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Phytochemical-rich antioxidant extracts of *Vaccinium vitis-idaea* L. leaves inhibit the formation of toxic Maillard reaction products in food models

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This is the peer reviewed version of the following article:

Račkauskienė, I., Pukalskas, A., Fiore, A., Troise, A.D. & Venskutonis, P.R. (2019) 'Phytochemical-rich antioxidant extracts of *Vaccinium vitis-idaea L*. leaves inhibit the formation of toxic Maillard reaction products in food models', *Journal of Food Science*.

which has been published in final form at https://doi.org/10.1111/1750-3841.14805. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving

1 **Full Title**
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3 of toxic Maillard reaction products in food models
4
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24 **Word count of text**
25 **7220** words
26
27 **Short version of title**
28 Lingonberry extract in Maillard reaction
29
30 **Choice of journal/section**
31 Food Chemistry (also relevant to Toxicology and Chemical Food Safety)
32
33 **Author disclosures**
34 We declare the there are no conflicts of interests
35

36 **ABSTRACT**: Thermal treatment of proteinaceous foods generates heat-induced Maillard
37 reaction substances including toxic advanced glycation end products (AGEs) and heterocyclic
38 amines (HAs). It is known that plant phenolic compounds may influence Maillard reaction. This
39 study investigated the impact of lingonberry leaf extracts on the formation of N^{ε}-
40 (carboxymethyl)lysine (CML) and N^{ε}-(2-furoylmethyl)-L-lysine (furosine) in milk model system
41 and HAs in meat-protein and meat model systems. In addition, lingonberry leaf extracts
42 obtained by different solvents were characterized by radical scavenging, Folin-Ciocalteu assays
43 and ultra-high pressure liquid chromatography quadruple-time-of flight mass spectrometry
44 (UPLC-qTOF-MS). Water extract (WE) stronger suppressed CML than furosine formation in milk
45 model system: CML levels were reduced by nearly 40%. Moreover, quinic acid and catechin
46 which were abundant in WE, were effective in inhibiting CML and furosine formation. WE and
47 acetone extract (AE) at 10 mg/mL significantly inhibited HAs formation in both model systems.
48 However, higher suppressing effect on HAs formation showed AE which had lower antioxidant
49 capacity and total phenolic content values than WE. WE contained higher amounts of
50 hydroxycinnamic acids, proanthocyanidins and flavonols, while AE was richer in flavan-3-ols and
51 arbutin derivatives. It indicates that the composition of phenolics might be a major factor for
52 explaining different effect of extracts from the same plant on HAs formation. In general, the
53 results suggest that lingonberry leaves is a promising source of phytochemicals for inhibiting
54 toxic Maillard reaction products and enriching foods with plant bioactive compounds.

55

56 **Keywords:** *Vaccinium vitis-idaea* L. leaves; Maillard reaction; CML; furosine; heterocyclic
57 amines

58 **Practical Application:**

59 The increased consumption in processed foods has been linked with the increased risks of
60 various diseases, while thermal food processing is required to develop flavour, insure safety
61 and extend shelf life. Therefore, developing effective technological means for inhibiting the
62 formation of heat-induced toxic substances is an important task. This study showed a potential
63 of lingonberry leaf extracts containing health beneficial phytochemicals to suppress the
64 formation of toxic Maillard reaction products during heating of milk and meat.

Introduction

Thermal treatment of raw milk or meat during processing is an essential step in reducing microbiological contamination causing foodborne diseases and extending product shelf life. On the other hand, thermal treatment results in the formation of undesirable compounds demonstrating toxicity and allergenicity. The majority of those modifications are linked to the Maillard reaction occurring between carbonyl groups of reducing sugars and free amino groups of amino acids (Nursten, 2005).

Thermal treatment of raw milk in such processing steps as separation, normalization, homogenization, pasteurization, sterilization or ultra-heat treatment induces Maillard reaction mainly between lactose and lysine residues and generates the advanced glycation end products (AGEs). The formation of AGEs increases with increasing heating time and temperature. The presence of AGEs in foods contributes to the increased inflammation and oxidative stress (Uribarri et al., 2007), which are linked to the recent epidemics of diabetes and cardiovascular diseases (Birlouez-Aragon et al., 2010; Sandu et al., 2005). A range of dietary AGEs compounds have been reported, while the present study is focused on N^{ε}-(carboxymethyl)lysine (CML) and indirect marker, N^{ε}-(2-furoylmethyl)-L-lysine (furosine). CML is one of the most studied AGE, which is present in both biological systems, such as plasma, urine, tissues, skin collagen and in many heat-processed foods (Nguyen, van der Fels-Klerx, & van Boekel, 2014). It is generated from the oxidation of Amadori product (AP) or direct reaction of lysine ε-amino group with glyoxal produced by degradation/oxidation of sugars during thermal treatment and lipid peroxidation. Furosine is an indirect marker of AGEs indicating early stage of Maillard reaction

86 and is formed from the acid hydrolysis of the APs (e.g. fructosyl-lysine) (Henle, Zehetner, &
87 Klostermeyer, 1995).
88 Thermal treatment of meat, particularly by deep-frying, oven-frying, roasting, grilling and
89 searing generates the formation of heterocyclic amines (HAs) possessing mutagenic and
90 carcinogenic properties. They are formed mainly as Maillard reaction products from amino
91 acids, creatin(in)e and reducing sugars (Skog, Johansson, & Jägerstad, 1998). Depending on the
92 cooking method, heating temperature and time, as well as on the content of precursors the
93 formation of different HAs are favoured during thermal treatment (Gibis & Weiss, 2015; Linghu,
94 Karim, & Smith, 2017; Yu et al., 2018). 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine
95 (PhIP) is the most abundant mutagenic HA of meat, while the chicken was reported as the most
96 susceptible type of meat for the formation of this HA (Gibis & Weiss, 2015). It was observed
97 that PhIP is carcinogenic in the rat colon, mammary gland, prostate, and breast (Shirai et al.,
98 1997). It was also shown that many HAs possess carcinogenic/mutagenic/genotoxic effects both
99 *in vitro* (Dumont et al., 2010) and *in vivo* (Chao et al., 2005; Shirai et al., 1997). Recent studies
100 have supported positive associations of HA's with colorectal adenoma risk (Budhathoki et al.,
101 2015; Khan et al., 2019) and increased oxidative stress in humans (Carvalho et al., 2015).
102 Phenolic compounds have been shown to possess inhibiting effects on the formation of
103 AGEs and HAs (Puangsombat, Jirapakkul, & Smith, 2011). These effects have been explained by
104 different reaction mechanisms, namely radical scavenging (Kikugawa, 1999), amine group
105 blocking (Guerra & Yaylayan, 2014), scavenging dicarbonyl intermediates (Totlani & Peterson,
106 2005) or reactive carbonyls produced from amino acid degradation (Cheng et al., 2009) and
107 lipid oxidation (Hidalgo, Delgado, & Zamora, 2017). For instance, catechin (Kokkinidou &

108 Peterson, 2014), (–)-epicatechin (Totlani & Peterson, 2006), (–)-epigallocatechin-3-gallate
109 (EGCG) (Bin, Peterson, & Elias, 2012) inhibited AGEs by trapping dicarbonyl compounds. EGCG
110 inhibited PhIP by scavenging reactive carbonyls from phenylacetaldehyde degradation (Cheng
111 et al., 2009). Moreover, the structure of phenolics was explored as an important factor in the
112 inhibition of HAs formation (Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014). Therefore,
113 phenolic antioxidant-rich natural plant extracts may be useful additives in proteinaceous foods
114 for inhibiting toxic Maillard reaction products.

