounds hydrolyzed by colon 46

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microbiota is lower than those of the free phenols readily absorbed in the small intestine [9], their abundance makes them a likely target to improve phenols nutritional availability. Therefore, several authors have studied the factors that trigger the passage from bound to conjugated phenolics during processing, such as maturation stage and fermentation [10], germination [11, 12], extrusion [13], baking [14], puffing [15], etc.

Some studies have investigated the changes in bioactive compounds and antioxidant capacity induced by baking, focusing mainly on heat damage and Maillard reaction products. Temperature, baking time and development of Maillard reactions can trigger the release of bound molecules as well as the loss of nutritional properties, along with an increase of Maillard reaction products and antioxidant capacity [16].

The reliable determination of phenolic acids is often a challenge, because the complex matrix and properties of foods hinder their retrieval. The free forms can be extracted using organic solvents, but the conjugated and bound forms require acid or base hydrolysis to free them from the cell wall matrix. Many solvents have been used to extract phenolic acids: ethanol–water [17], acidified methanol–water [18], isopropanol, acetonitrile, acetone–water [19], ethanol–acetic acid [20], methanol–acetone–water [21], etc.; in particular, this last mixture has been broadly adopted because of its effectiveness [4, 22, 23].

Einkorn (*Triticum monococcum* L. subsp. *monococcum*) is a diploid hulled wheat, allied to durum and bread wheat. The renewed interest for this cereal is linked to its high protein, carotenoid and tocol contents [15], as well as to its thrifty nature and disease resistance that favour low-input or organic management and propose it as an environment-friendly crop [24].

Aim of our research was to study the changes in phenolic composition and content, antioxidant capacity and heat damage of einkorn water biscuits (WB) during the baking process carried out for different times.

Materials and methods

Materials

Monlis, the most widespread Italian einkorn cultivar, was cropped in 2018–2019 at Sant'Angelo Lodigiano (45°13′40" N 9°25′21" E, Po plain, Italy) in 10 m^2 plots arranged in a randomised complete block design with three replications, following standard cultural practices. After harvesting, the kernels from the three replications were combined and stored at 5 °C. Before processing, the seeds were de-hulled with an Otake FC4S thresher (Satake, Japan). Whole meal flour for biscuit preparation was obtained from kernels ground with a Cyclotec 1093 lab mill (FOSS Tecator, Denmark).

Water biscuits preparation

The WB were manufactured using only whole meal flour (30 g dry weight basis for each WB) and water, to unambiguously determine the role of flour in phenolic acid changes. Optimum water quantity and mixing time of each sample were determined by Brabender farinograph analysis. Dough mixing was carried out with a cookie dough micromixer (National Mfg. Co, Lincoln, Nebraska, USA). The dough was transferred to ungreased cookie sheets covered with parchment paper, rolled to 7 mm thickness using gauge strips and a rolling pin, and cut with a cookie cutter (inside diameter: 60 mm). Baking was carried out at 205 °C for increasing times (10 min steps) from 25 to 75 min, to study the changes under different heat conditions; acceptable water biscuits are obtained after 25 and 35 min, while after 55 min they become hard and after 75 min scorched. Four WB were prepared for each accession. Baked WB were collected, stored at − 20 °C until analysis, and finely ground with a commercial heavy-duty blender (Waring, Torrington, USA) just before analysis.

Chemical analysis

Dry matter (DM) content was determined following method 44–15.02 [25]. Water activity (a_w) was evaluated using the Aqua Lab model Series 3 TE instrument (Decagon Devices, Inc, Pullman, WA, USA). Levels of furosine, glucosylisomaltol (GLI), hydroxymethylfurfural (HMF) and furfural in WB were determined by HPLC as reported by Hidalgo and Brandolini [26]. Soluble conjugated and insoluble-bound phenolic acids were analysed by RP-HPLC as described by Brandolini et al. [4]. The phenolic extracts were prepared as outlined in Moore et al. [23], with little modifications. Exactly 0.5 g of sample were mixed with 15 mL of a methanol/acetone/water (7:7:6) solution. After 15 min in an ice bath under discontinuous vortexing and after centrifugation (11,200 g, 10 min, 8 °C) with a Centrikon K24 centrifuge (Kontron Instruments, Bletchley, UK), the supernatant was recovered; the extraction from the sediment was repeated twice more, the three extracts were pooled and evaporated under vacuum at 35 °C for 18 min with a rotator evaporator Laborota 4000 (Heidolph, Milan, Italy). To recover the soluble conjugated and the insoluble-bound phenolic compounds, the pooled supernatants and the sediments were separately hydrolysed with 15 mL of 4 M NaOH under nitrogen for 4 h at room temperature and continuous shaking. The samples were then brought to pH 1.5–2.0 with 6 M HCL and extracted twice with 20 mL of ethyl ether/ethyl acetate (1:1 v/v). The extracts were clarified with anhydrous sodium sulphate,

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144 filtered with glass fibre 110 mm (Whatman, Maidstone, 145 England), evaporated as previously outlined, suspended 146 in 2 mL of methanol:water (1:1 v/v) and filtered with a 147 0.22 mm PTFE membrane (Millipore, Carrigtwohill Co, 148 Cork, Ireland) for HPLC injection. All the analyses were 149 performed twice.

