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**Use of winemaking by-products as an ingredient for tomato puree: the effect of particle size on product quality**

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31 **USE OF WINEMAKING BY-PRODUCTS AS AN INGREDIENT FOR TOMATO PUREE: THE**  
32 **EFFECT OF PARTICLE SIZE ON PRODUCT QUALITY**

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43 **Running title:** Grape skin as ingredient for tomato puree

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46 ABSTRACT

47 Formulations of tomato puree with grape skin fibres (Chardonnay variety) having varying particle sizes were  
48 studied. The contents of flavonoids (by HPLC-DAD) and proanthocyanidins (*n*-butanol/HCl assay), reducing  
49 capacity (ferric ion reducing antioxidant power, FRAP) and anti-glycation activity by a bovine serum  
50 albumin (BSA)/fructose model system were analysed *in vitro*. A liking test was performed with consumers.  
51 Stabilization was carried out by either an intensive autoclave treatment or an optimized microwave-treatment  
52 achieving 6D-reduction of the target microorganism (*Alicyclobacillus acidoterrestris*). In the fortified tomato  
53 purees, proanthocyanidins' solubility decreased, but it was partly restored by autoclave treatment, which also  
54 caused deglycosylation of flavonol glycosides. Microwave treatment did not show any effect on phenolics.  
55 The reducing capacity and ability to inhibit protein glycation greatly increased in the fortified purees. The  
56 particle sizes of solids in the formulations played a major role with respect to the consumers' liking, with the  
57 smallest ones showing maximum ratings.

58

59 KEYWORDS

60 Tomato, grape skins, reducing capacity~~antioxidant activity~~, *in vitro* anti-glycation activity, liking

61

## 62 1. Introduction

63 The food industry is facing the challenge of developing new foods having increased health benefits and  
64 meeting consumers' appreciation. In fact, with the surge in the incidence of cardiovascular diseases, cancer  
65 and type-2 diabetes, there is a need to develop new dietary strategies, especially with reference to the  
66 potential health properties of underutilized by-products of food processing ([Schieber, Stintzing, & Carle,  
67 2001; Hokayem et al., 2013](#)~~[Roekenbach, Rodrigues, Gonzaga, Caliani, Genovese, Gonçalves, & Fett, 2011](#)~~).

68 Grape (*Vitis vinifera*) pomace, the by-product of winemaking, is a bioresource available on large-scale as  
69 grape constitutes one of the main fruit crops in the world. Grape pomace contains both phenolics and dietary  
70 fibres, thus it can be referred to as “antioxidant dietary fibre”. Because of the close relationship between  
71 antioxidant and dietary fibre and their common fate in the gut, it has been proposed that these food  
72 components have a joint role in prevention of human diseases (Perez-Jimenez et al., 2008). ~~[Grape and wine  
73 phenolics have been demonstrated to inhibit human low density lipoprotein oxidation in vitro. In vivo studies  
74 on human adults](#)~~ have demonstrated that grape pomace has a positive effect in the prevention of  
75 cardiovascular diseases (Perez-Jimenez et al., 2008; ~~[Saura-Calixto et al., 2010](#)~~). ~~[Grape skin extracts from  
76 Vitis rotundifolia and Vitis vinifera can also inhibit fructose and methylglyoxal mediated protein glycation  
77 in vitro, thus having a potential role in preventing hyperglycaemia's complications \(Farrar, Hartle, Hargrove,  
78 Greenspan, 2007; Sri Harsha, Gardana, Simonetti, Spigno, & Lavelli, 2013\). The anti-diabetic efficiency of  
79 An anti diabetes effect has been demonstrated when ggrape polyphenols derived extracts with high amounts  
80 of proanthocyanidins wasere tested supplemented in type-2 diabetic patients to the diet of high fructose fed  
81 rats, resulting in improved insulin resistance and suppressed oxidative stress \(\[Hokayem et al., Dandona,  
82 Aljada, Chaudhuri, Mohanty, & Garg, 2013\]\(#\)05\).](#)~~

83 These results have boosted the use of grape pomace as an ingredient for new functional foods, such as bread  
84 (Mildner-Szkudlarz, Zawirska-Wojtasiak, Szwengiel, & Pacynski, 2011), fish products (PazosTorres,  
85 Medina, 2005; Ribeiro, Cardoso, Silva, Serrano, Ramos, & Santos, 2013), meat products (Sayago-Ayerdi,  
86 Brenes, & Goni, 2009) and yogurt (Tseng & Zhao, 2013). The development of foods that provide additional  
87 health benefits beyond basic nutrients is also a trend in the fruit processing industry (Augusto, Falguera,  
88 Cristianini, & Ibarz, 2011).