115 Lingonberry (*Vaccinium vitis-idaea* L., Ericaceae) is one of the most important wild berry
116 plant in the forests of the Nordic countries. Lingonberry leaves have been used in traditional
117 medicine for their diuretic, astringent, and antiseptic properties to treat urinary tract infections.
118 Lingonberry leaves contain many classes of flavonoids such as flavan-3-ols, flavanols,
119 hydroxycinnamic acids, and proanthocyanidins (Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen,
120 2009; Ieri, Martini, Innocenti, & Mulinacci, 2013; Liu et al., 2014). According to European
121 Commission Novel Food Catalogue lingonberry leaves have been authorized as food
122 supplements. In addition, *V. vitis-idaea* berry extracts demonstrated antiglycation activity
123 (Beaulieu et al., 2010), while there are no reports on antiglycation properties or HAs inhibition
124 by lingonberry leaf extracts.

125 So far as milk and meat are very important foods for adequate human nutrition, particularly
126 in supplying high value proteins and various bioactive micronutrients, the application of plant
127 antioxidants for reducing the formation of undesirable toxic compounds during heat treatment
128 might be a promising approach in achieving better equilibrium between the positive and
129 negative nutritional aspects in the consumption of heat processed proteinaceous foods.

130 Therefore, this study was aimed at investigating the effect of lingonberry leaf extracts on the
131 formation of CML and furosine in milk model system and HAs in meat-protein and meat model
132 systems. The antioxidative potential of different lingonberry leaf extracts obtained by different
133 solvents from deodorized and non-deodorized material was characterized by radical scavenging
134 capacity, the total phenolic content and composition of phenolic compounds.
135

136 **Materials and methods**

137 **Chemicals**

138 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2-azinobis-3-ethyl benzothiazoline-6-sulphonic acid
139 (ABTS), 6-hydroxy-2,5,7,8,-tetramethylchromane-2-carboxylic acid (Trolox), 2,2'-azobis(2-
140 methylpropionamidine) dihydrochloride (AAPH), diethylene glycol, sodium borohydride, boric
141 acid, nonafluoropentanoic acid (NFPA), trichloroacetic acid (TCA), D (+)-lactose monohydrate,
142 acetonitrile (HPLC grade), rutin, quercetin-3-*O*-glucoside, quinic acid, *p*-coumaric, caffeic and
143 gallic acids, Folin–Ciocalteu phenol reagent and Milli-Q water were from Sigma-Aldrich (St.
144 Louis, MO, USA). (+)-Catechin reference was purchased from Chromadex (Irvine, California,
145 USA), chlorogenic acid, L-phenylalanine and glucose were from Roth (Karlsruhe, Germany);
146 fluorescein sodium salt (FL) from Fluka Chemicals (Steinheim, Germany). Heterocyclic amines,
147 MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinolone), MeIQx (2-amino-3,8-
148 dimethylimidazo[4,5-f] quinoxaline), Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole)
149 and PhIP were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). CML, d_2-
150 CML and furosine were purchased from PolyPeptide Laboratories (Strasbourg, France).
151 Creatinine was from Alfa Aesar (Karlsruhe, Germany). Bond Elut PRS LRC cartridges and Bond-

152 Elut C18 cartridges were purchased from Agilent Technologies (Lake Forest, CA, USA).
153 Chromabond XTR cartridges were from Macherey-Nagel GmbH & Co. (Bethlehem, PA, USA).
154 Oasis HLB cartridges for CML and furosine stable isotope dilution assay were purchased from
155 Waters Corporation (Milford, CA, USA).
156

157 **Plant material and preparation of extracts**

158 *Vaccinium vitis-idaea* L. leaves were collected in the forest of Mažeikiai (Telšiai district,
159 Lithuania) from June to mid-September. The plants were dried at 40 °C and ground in an ultra-
160 centrifugal rotor mill Retsch ZM200 (Retsch GmbH, Haan, Germany) using 0.5 mm sieve.
161 Powdered lingonberry leaves (200 g) were placed in a 3 L round-bottom flask, connected with
162 Clevenger-type apparatus, diluted with 1.5 L of water and hydrodistilled during 3 h for
163 removing volatile compounds. Then, water phase was separated from the solids by filtering
164 through the cotton and freeze-dried. The product obtained is indicated as water extract (WE).
165 The solid residues, which are called deodorized lingonberry leaves, were dried at 40 °C in a
166 drying oven. Then, raw and deodorized lingonberry leaves were subjected to pressurised liquid
167 extraction with methanol and acetone in a Dionex ASE 350 apparatus (Sunnyvale, CA, USA) at
168 the following parameters: 70 °C, 10 MPa, 3 cycles, 5 min each, 100% flush volume, purging with
169 nitrogen. Organic solvents were evaporated at 40 °C in a rotary vacuum evaporator (Büchi,
170 Flawil, Switzerland). Acetone, as an aprotic lower polarity solvent, and methanol, as a protic
171 high polarity solvent, were selected in order to evaluate what kind of compounds may be
172 recovered by using these solvents and what extracts (containing lower or higher polarity
173 phytochemicals) possess the strongest radical scavenging properties. In total, five extracts were

174 produced: three extracts from the raw (WE, ME, AE) and two extracts from the deodorized
175 lingonberry leaves (DME and DAE).
176

177 **Evaluation of antioxidant potential of** lingonberry leaves

178 **Folin-Ciocalteu assay**

179 Total phenolic content was determined using Folin-Ciocalteu method (Singleton, Orthofer, &
180 Lamuela-Raventós, 1999). Extract solution (0.2 mL) was added to 1.0 mL of Folin–Ciocalteu
181 reagent (1:10, v/v) and was shaken for 5 min; then 0.8 mL of 7.5 mg/g sodium carbonate
182 solution was added and after incubation in the dark for 1.5 h, the absorbance was read at 760
183 nm in a HALO RB-100 UV–Vis spectrophotometer (Dynamica, Switzerland). The results were
184 expressed as mg gallic acid equivalents (GAE) in g of extract dry weight (edw) and in plant dry
185 weight (pdw).
186

187 **DPPH$^{\bullet}$ scavenging assay**

188 DPPH$^{\bullet}$ scavenging was determined according to Brand-Williams, Cuvelier, & Berset (1995).
189 Briefly, the decrease in absorbance was recorded at 517 nm in a FLUOstar Omega microplate
190 reader (BMG Labtech, Durham, NC) over 35 min after the addition of 7.5 µL of extract or trolox
191 solution in 96-well microplates with 300 µL of methanolic DPPH$^{\bullet}$ solution (60 µM). The results
192 were expressed as effective concentrations EC_{50} (mg/mL) required to decrease the initial DPPH$^{\bullet}$
193 concentration in the reaction mixture by 50%.
194

195 **ABTS$^{\bullet+}$ scavenging assay**

196 The $ABTS^{•+}$ scavenging (decolouration) capacity was determined according to Re et al.
197 (1999). Briefly, after adjusting the absorbance of $ABTS^{•+}$ solution to 0.70±0.02 at 734 nm with
198 phosphate buffer (pH 7.4), the measurements were performed in microplate reader after
199 mixing 3 µL of extract or trolox solutions with 297 µL of diluted $ABTS^{•+}$ solution. Trolox
200 equivalent antioxidant capacity (TEAC) was used to express $ABTS^{•+}$ scavenging capacity in mM
201 TE/g edw and pdw.
202

203 **Oxygen radical absorbance capacity (ORAC) assay**

204 The ORAC assay was performed as described by Huang, Ou, Hampsch-Woodill, Flanagan, &
205 Prior (2002). Briefly, 25 µL of extract or trolox solution were mixed with 150 µL of 96 mM FL in
206 microplate, which further was incubated at 37 °C for 15 min. Then, 26 µL of 240 mM AAPH was
207 added and microplate was shaken for 30 s. The fluorescence was recorded (λ_{ex} = 493 nm, λ_{em} =
208 515 nm) every 66 s during 80 min. The ORAC values are expressed as mM TE/g edw and pdw.
209