150 Structural characterization of unknown 151 chromatographic peak

152 The chromatographic peak eluting at 20.0 min during the 153 RP-HPLC pattern of soluble conjugated phenolic acids was 154 collected from ten runs and dried under vacuum at 20 °C. 155 After dilution with water, the collected fraction was submit-156 ted to RP-HPLC/HR-MS using the same chromatographic 157 conditions [4] and by coupling the HPLC separation module 158 to a Q Exactive hybrid quadrupole-Orbitrap mass spectrom-159 eter through an HESI-II probe for electrospray ionisation 160 (Thermo Scientific, San Jose, CA, USA). The ion source and 161 interface conditions were: spray voltage + 3.0 kV, sheath gas 162 flow 60 arbitrary units, auxiliary gas flow 20 arbitrary units 163 and temperature 300 °C, capillary temperature 350 °C. Posi-164 tive mass calibration was performed with Pierce LTQ ESI 165 Positive Ion Calibration Solution (Thermo Scientific Pierce, 166 Rockford, IL, USA). The RP-HPLC eluate was analyzed by 167 Full MS and tandem MS analysis (MS^2). The resolution was 168 set at 70,000 and 17,500 and the AGC targets were 1×10^6 169 and 1×105 for Full MS and MS^2 scan types, respectively. 170 The maximum ion injection times were 50 ms. The MS data 171 were processed using Xcalibur software (Thermo Scientific, 172 San Jose, CA, USA).

173 The vacuum-dried fraction was also submitted to nuclear 174 magnetic resonance (NMR) spectroscopy. To this pur-175 pose, the vacuum-dried fraction was dissolved in 0.6 mL 176 methanol-d_4 or acetone-d_6 (Sigma-Aldrich, Milan, Italy) 177 and immediately transferred to a 5 mm NMR tube (Wil-178 mad 535-PP). ^{1}H-NMR spectra were recorded at 25 °C on 179 a Bruker AV600 spectrometer (Bruker Spectrospin AG, 180 Rheinstetten, Germany), operating at 600.10 MHz for the 181 ^{1}H nucleus and equipped with a standard triple-resonance 182 probe with z-axis gradients and temperature control unit. 183 ^{1}H-chemical shifts were measured in δ (ppm), using as ref-184 erence the acetone-d_5 residual peak, set at δ 2.096 [27]. All 185 2D spectra were obtained in phase-sensitive mode using 186 standard pulse sequences. 2D-NOESY experiments were 187 performed using 0.6 s mixing time, while the spin-lock dura-188 tion for the 2D-TOCSY experiment was set at 80 ms. Other 189 relevant acquisition parameters for all experiments: time 190 domain: 4 K; number of scans: 16–48; relaxation delay for 191 2D-TOCSY: 2.5 s and for 2D-NOESY: 3 s. Raw data were 192 Fourier-transformed after apodization with a 90°-shifted 193 sine-bell-squared function, zero-filling to 2 K × 2 K real data 194 points and baseline corrected. Spectra were processed using 195 Bruker software TOPSPIN v.1.3. Spectral data are reported 196 for the compound dissolved in acetone-d_6.

Antioxidant capacity 197

198 The samples were extracted as in Yilmaz et al. [28]. 199 Briefly, exactly 0.5 g sample were weighed and extracted 200 with 5 mL of methanol:water (80:20 v:v) acidified with 201 1% HCl (MeOH:HCl) under agitation using Vortex (30 s) 202 and a multi-rotator stirrer PTR-35 (Grant-bio, England) for 203 30 min at 4 °C in the dark. The mixtures were centrifuged 204 at 11,200 g for 10 min at 8 °C and the supernatants recov-205 ered; after re-extracting the residues, the supernatants were 206 pooled, giving 10 mL crude extracts.

207 The saturated butanol (BuOH) extracts were obtained 208 performing a single extraction with 0.5 g of sample and 209 10 mL of solvent under agitation with Vortex (30 s) and 210 orbital stirrer (2 h) at 4 °C in the dark followed by centrifu-211 gation as formerly described.