210 **Analysis of phenolic compounds**

211 Phenolic compounds were analyzed by ultra-high pressure liquid chromatography with
212 quadruple-time of flight mass spectrometry (UPLC-qTOF-MS) using an Acquity UPLC system
213 (Waters Corporation) and Brüker maXis UHR-TOF mass spectrometer (Brüker Daltonics,
214 Bremen, Germany). Separation was performed using 1.7 µm Acquity BEH-C18 (50 × 2.1 mm)
215 column at a flow rate of 0.4 mL/min (injection volume 2 µL). The eluents were 0.1% formic acid
216 (A) and acetonitrile (B) at the following gradient: 0–2 min, 10–22% B, 2–7 min, 22%–50% B, 7–
217 10 min, 10% B. Mass spectra were acquired in a negative electrospray ionization mode (ESI) by

218 full scan acquisition covering a range of m/z 100-800 at the following parameters: capillary
219 voltage, 4000 V; collision cell energy, 8 eV, dry gas temperature, 200 °C with a flow rate of 10
220 L/min; nebulizer pressure, 2 bar; drying and nebulizing gas, nitrogen. Peak identification was
221 performed by comparing retention times (t_R) with those of standards or and accurate m/z
222 values from which the molecular ion formulas were calculated for each compound. Mass
223 fragmentation was performed with nitrogen as a collision gas at 40–45 eV collision energy.
224

225 **Determination of effects of** lingonberry leaf **extracts on** Maillard reaction products

226 **Preparation of milk model system and determination of furosine and CML**

227 Milk model system was prepared by dissolving (w/w) 1.2% skimmed milk powder, 2.5% whey
228 protein (both from Lactalis Ingredients, Bourgbarré, France) and 5% lactose monohydrate in
229 87.9% of water, and then homogenizing (60 °C, 16 MPa, 3 passes) with 3.3% of palm oil (Kerfoot
230 Groop, Yorkshire, UK) and 0.1% of soy lecithin Solec (DuPont, Dangé Saint Romain, France). WE,
231 quinic acid and catechin were added at 0.05, 0.1 and 0.3 mg/mL concentrations, while the
232 controls (also in other model systems) were without any additives. The samples were heated at
233 140 °C for 0, 30 and 45 s. Furosine and CML were extracted and determined as described by
234 Delatour et al. (2009) and Fenaille et al. (2006) with slight modifications reported by
235 Račkauskienė et al. (2015).
236

237 **Preparation of meat-protein model system and determination of HAs**

238 Meat-protein model system consisting of 0.4 mM phenylalanine, 0.4 mM creatinine and 0.2
239 mM glucose in 3 mL of diethylene glycol (14% of water) was used as described by Wong, Cheng,

240 & Wang (2012). WE and AE powders were added to the mixture at 0.1, 1, 5, and 10 mg/mL
241 levels. The samples were heated at 128 °C in the closed stainless steel vials (homemade from
242 stainless steel type EN 1.4401) for 120 min, afterwards cooled in an ice-water bath for 10 min
243 and stored at −18 °C. After extraction of HAs (Wong, Cheng, & Wang, 2012) the eluate was
244 dried under nitrogen and re-dissolved in 400 µL of methanol before analysis.

245 PhIP was determined using the same UPLC system as for phenolic compounds. Separation of
246 HAs was performed using Acquity BEC-C18 column thermostated at 40 °C. The mobile phase
247 was composed of 30 mM formic acid/ammonium formate (pH 4.75) in deionized water (A) and
248 acetonitrile (B) delivered at 0.4 mL/min (injection volume 5 µL) at the following gradient: 0–
249 0.15 min, 5% B; 0.15–2.5 min, 5–30% B; 2.5–3.0 min, 30–60% B; 3–5 min, 60% B and 5–7 min
250 60–100% B. Positive ESI was used for mass analysis and MS parameters were the same as
251 described above. The full scan MS data were recorded in the range of 100 to 800 m/z. PhIP was
252 quantified using calibration curve prepared from its solutions in methanol (1 - 800 ng/mL) and
253 following the whole procedure described above. The limits of detection (LOD) and
254 quantification (LOQ) were 1 and 3 ng/mL, respectively, while average PhIP recovery was
255 approx. 61%.

256

257 **Preparation of meat model system and determination of HAs**

258 A meat model system consisting of 0.1 g of homogenized freeze-dried beef meat purchased
259 in the local market (Kaunas, Lithuania) and 1 mL of diethylene glycol (14% of water) was used
260 (Messner & Murkovic, 2004). WE and AE powders were added to the mixture at 0.1, 1, 5, and
261 10 mg/mL concentrations. The samples were placed in the stainless steel vessels, which were

262 closed and heated at 180 °C for 30 min. After heating the vessels were cooled on ice (10 min) to
263 terminate the reaction and the samples were stored at −18 °C prior to extraction. The samples
264 were then transferred to 50 mL glass beakers with 15 mL of 1 M NaOH and homogenized by
265 magnetic stirring for 60 min at 500 rpm. Next, 5 mL of alkaline solution were mixed with
266 diatomaceous earth in Chromabond XTR cartridges, and extracted (Wong et al., 2012). The
267 eluate was dried under nitrogen and re-dissolved in 100 µL of methanol before analysis.
268 HAs were analysed on a Waters Acquity UPLC H-Class system equipped with triple quadruple
269 spectrometer Waters Xevo TQ-S in a positive ESI mode. The compounds were separated in an
270 Acquity BEH-C18 column at a flow rate of 0.8 mL/min (injection volume 2 µL). The mobile phase
271 was 30 mM formic acid-ammonium formate buffer (A) and acetonitrile (B) eluted at the
272 following gradient: 0–0.1 min, 5% B; 0.1–1.5 min, 5–30% B; 1.5–1.8 min, 30–60% B; 1.8–1.85
273 min, 60% B; 1.85–2.4 min, 60–95% B; 2.4–2.9 min, 5% B. The quantitation was carried out in
274 multiple reaction monitoring (MRM) mode using transitions as follows: 225 → 210 for PhIP; 213
275 → 198 for MeIQ; 214 → 199 for MeIQx; 212 → 195 for TrP-P-1. MRM conditions were
276 automatically optimized with 100 ng/mL HAs using the Intellistart function system. MS/MS
277 parameters were as follows: cone voltage, 50 V; capillary voltage, 3.0 kV; desolvation
278 temperature, 350 °C; cone gas flow rate, 150 L/h (Nitrogen); desolvation gas flow rate, 650 L/h
279 (Nitrogen); collision gas flow rate, 0.13 mL/min (Argon); collision energy, 35 eV. Data
280 acquisition was carried out by MassLynx 4.1 software. Quantitative analysis was performed
281 using Waters TargetLynxTM software. Calibration curves ranged from 1 to 500 ng/mL. The LOD
282 and LOQ for standard solutions were calculated based on signal-to-noise ratios of 3:1 and 10:1,