212 The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sul-213 phonic acid)] radical cation scavenging capacity was ana-214 lysed as described by Re et al. [29], with the minor changes 215 reported by Yilmaz et al. [28]. The FRAP (reduction of 216 the Fe(III)e2,4,6-tripyridyl-s-triazine complex to the fer-217 rous form at low pH) test was determined following Benzie 218 and Strain [30], with the small modifications described by 219 Yilmaz et al. [28]. The antioxidant capacity was expressed 220 as mmol TE/kg DM.

Results and discussion 221

222 To better understand the relationships among content of 223 soluble conjugated and insoluble-bound phenolic acids, 224 antioxidant capacity and heat damage, their evolution was 225 studied during baking of WB prepared from Monlis whole 226 meal flour (Fig. 1a–d, respectively). Furosine increased up 227 to 50 min and then steeply declined, HMF started grow-228 ing after 50 min, in correspondence to furosine decrease, 229 while GLI and furfural somewhat augmented only after long 230 baking times. The evolution of these heat-damage markers 231 was similar to that reported by Hidalgo and Brandolini [26] 232 during baking of WB made from refined bread wheat flour; 233 nonetheless, the levels of GLI, furosine, HMF and furfural 234 were generally higher (Fig. 1d) in the einkorn WB studied in 235 the present work. Ait Ameur et al. [31] studied the kinetics 236 of HMF in the advanced stages of baking and observed that 237 HMF was very sensitive to a_w, reporting that a low a_w (0.51) 238 at baking temperatures of 200–250 °C was critical for HMF 239 formation. The a_w in the whole meal flour WB was 0.80 after 240 25 min and decreased to 0.48 at 35 min, while the a_w in the 241 white flour WB remained stable at 0.8 up to 55 min [26].

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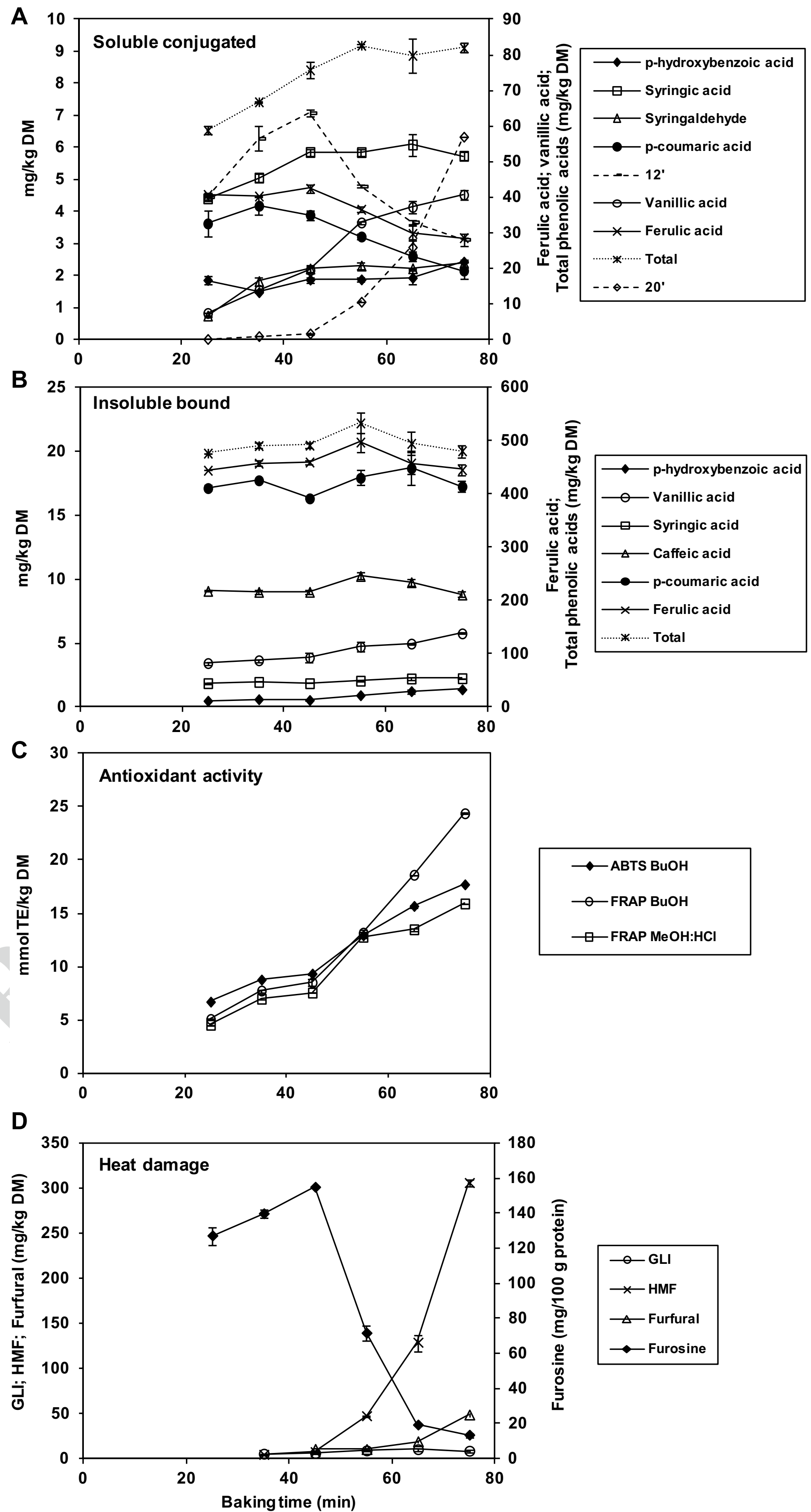