283 respectively. The LOD and LOQ values (ng/mL) were 1 and 2 for PhIP, 2 and 5 for MeIQ, 2 and 5
284 for MeIQx, and 2 and 6 for Trp-P-1, respectively. The recovery ranged from 67 to 86%.
285
286 **Statistical analysis**
287 Antioxidant characteristics are reported as means ± standard deviations (SD) from 3
288 replicate measurements. Milk, meat-protein and meat model systems were prepared in
289 duplicate, while extraction procedure was performed in duplicate for each replicate. Each
290 replicate was analysed in triplicate in LC-MS/MS and UPLC systems. Multivariance analysis with
291 Turkey test as a post hoc analysis was used for statistical data assessment ($P < 0.05$).
292
293 **Results and discussion**
294 **Evaluation of antioxidant properties of** lingonberry leaves
295 **Extraction yields and** total phenolic content **of** lingonberry leaf **extracts**
296 In case of raw lingonberry leaves the highest and the lowest extraction yields were obtained
297 with methanol and acetone, respectively, whereas deodorized lingonberry leaves gave 2-fold
298 lower extract yields than raw lingonberry leaves (Table 1). In general, the yields decreased in
299 the following order: ME > WE > AE > DME > DAE. The differences in the ME and WE yields were
300 not large; both solvents are high polarity compounds, whereas methanol is known as one of the
301 most effective solvents for the extraction of low sugar content plant materials. Moreover,
302 methanol extraction was performed at the increased pressure, which facilitates diffusion and
303 solubilization processes. Acetone is less effective solvent for higher polarity constituents, while
304 DME and DAE were prepared from the solid hydrodistillation residue, which has already been

305 extracted by water. The highest amounts of total phenolics expressed in mg GAE/g pdw were
306 recovered with ME followed by WE, AE, DME, and DAE. More than 2-fold lower total phenolic
307 content was determined for the deodorized than for the raw lingonberry leaf extracts. Recent
308 study (Bujor, Ginies, Popa, & Dufour, 2018) reported total phenolic content for lingonberry
309 leaves in the range of 85.3 - 114.6 mg GAE/g DM, which is in agreement with our data
310 (57.42±1.19–91.15±1.3 mg GAE/g pdw).
311

312 **Radical scavenging properties of** lingonberry leaf **extracts**

313 $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging and ORAC values are presented in Table 1. DAE was the weakest
314 $DPPH^{\bullet}$ scavenger while the differences between EC_{50} values of WE, ME, AE, and DME were not
315 significant in this assay. It was also observed that lingonberry leaf extracts scavenged $DPPH^{\bullet}$
316 rather slowly; the reaction between antioxidants and radical was completed in approx. 35 min.
317 In $ABTS^{\bullet+}$ and ORAC assays the antioxidant capacity of extracts was decreasing in the following
318 order: WE > ME > AE > DME > DAE. A significant correlation exists between total phenolic
319 content (mg GAE/g pdw) and $DPPH^{\bullet}$ scavenging ($r = -0.70$), $ABTS^{\bullet+}$ scavenging ($r = 0.87$) and
320 ORAC ($r = 0.95$) values.

321 In general, high polarity protic solvents methanol and water gave higher yields, total
322 phenolic content and antioxidant capacity values than lower polarity solvent acetone. It
323 indicates that lingonberry leaves contain higher amounts of polar polyphenolic compounds.
324 Therefore, extraction yields, total phenolic content and antioxidant capacity values of extracts
325 obtained from the deodorized material were remarkably lower in comparison to the extracts
326 from the raw material; the main part of water-soluble compounds remains in WE after hydro-

327 distillation of lingonberry leaves. It should be noted that comprehensive *in vitro* evaluation of
328 radical scavenging capacity of different lingonberry leaf extracts is reported for the first time.
329
330 **Comparison of phenolic profiles of different** lingonberry leaf **extracts**
331 The UV chromatograms and the relative amounts (based on UPLC-qTOF-MS peak areas) of
332 phenolic compounds in different lingonberry leaf extracts are presented in Figure 1 and Table 2.
333 Twenty phenolic compounds were positively or tentatively identified in lingonberry leaf extract.
334 Authentic standards were used for identification of catechin, quinic, chlorogenic, caffeic, citric
335 and *p*-coumaric acids, rutin and quercetin-3-O-glucoside. Other compounds were tentatively
336 identified by comparing the exact masses, fragmentation patterns, t_R by using databases such
337 as METLIN and Human Metabolome (HMDB) (Benton, Wong, Trauger, & Siuzdak, 2008) and
338 previously reported data for those compounds in lingonberry leaves (Ek, Kartimo, Mattila, &
339 Tolonen, 2006; Liu et al., 2014). It should be noted that so far as standards were not available
340 for these compounds their identification is assumed as tentative. ME and AE demonstrated
341 lower recovery of phenolics, expressed in AU×10^{-7}/mg edw, than WE by 37 and 51%,
342 respectively. Lower recovery of phenolics by acetone may be explained by the prevalence of
343 higher polarity compounds in lingonberry leaves, which are better soluble in protic solvents.
344 Finally, DME and DAE contained lower amounts of total phenols by 53 and 81% than WE,
345 respectively. The content of phenolics in deodorized plant extracts was considerably lower
346 comparing with the extracts from the raw lingonberry leaves, because the main part of polar
347 compounds was dissolved in water during hydro-distillation. It indicates that after water

348 extraction of raw lingonberry leaves some part of extractable phenolics still remained in
349 deodorized lingonberry leaves and were recovered by methanol and acetone.
350 The main classes of phenolics in WE were hydroxycinnamic acids and flavonols while arbutin
351 derivatives and flavan-3-ols were more abundant in ME and AE. The dominant flavonols in all
352 extracts were pentosides and hexosides of quercetin, which is in agreement with previous
353 studies (Ek et al., 2006, Liu et al., 2014). Exact mass and fragmentation patterns indicated that
354 the compounds ***16*** and ***17*** may be quercetin-3-*O*-xyloside and quercetin-3-*O*-arabinoside. This
355 assumption is also supported by the order of their elution, which was determined in previous
356 study (Liu et al., 2014). Caffeic acid ***7***, *p*-coumaric acid ***8*** and quercetin-pentoside ***16*** were found
357 only in WE, DME and DAE indicating that detectable amounts of these compounds were
358 released after hydro-distillation. Also, citric acid ***4***, chlorogenic acid ***6***, *B*-type proanthocyanidin
359 ***12*** and rutin ***14*** were found only in WE.
360 The differences in the yields, antioxidant capacity and total phenols for WE and ME were not
361 considerable. This is a positive result indicating that green and cheap solvent water may be
362 used for preparing phenolics-rich extracts from lingonberry leaves on industrial scale. However,
363 the profile of phenols in WE was different compared to ME and AE, while in the latter extracts it
364 was quite similar. WE and AE were chosen for further evaluation of their possible inhibition of
365 toxic Maillard reaction products.
366
367 **Effects of** lingonberry leaves **on** Maillard reaction products
368 **The effect of WE, quinic acid and catechin on furosine and CML formation**

369 In this experiment, the antiglycation activity of WE, quinic acid and catechin was accessed
370 through the furosine and CML determination in milk model system. Since WE exhibited the
371 highest antioxidant potential it was chosen for this experiment. To identify possible active
372 components in the lingonberry leaves, the standards of quinic acid and catechin, were further
373 evaluated. Quinic acid was among the major constituents in WE, while catechin was the
374 strongest radical scavenger as it was determined by the on-line HPLC-DPPH$^{\bullet}$ scavenging method
375 (data not shown).

376 The effect of WE on furosine and CML formation is represented in Figure 2A1 and A2,
377 respectively; the former was better inhibited at lower, while the latter one at higher
378 concentrations of WE. For instance, at 0.05 and 0.1 mg/mL WE reduced furosine level by 32 and
379 21% after 30 s of heating and by 18 and 27% after 45 s of heating, respectively. CML formation
380 was reduced by 38 and 19% after 30 s of heating and by 42 and 38% after 45 s of heating at 0.1
381 and 0.3 mg/mL WE addition, respectively.