Fig. 1 Evolution of soluble conjugated phenolic acids (**a**), insoluble-bound phenolic acids (**b**), ABTS and FRAP antioxidant capacity of saturated butanol (BuOH) and/or methanol acidified with hydrochloric acid (MeOH:HCl) extracts (**c**), and glucosylisomaltol (GLI), hydroxymethylfurfural (HMF), furfural, furosine (**d**) during baking at different times of Monlis water biscuits. The dotted lines refer to the unknown compounds with peaks at 12 and 20 min retention times; the values correspond to area/100,000. The error bars represent the standard error

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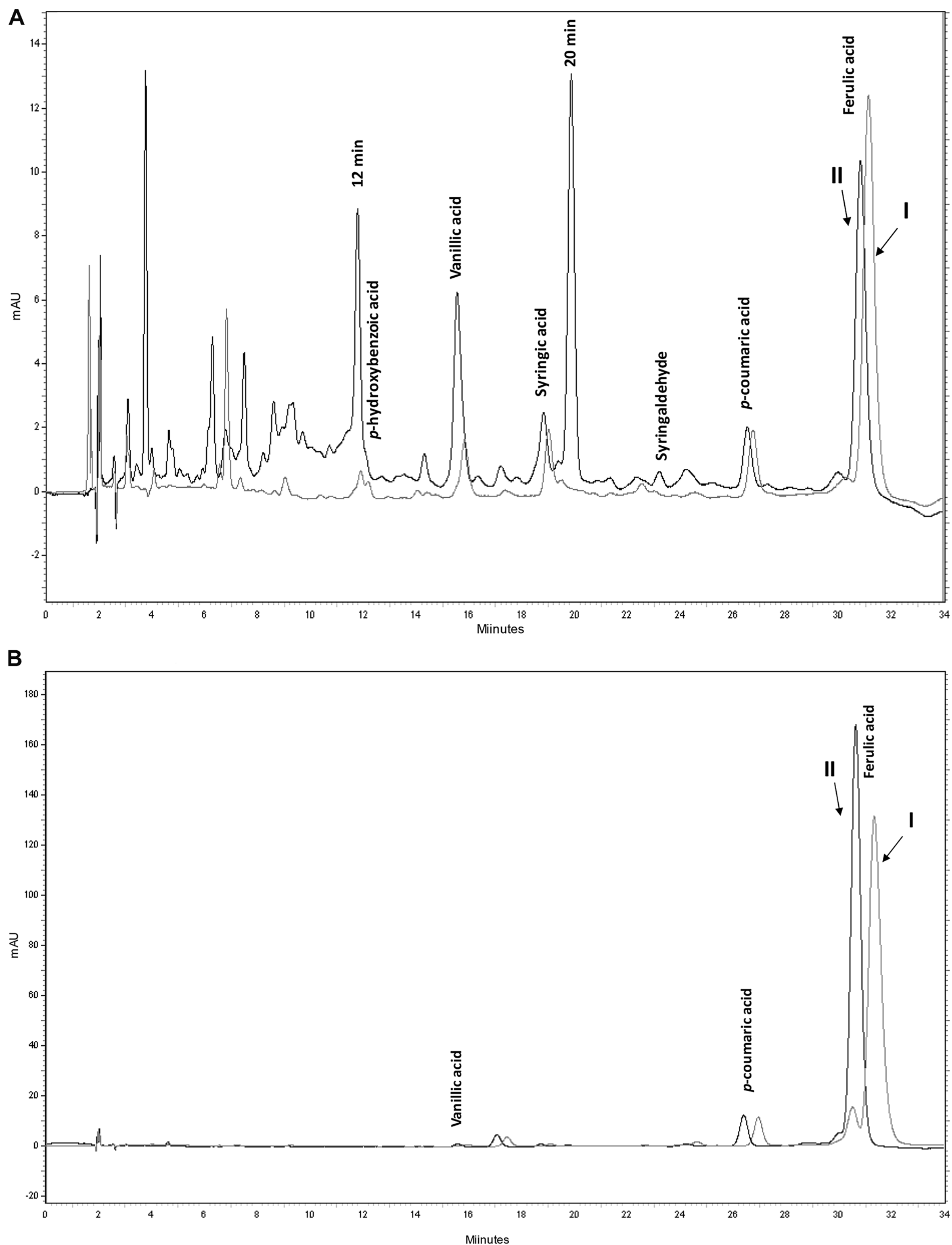

Fig. 2 Chromatogram of the soluble conjugated (**a**) and insoluble-bound (**b**) phenolic acids extracted with methanol/acetone/water from Monlis whole meal flour (I) and from water biscuits baked for 55 min (II)

Two major unknown peaks appeared at 12 and 20 min retention times in the RP-HPLC pattern of the soluble conjugated extracts (Fig. 2a). The former compound increased until 45 min and then rapidly diminished, while the latter compound continued growing (Fig. 1a). Their evolution mirrored those of furosine and HMF, respectively (Fig. 1d). Therefore, the importance of the compound eluting at 12 min and its possible contribution to the antioxidant capacity are

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probably limited to foods from low heat-stress treatments. On the other hand, the compound eluting at 20 min may be a major player in determining the antioxidant properties of bakery products under severe heat-stress conditions. Its spectrum differed from that of HMF (maximum absorbance wavelength at 327 nm instead of 284 nm, respectively). To identify this compound, the eluting fraction corresponding to the unknown peak was collected from RP-HPLC and was analysed by RP-HPLC/HR-MS. Surprisingly, the resulting chromatographic pattern showed a peak eluting at 22.5 min in addition to the expected one eluting at 20.0 min (not shown). Both peaks had the same UV spectrum (maximum absorbance at 327 nm) and the same accurate mass (MH^+: 167.0630), corresponding to a $C_9H_{10}O_3$ molecular formula (calculated: 166.06298 Da). The MS^1 spectra of the two chromatographic peaks overlapped completely and three major fragments (MH^+: 149.0598, 121.0651, 93.0706) were recorded.

The ^{1}H-NMR spectrum (600 MHz, acetone-d6; Fig. 3a) confirmed the presence of two molecules at an approximately 75:25 molar ratio. The ^{1}H-NMR spectrum of the major component (75%) exhibited two trans-olefinic protons at δ 7.40 and 6.55 ($^3J_{H,H}$ = 16.0 Hz), two aromatic signals at δ 6.84 and 6.48 ($^3J_{H,H}$ = 3.4 Hz), one oxygenated methylene group as a singlet at δ 4.62, and one methyl singlet at δ 2.32. The ^{1}H-NMR spectrum of the minor component (25%) exhibited two cis-olefinic protons at δ 6.67 and 6.22 ($^3J_{H,H}$ = 13.4 Hz), two aromatic signals at δ 7.81 and 6.46 ($^3J_{H,H}$ = 3.4 Hz), one oxygenated methylene group as a singlet at δ 4.59, and one methyl singlet at δ 2.29. The small value of the vicinal coupling constant between the aromatic protons suggested a furyl moiety. For both components, the