382 The effect of quinic acid and catechin on furosine and CML formation is presented in Figure
383 2B and C, respectively. Quinic acid and catechin reduced the levels of furosine in milk model
384 system after 30 s heating by 35% at all concentrations; while after 45 s heating, its level was
385 reduced by approx. 10 and 40% at 0.05 mg/mL and 0.3 mg/mL concentrations, respectively. The
386 formation of CML was even more efficiently inhibited than furosine. The average decrease of
387 CML at both heating times was approx. by 31, 29, and 43%, and 31, 39, and 51% when 0.05, 0.1
388 and 0.3 mg/mL of quinic acid (Figure 2B2) and catechin (Figure 2C2) were added, respectively.
389 The reduction of CML and furosine by catechin can be explained through two pathways: first,
390 through the reaction between amino groups and *o*-quinoidal moieties firming via B ring

391 oxidation; and second, through the trapping of carbonyl-containing sugar fragments at highly
392 activated A ring. There are no reports showing suppressing effect of quinic acid on Maillard
393 reaction products formation in milk. However, the protecting effect against protein
394 carbonylation was reported previously (Yoshimura et al., 2016). Quinic acid is a cyclic hydroxyl-
395 acid that is present in various fruits and vegetables; therefore, it would be interesting to
396 continue the studies for clarifying reducing effect of quinic acid on the formation of Maillard
397 reaction products.

398 In general, the inhibition effect of WE on Maillard reaction products formation depended on
399 heating time and extract concentration. The reduction of both Maillard reaction products can
400 be associated with the above-described ways. Given their antiglycation activity and relatively
401 high abundance in leaves, catechin and quinic acid may be suggested as the main active
402 compounds responsible for CML and furosine inhibition. Catechin, quercetin-3-*O*-galactoside
403 and cyanidin-3-*O*-glucoside were reported as the main contributors to the antiglycation
404 properties of *V. vitis-idaea* berry extracts (Beaulieu et al., 2010).

405 **The effect of WE and AE on the formation of HAs**

406 In this experiment, the effects of WE and AE of lingonberry leaves on the formation on HAs
407 were tested in two model systems: meat-protein and meat. In a meat-protein model system,
408 the interference of meat matrix components (lipids, proteins, water) was eliminated, by using
409 only the substances, which are necessary for the formation of HAs. As only phenylalanine was
410 used, mainly PhIP formation was generated; therefore the effect of lingonberry leaf extracts on
411 the formation of PhIP was evaluated in meat-protein model system. Meat model system

412 consisted of a real meat-like matrix, from which only the water factor was eliminated; in this
413 case the formation of HAs was evaluated measuring several typical to roasted beef HAs.
414 The effect of lingonberry leaves extracts on PhIP formation in meat-protein model system is
415 shown in Figure 3. The amounts of PhIP significantly increased when WE were added in the
416 range of 0.1-5 mg/mL and decreased by 20% with the addition of 10 mg/mL; while significant
417 effect of AE was observed only at 10 mg/mL when PhIP level was reduced by 40%. Better
418 inhibitory effect observed in case of AE, possessing weaker radical scavenging and total
419 phenolic content than WE, may seem somewhat unexpected. According to our results and
420 previously reported observations (Hidalgo & Zamora, 2018), it may be assumed that different
421 effect of WE and AE on PhIP formation could be due to the different composition of phenolics:
422 WE contained more hydroxycinnamic acids, proanthocyanidins and flavonols than AE. The
423 amounts of hydroxycinnamic acids as quinic, protocatechuic, chlorogenic, caffeic and *p*-
424 coumaric were in traces or even undetectable in AE. Phenolic acids, e.g. chlorogenic (Cheng,
425 Chen, & Wang, 2007), were shown to be ineffective inhibitors or even enhancers of PhIP
426 formation. Moreover, AE comparing to WE contained higher amounts of flavan-3-ols such as
427 catechin and chinchonain *1* and arbutin derivative (2-O-caffeoylarbutin). Green tea catechins in
428 many studies were reported as effective inhibitors of HAs (Cheng et al., 2009; Quelhas et al.,
429 2010). Those differences in the composition of phenolics explain different effect of lingonberry
430 leaf extracts on PhIP formation in the analysed meat-protein model system.
431 The effects of lingonberry leaves extracts on the formation of four HAs in meat model
432 system are presented in Figure 4. In this model four HAs were quantified; the dominant
433 compound was PhIP (266.84±6.67 ng/g of beef dry weight), whereas MeIQ, MeIQx and Trp-P-1

434 were found in remarkably lower amounts. Trp-P-1 is a carboline type HA and in cooked meat
435 usually forms at higher temperatures (> 200 °C); however, probably due to prolonged heating
436 (120 min) it was also formed in the used beef-model system. WE and AE reduced PhIP
437 concentration dose-dependent by 3.24, 10.55, 39.29 and 14.42, 26.85, 37.43%, respectively.
438 Consequently, the opposite effects of WE addition on the formation of PhIP were observed in
439 the more real-like meat model system: WE did not promote PhIP formation. It may be
440 explained by the different formation mechanisms of PhIP in chemical and real-like meat model
441 systems, as was reported previously (Cheng et al., 2007). However, the levels of MeIQ, MeIQx
442 and Trp-1 were increased in the samples with lingonberry leaf extracts and WE showed
443 stronger promoting effect than AE. The addition of 10 mg/mL of WE showed the highest
444 promoting effect on the formation of MeIQ, MeIQx and Trp-1 and the highest reducing effect
445 on PhIP formation. These results might be explained by different HAs formation mechanisms,
446 which largely depend on their structures. For instance, MeIQ belongs to imidazoquinoline,
447 while MeIQx to imidazoquinoxaline class of Has. The precursors of MeIQ and MeIQx are
448 pyridine and pyrazine free radicals, respectively, forming *via* Maillard reaction between sugars
449 and amino acids during Strecker degradation. Catechin and EGCG influenced the formation of
450 pyrazinium radicals by reacting with imine intermediates: moreover, at low concentrations (5-
451 20 mM) it increased and at high concentrations (≥50 mM) decreased their formation. Thus, the
452 formation of MeIQ and MeIQx could be enhanced through the generation of pyridine and
453 pyrazine radicals. As PhIP belongs to imidazopyridine type compound, it is formed from the
454 condensation of phenylacetaldehyde and creatinine. EGCG has been shown as an effective

455 inhibitor of phenylacetaldehyde, which is an important intermediate product in PhIP formation
456 (Cheng et al., 2009).

457 Based on the obtained data, we can assume that WE may act as a generator of Trp-P-1,
458 MeIQ and MeIQx precursors, and as a scavenger of intermediates in the formation of PhIP in
459 meat model system. In general, AE was more potential additive for reducing HAs formation in
460 both model systems.

461

462 **Conclusions**

463 The results of our study indicate that lingonberry leaf extracts possess the ability of tuning
464 Maillard reaction in food models. WE inhibited CML and furosine formation in milk model
465 system during its heating; moreover the inhibitory effect was stronger for the formation of CML
466 than furosine. This finding suggests that WE is stronger inhibitor of the advanced stage Maillard
467 reaction products compared to the early stage products. Phenolic compounds such as catechin
468 and quinic acid, which were abundant in WE, showed strong suppressing effect on CML and
469 furosine formation in milk model system; consequently, these compounds may be important
470 lingonberry leaves extract constituents responsible for CML and furosine inhibition. Results of
471 extract's effects in meat models systems suggest that inhibition effect is more related to the
472 phenolic compounds profile than to the radical scavenging capacity or total phenolic content.
473 Accordingly, it may be preliminary hypothesized that hydroxycinnamic acids might act as
474 enhancers, while flavan-3-ols and arbutin derivatives as inhibitors of HAs. Moreover, effective
475 phytochemical composition of such extracts could be purposively designed by using different
476 organic solvents and procedures for extractions. In general, the results obtained show that

477 lingonberry leaf extracts are promising ingredients for their applications in foods both for
478 controlling the formation of Maillard reaction products during thermal processing and for
479 enriching them with natural antioxidants possessing health benefits.