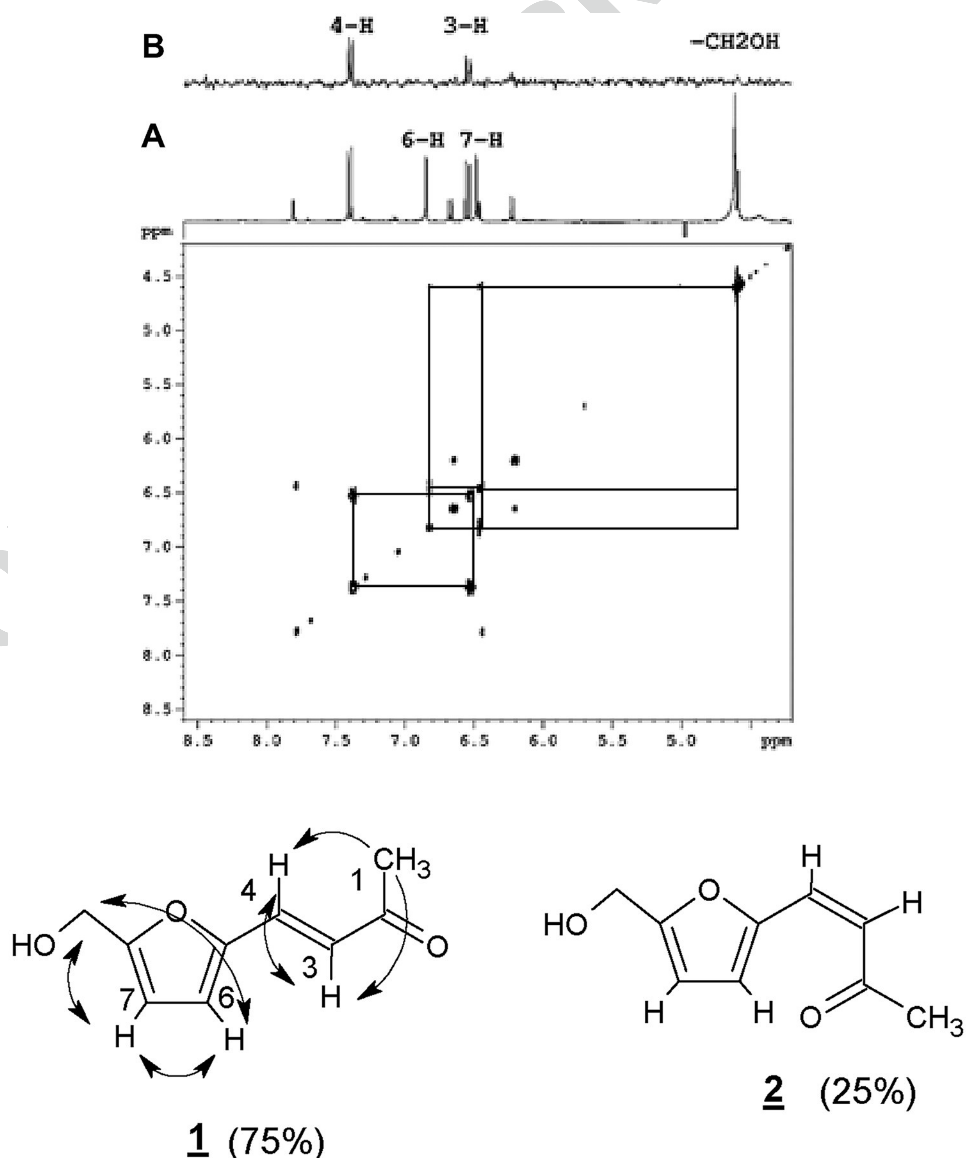

Fig. 3 **a** ^{1}H 2D-NMR TOCSY spectrum of the 20.0/22.5 min. HPLC peaks (acetone-d_6, 600 MHz, spin-lock: 80 ms, 25 °C). The intra-residue interactions for isomer 1 are made evident with boxes. The 1D-NMR spectrum is shown on top; (**b**) trace of a 2D-NOESY spectrum (t_{mix} = 0.6 s, 25 °C) extracted at δ 2.32, showing the through-space interactions between 1-CH_3 and 4-H,3-H protons of isomer 1. Structures and numbering of isomers 1 and 2 are shown at bottom. The observed NOESY and TOCSY interactions are depicted as single and double arrows, respectively

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282 1H connectivity and spatial proximity were established by analysis of 2D-TOCSY/COSY and 2D-NOESY experiments, respectively. The hydroxymethylene group (CH_2OH) showed a long-range connectivity with 6-H and 7-H of the furyl moiety (Fig. 3a), while the methyl group was detected by means of the NOESY interaction observed for the olefinic protons (Fig. 3b). Chemical shifts and coupling constants were consistent with published data for 4-[5-(hydroxymethyl)-2-furyl]-3(E)-buten-2-one [32]. Some minor discrepancies might be explained by the different solvents used for the NMR experiments (acetone-d_6 vs. chloroform-d). Finally, the UV maximum absorbance, with a measured 43 nm bathochromic effect compared to HMF (λ_{max} = 283 nm), confirmed the presence of an α,β-unsatured ketone-conjugated furane chromophore. Hence, on the basis of RP-HPLC/HR-MS and NMR data, the chromatographic peak eluting at 20.0 min should be assigned to 4-[5-(hydroxymethyl)-2-furanyl]-3(E)-buten-2-one (Fig. 3, compound 1). The additional peak at 22.5 min was the less thermodynamically stable isomer 4-[5-(hydroxymethyl)-2-furanyl]-3(Z)-buten-2-one (Fig. 3, compound 2), likely to result from the isomerisation of 3-buten-2-one,4-[5-(hydroxymethyl)-2-furanyl]-(3E) during vacuum drying. These two molecules originate from the aldol condensation and subsequent dehydration of HMF with acetone during the extraction of phenolic compounds. Such a reaction has been thoroughly studied in the production of fuels from biomass-derived carbohydrates [33] and there is a general agreement about the positive role of metal oxides as Lewis acids in the aldol condensation catalysis [34]. As whole meal flours are generally rich in divalent ions [35], given the basic conditions used during the extraction process (see "Materials and methods"), it is feasible to assume that the WB prepared from whole meal flour provide the right catalytic conditions to promote an aqueous-phase aldolic condensation of HMF with the acetone used as an extraction solvent (see scheme in Fig. 4) [36].