480 In addition, the results supports the need of further studies for the evaluation of the
481 mechanisms of the involvement of plant phytochemicals in the Maillard reaction to ensure
482 optimal application of natural plant origin ingredients in suppressing the formation of heat-
483 induced toxic compounds in foods.

484

485 **Acknowledgments**

486 The study was supported by Research Council of Lithuania, grant no. S-MIP-17-100.

487

488 **Author Contributions**

489 I. Račkauskienė performed the major part of experimental work (data acquisition, statistical
490 analysis and data interpretation) and prepared the manuscript draft and finalized it after
491 approval by the other co-authors. P.R. Venskutonis conceptualized and designed the study,
492 critically reviewed the data and finally approved the manuscript. A. Pukalskas, A. Fiore and A.D.
493 designed the study, performed part of experimental work, interpreted data, critically reviewed
494 it and finally approved the manuscript.

495

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650 **Table 1–The yield,** total phenolic content **and antioxidant activity of different extracts of** lingonberry leaves**.**

LL Extract	**Yield (g/100 g pdw)**	**TPC (mg GAE/edw)**	**TPC (mg GAE/g pdw)**	**DPPH, EC_{50} (mg/mL)**	**ABTS (mM TE/g edw)**	**ABTS (mM TE/g pdw)**	**ORAC (mM TE/g edw)**	**ORAC (mM TE/g pdw)**
WE	32.96±1.90^{d}	252.22±4.40^{c}	83.12±1.45^{d}	2.40±0.01^{a}	11.16±0.55^{d}	3.68±0.18^{d}	10.63±0.88^{e}	3.50±0.29^{e}
ME	36.38±2.51^{e}	250.56±3.62^{c}	91.15±1.31^{e}	2.36±0.00^{a}	6.05±1.55^{c}	2.20±0.57^{c}	7.44±1.05^{d}	2.71±0.38^{d}
AE	23.69±2.60^{c}	242.42±5.05^{c}	57.42±1.19^{c}	2.52±0.01^{a}	4.33±0.23^{b}	1.03±0.05^{b}	4.43±0.23^{b}	1.44±0.10^{c}
DME	14.09±0.60^{b}	141.80±3.91^{b}	19.98±0.55^{b}	2.54±0.00^{a}	3.07±0.63^{b}	0.43±0.09^{a}	6.08±0.42^{c}	0.62±0.03^{b}
DAE	9.57±0.50^{a}	96.52±8.53^{a}	9.24±0.82^{a}	5.11±0.58^{b}	1.32±0.53^{a}	0.13±0.05^{a}	1.62±0.09^{a}	0.16±0.00^{a}

651 Data represent mean ± SD of triplicate analyses: different superscript letters ($^{a–e}$) with in a column indicate significant differences

652 between the extracts ($P < 0.05$). Abbreviations: LL, lingonberry leaves; TPC, total phenolic content; WE, water extract; ME, methanol

653 extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone extract; edw, extract dry weight; pdw,

654 plant dry weight.

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Table 2– Compounds identified in the extracts of *Vaccinium vitis-idaea* L. leaves and their UPLC/qTOF–MS/MS data and the content

No.	Compound	t_R (min)	$[M-H]^-$ (*m/z*)	Molecular ion formula	MS/MS (*m/z*)	Lingonberry leaves extracts				
						WE	ME	AE	DME	DAE
1	Catechin[1]	1.28	289.0719	$C_{15}H_{13}O_6$	245.0826(100), 179.0306(25)	31.85±0.23^{c}	32.18±0.56^{c}	36.48±0.31^{d}	25.37±0.08^{b}	5.93±0.15^{a}
2	Epicatechin[2]	1.55	289.0716	$C_{15}H_{13}O_6$	245.0825(60), 179.0306(19)	14.62±0.35^{a}	32.18±0.57^{b}	14.33±0.16^{a}	18.56±9.5^{ab}	1.53±0.01^{a}
	Total Flavan-3-ols					***46.47***	***64.36***	***50.81***	***43.93***	***7.46***
3	Quinic acid[1]	0.35	191.0564	$C_7H_{11}O_6$	–	42.81±2.52^{c}	25.46±0.56^{b}	2.00±0.02^{a}	1.61±0.12^{a}	TR
4	Citric acid[1]	0.43	191.0201	$C_6H_7O_7$	111.0083(20)	46.48±0.32	ND	ND	ND	ND
5	Caffeoyl-shikimic acid[2]	1.08	335.0775	$C_{20}H_{15}O_5$	179.0340(55), 161.0237(100)	2.85±0.02^{e}	2.22±0.10^{d}	1.96±0.02^{c}	1.25±0.02^{b}	0.72±0.05^{a}
6	Chlorogenic acid[1]	1.33	353.0874	$C_{16}H_{17}O_9$	–	3.44±0.05^{a}	TR	TR	TR	ND
7	Caffeic acid[1]	1.47	179.0353	$C_9H_7O_4$	–	2.62±0.04^{b}	ND	ND	1.04±0.00^{a}	0.98±0.08^{a}
8	*p*-Coumaric acid[1]	1.87	163.0402	$C_9H_7O_3$	–	1.56±0.16^{b}	ND	ND	0.92±0.02^{a}	1.57±0.04^{b}
	Total hydroxycinnamic acids					***99.76***	***27.68***	***3.96***	***4.82***	***3.27***
9	Arbutin[2]	0.49	271.0824	$C_{12}H_{15}O_7$	108.0218(100), 109.0275(8)	33.18±0.03^{d}	31.78±0.67^{d}	23.00±0.43^{c}	17.48±0.08^{b}	9.61±0.05^{a}
10	2-*O*-caffeoylarbutin[2,3]	1.70	433.1147	$C_{21}H_{21}O_{10}$	323.0778 (5), 179.0350 (30), 161.0245 (100), 135.0449 (10)	24.11±1.01^{c}	29.85±0.35^{d}	34.34±0.24^{e}	21.92±0.07^{b}	10.21±0.23^{a}
11	Caffeoyl acetyl arbutin[3]	2.56	475.1242	$C_{23}H_{23}O_{11}$	179.0347(15), 161.0244(100)	8.51±0.14^{b}	8.82±0.00^{b}	10.97±0.22^{c}	10.49±0.31^{c}	6.05±0.08^{a}
	Total Arbutin derivatives					***65.8***	***70.45***	***68.31***	***49.89***	***25.87***
12	B-type proanthocyanidin[2]	1.34	577.1336	$C_{30}H_{25}O_{12}$	407.0757(68), 289.0709(100), 245.0807(21), 161.0233(15), 125.0240(35)	8.35±0.35^{a}	TR	TR	TR	ND
13	A-type proanthocyanidin[2,3]	1.95	575.1195	$C_{30}H_{23}O_{12}$	539.0998(65), 407.0791(48), 285.0387(100), 125.0245(58)	3.67±0.03^{c}	0.66±0.03^{b}	0.66±0.19^{b}	0.49±0.00^{ab}	0.27±0.07^{a}
	Total proanthocyanidins					***12.02***	***0.66***	***0.66***	***0.49***	***0.27***
14	Rutin[1]	1.84	609.1459	$C_{27}H_{29}O_{16}$	300.0267(100), 301.0332(30)	1.53±0.03^{a}	TR	TR	TR	ND
15	Quercetin-3-*O*-glucoside[1]	1.95	463.0881	$C_{21}H_{19}O_{12}$	301.0339(66), 300.0272(100)	3.33±0.05^{b}	3.31±0.05^{b}	3.15±0.20^{b}	3.26±0.11^{b}	1.12±0.09^{a}

16	Quercetin pentoside 1[2,3]	2.10	433.0773	$C_{20}H_{17}O_{11}$	301.0337(56), 300.0266(100), 271.0225(5)	20.43±0.07^{c}	ND	ND	18.20±0.32^{b}	7.70±0.25^{a}
17	Quercetin pentoside 2[2,3]	2.25	433.0769	$C_{20}H_{17}O_{11}$	301.0345(58), 300.0274(100), 271.0238(5)	7.71±0.28^{b}	7.44±0.11^{b}	7.68±0.16^{b}	9.34±0.26^{c}	4.17±0.17^{a}
18	Quercetin-deoxyhexoside[2,3]	2.28	447.0931	$C_{21}H_{19}O_{11}$	301.0338(23), 300.0252(100), 271.0227(86)	15.95±0.08^{e}	15.24±0.18^{d}	12.38±0.14^{c}	10.80±0.07^{b}	7.70±0.00^{a}
19	Quercetin-3-*O*-(HMG)-rhamnoside[3]	2.67	591.1355	$C_{27}H_{27}O_{15}$	529.1334(27), 489.1028(100), 447.0924(72), 301.0269(65)	29.80±0.10^{d}	0.41±0.03^{b}	0.61±0.02^{c}	0.29±0.01^{a}	0.30±0.01^{a}
20	Kaempferol-3-*O*-(HMG)-rhamnoside[3]	2.95	575.1392	$C_{27}H_{27}O_{14}$	515,1377(15), 473.1075(40), 431.0969(38), 285.0391(100)	1.78±0.02^{d}	1.07±0.02^{b}	1.46±0.08^{c}	1.02±0.06^{b}	0.38±0.01^{a}
	Total flavonols					***80.53***	***27.47***	***25.28***	***42.91***	***21.37***
	Total phenols					**304.58**	**190.62**	**149.02**	**142.04**	**58.24**

658 Data represent mean (AU×10^{-7}/mg edw) ± SD of triplicate analyses: different superscript letters ($^{a–e}$) with in a row indicate significant
659 differences between the extracts (*P* < 0.05). [1]Compounds identified by comparing with standard. [2]Compounds identified tentatively
660 by calculated molecular formula and fragmentation patterns using METLIN and/or HMDB databases. [3]Compounds identified
661 tentatively by calculated molecular formula and fragmentation patterns comparing to literature data (Ek et al., 2006; Liu et al., 2017).
662 Abbreviations: WE, water extract; ME, methanol extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized
663 acetone extract; edw, extract dry weight; NI, not identified; –, not performed; ND, not detected; TR, traces.
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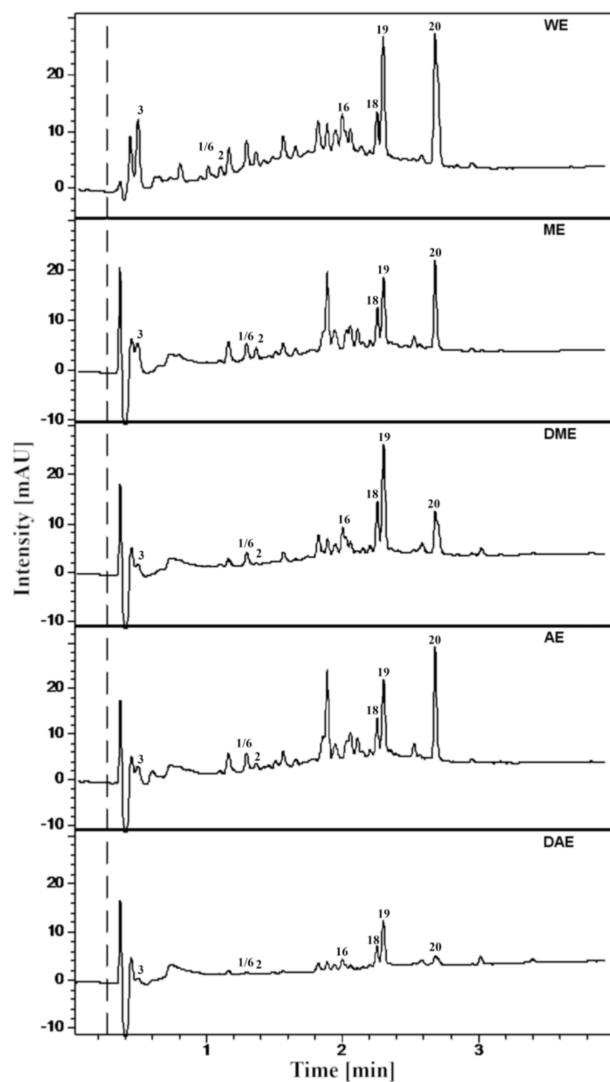


667

668 **Figure 1**–UPLC-UV chromatograms at 254 nm of lingonberry leaf extracts. Peak numbers 669 correspond to the compounds listed in Table 2. Abbreviations: WE, water extract; ME, methanol 670 extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone 671 extract.