The two isomers represent, therefore, chemical artefacts, which must be considered when using acetone for assessing the phenolic acid composition of baked products. A clear-cut evidence that compounds 1 and 2 derive from HMF during the extraction is reported in Fig. 5, which compares the HPLC analysis of extracts performed with and without acetone (traces A and B, respectively). It is evident that compound 1 was detected only in the acetone extract, at a concentration inversely proportional to that of HMF. 327

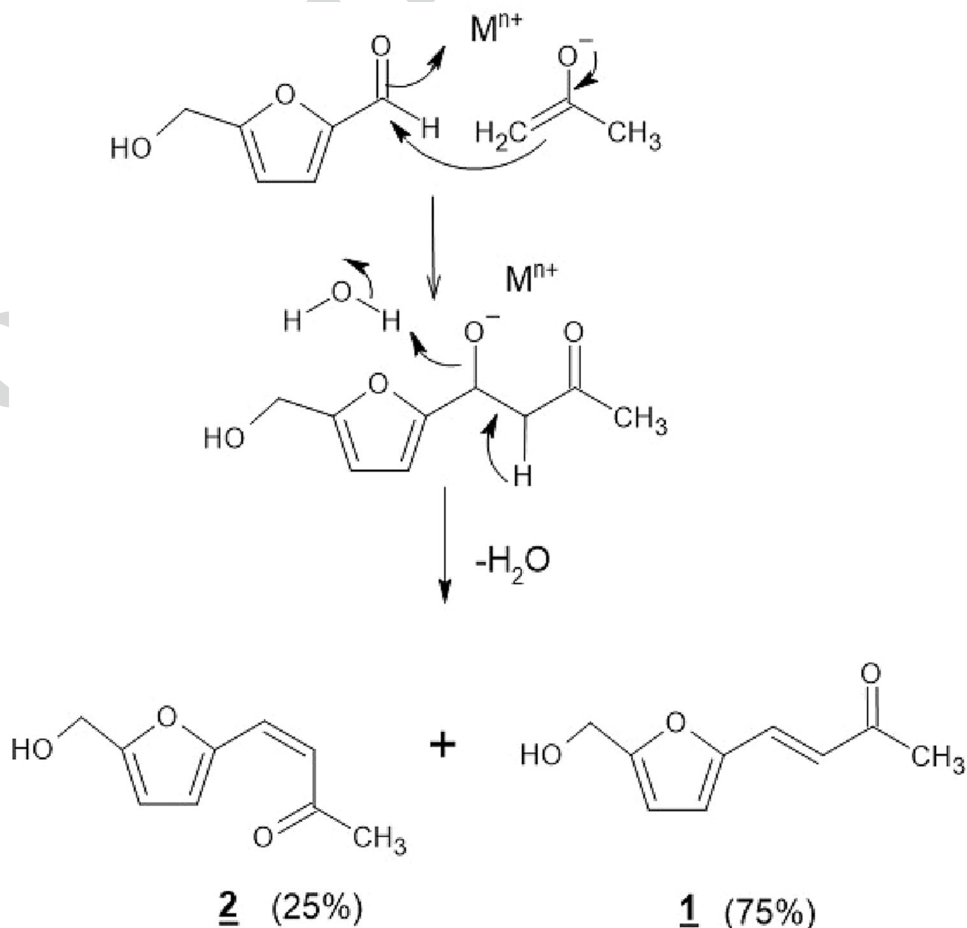

Fig. 4 Proposed mechanism of base-catalysed aldol condensation between HMF and acetone. Isomers 1 and 2 readily interconvert during workup through double bond isomerisation

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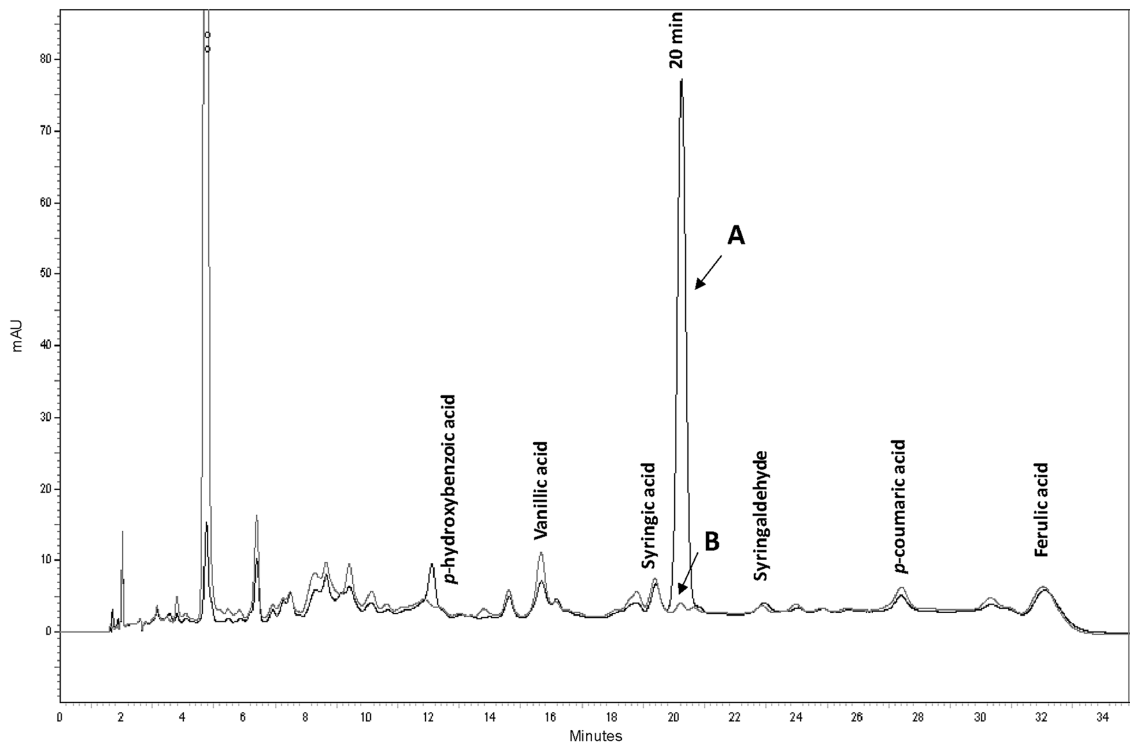

Fig. 5 Chromatogram of the soluble conjugated phenolic acids extracted with (**a**; methanol/acetone/water) and without (**b**; methanol/water) acetone from water biscuits baked for 75 min

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The conjugated phenolic acids (Fig. 2a) identified in the WB were ferulic, vanillic, syringic, *p*-coumaric, *p*-hydroxybenzoic and syringaldehyde, while the bound phenolic acids (Fig. 2b) were ferulic (by far the most abundant), *p*-coumaric, syringic, and *p*-hydroxybenzoic acids. These phenols (plus caffeic acid at low concentration) were also observed in similar products [15]. In the soluble phenolic extracts, ferulic and *p*-coumaric acid initially increased, but then decreased significantly after 45 min, while *p*-hydroxybenzoic acid, syringic acid and syringaldehyde increased slightly and vanillic acid kept growing steadily throughout all the baking time; the insoluble-bound fraction composition, instead, generally did not change, although vanillic acid steadily increased during baking. These results are in line with those reported by Hidalgo et al. [15] for WB baked for 25 and 35 min. The same authors noticed that stronger heating treatments, such as those applied during kernels puffing, led to higher increases of the soluble conjugated compounds, but still did not influence the bound fraction. The authors suggested that the increase in soluble conjugated phenolic acids might be related to heat-induced rupture of certain ester bonds, and thus to an increase in extractable compounds.

The insoluble-bound phenolic acids syringic, caffeic, *p*-coumaric and ferulic did not show significant variations during baking, whereas the *p*-hydroxybenzoic and vanillic acids increased slightly from 45 min onwards. Hence, the slight increase of some phenolic acids in the soluble conjugated fraction does not seem to be related to a partial degradation of insoluble-bound phenolics.

The in vitro antioxidant capacity of saturated butanol and acidified methanol–water extracts augmented significantly as the baking time passed from 25 to 75 min (Fig. 1c). This increase does not seem justified by the changes in concentration of phenolic acids or of other antioxidant molecules such as carotenoids and tocols, whose levels diminish during processing [37, 38]. Therefore, the most likely explanation is the formation of antioxidant compounds during baking as a consequence of the Maillard reaction, as suggested for bread crust [39, 40] and for biscuits [41, 42].

Conclusions

This research demonstrates the high stability of soluble conjugated and insoluble-bound phenolic acids even after long baking times. The increased antioxidant capacity after baking is more a consequence of heat damage than of conjugated and bound phenols liberation. The use of acetone during extraction should be considered with caution or even avoided, as it might lead to artefacts arising from undesired aldol condensation products with furan

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377 aldehydes. Nevertheless, the effectiveness of other sol-
378 vents for extracting free phenols from water biscuits and,
379 in general, thermally processed cereal products needs fur-
380 ther experimental evidence.
381

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384 ## Compliance with ethical standards

385 **Conflict of interest** The authors declare that they have no conflict of
386 interest.

387 **Ethical approval** This article does not contain any studies with human
388 or animal subjects.

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