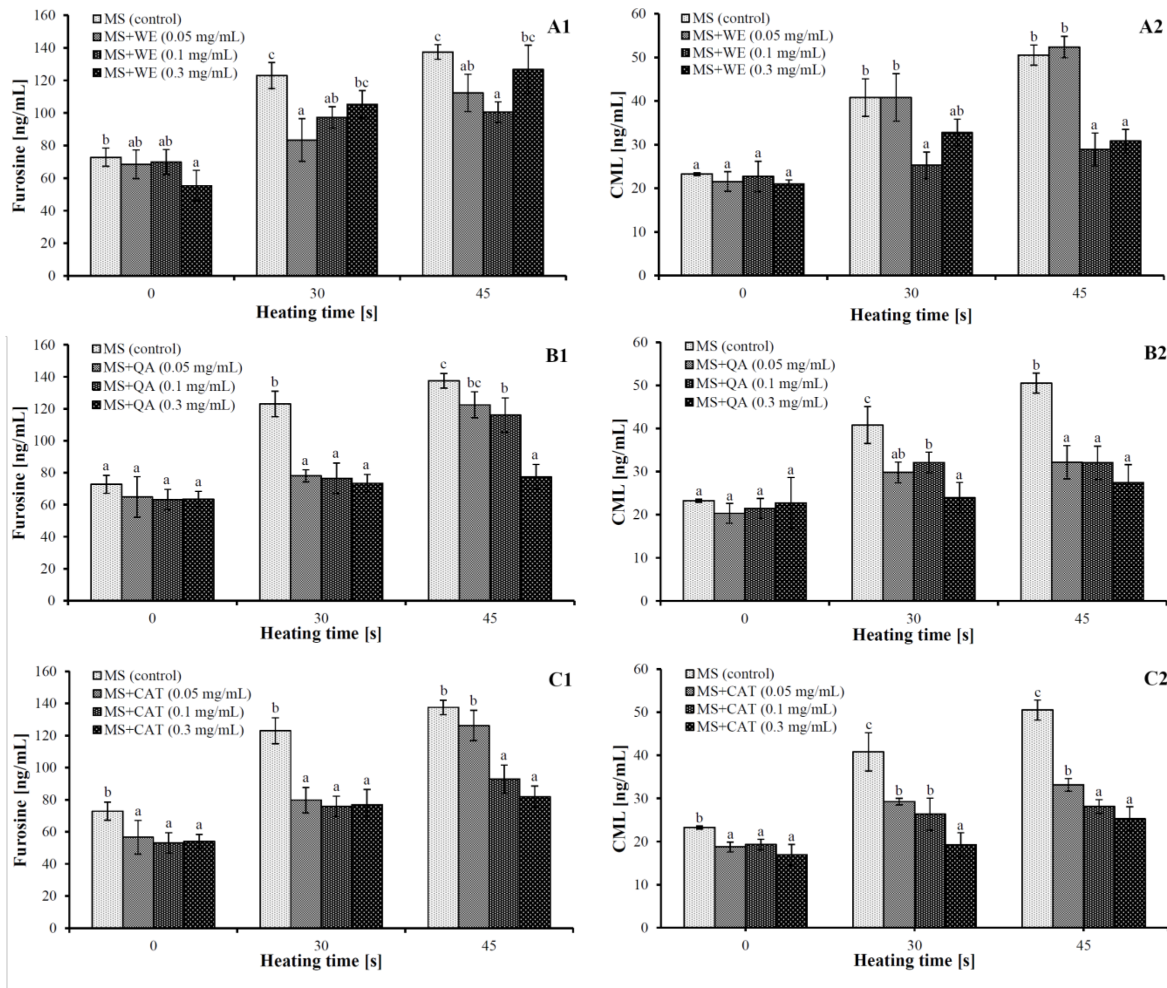
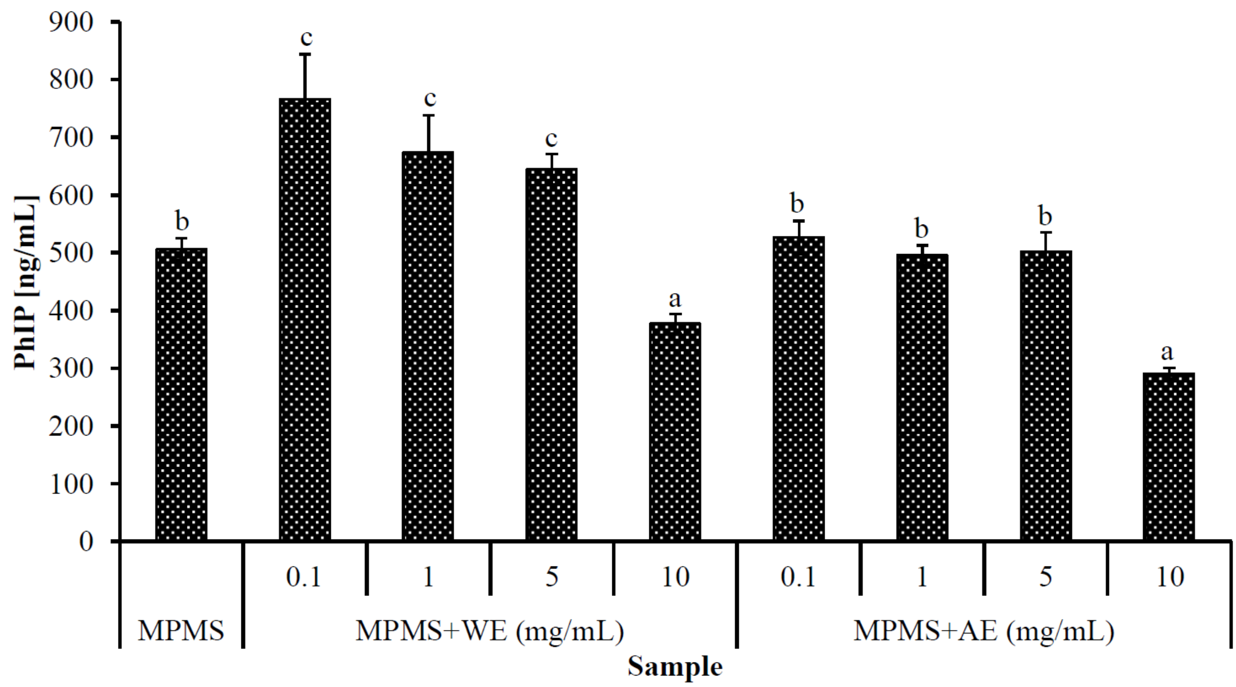


Figure 2–Effects of WE, quinic acid and catechin on furosine and CML formation in milk model system at different heating times: the different letters on the columns at the same heating time indicate the significant differences (*P* < 0.05). Abbreviations: MS, milk model system; QA, quinic acid; CAT, catechin; WE, water extract.



677

678 **Figure 3**–Effects of WE and AE of lingonberry leaves on PhIP formation in meat-protein model

679 system. The different letters on the columns indicate the significant differences ($P < 0.05$).

680 MPMS: meat-protein model system; WE: water extract; AE: acetone extract.

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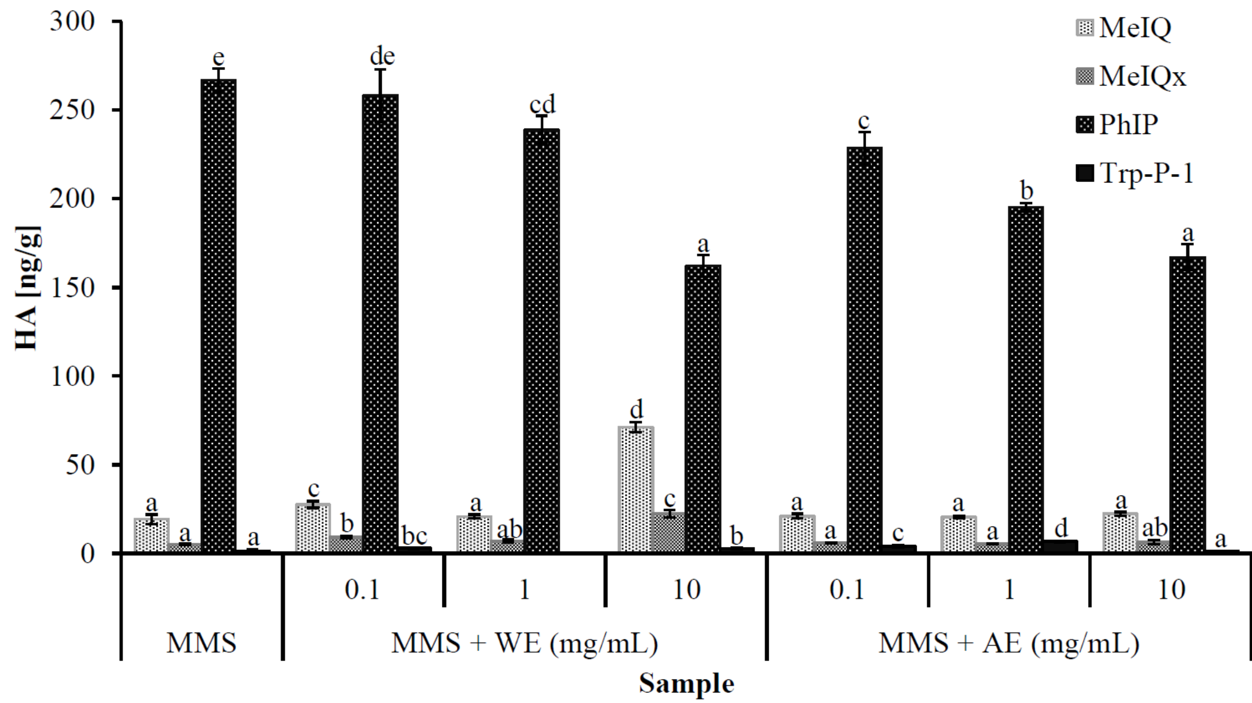

682 **Figure 4**–Effects of WE and AE of lingonberry leaves on HAs formation in meat model system.

683 The different letters on the columns at the same HA indicate the significant differences (*P* <

684 0.05). MMS: meat model system; WE: water extract; AE: acetone extract.