89 The aim of the present study was to assess the prospective use of a phytochemical- and fibre-rich ingredient  
90 recovered from winemaking by-products for the development of a new tomato-based product. Technological  
91 challenges raised by fortification were studied, such as: the choice of the particle size of the suspension, the  
92 incorporation of an adequate level of the new ingredient, the choice of pasteurization conditions, the  
93 processing effect on phenolic stability and the need to address consumers' liking.

## 94 **2. Materials and methods**

### 95 *2.1. Chemicals*

96 Standards of catechin, quercetin 3-O-rutinoside (rutin), quercetin 3-O-glucuronide, quercetin 3-O-glucoside,  
97 kaempferol 3-O galactoside, kaempferol 3-O glucuronide, kaempferol 3-O glucoside, quercetin, kaempferol  
98 and naringenin were purchased from Extrasynthese (Lyon, France). The integrated total dietary fibre assay  
99 procedure kit was purchased from Megazyme International Ireland Ltd (Bray, Ireland). All other chemicals  
100 were purchased from Sigma Aldrich Italia (Milan, Italy).

### 101 *2.2. Grape skins*

102 Grape pomace samples of the Chardonnay (Ch) variety were kindly provided by a winery located in  
103 Northern Italy. At the winery, Ch grapes were pressed with separation of grape solids and must. Then grape  
104 stalks were separated with a mechanical destemming and the remaining material was sieved (with a 5 mm  
105 sieve) to separate the skins from the seeds and frozen to inhibit microbial growth. The skins were transported  
106 frozen to the lab, dried at 50 °C for about 8 h. The powders obtained were sieved by using the Octagon  
107 Digital sieve shaker (Endecotts L.t.d., United Kingdom), with three certified sieves (openings: 125, 250 and  
108 500µm), under continuous sieving for 10 min at amplitude 8. Three fibrous fractions having different  
109 particle sizes were collected, namely: ChL (250µm < ChL ≤ 500µm), ChM (125µm < ChM ≤ 250µm) and  
110 ChS (ChS ≤ 125µm). These fractions were stored under vacuum, in the dark, at 4 °C.

### 111 *2.3. Tomato puree*

112 Two tomato puree samples, namely PV and PR were provided by Conserve Italia Soc. Coop. (San Lazzaro di  
113 Savena, Italy). At the industrial plant, tomatoes were homogenized and heated to approximately 95 °C by  
114 steam injection to inactivate endogenous enzymes (hot-break). The homogenate was then passed hot through  
115 a 0.5 mm-screen (PV) or a 1 mm-screen (PR) pulper/finisher to remove seeds and skin fragments and

116 deaerated under vacuum. The finished purees were then concentrated at 80 °C and under reduced  
117 atmospheric pressure using a tubular heat exchanger (the final moisture contents were  $89.1 \pm 0.2$  and  $89.8 \pm$   
118  $0.2$  for PV and PR, respectively). The purees were then aseptically stored in tank under nitrogen for 6 months  
119 before bottling. After bottling, the purees were autoclaved at 115 °C for 5.5 min.

#### 120 *2.4. Preparation of the fortified tomato purees*

121 An amount of 3.2 g of the ChL, ChM and ChS fractions was added to 96.8 g of the PV and PR tomato  
122 purees. Each puree was filled into different glass bottles (250 mL capacity). A set of the bottled fortified  
123 purees was then submitted to microwave heating (8 min at 900 watt). During heating, the temperature of the  
124 tomato puree was monitored continuously by using a thermocouple set in the geometric centre of one of the  
125 bottles (the slowest heating point).

126 To calculate the pasteurization effectiveness during microwave heating, *Alicyclobacillus acidoterrestris* was  
127 used as a target (Silva & Gibbs, 2004). Different heating conditions were tried and the resulting  
128 time/temperature curves were obtained. D values for the target microorganism were calculated as a function  
129 of temperature using the Bigelow's model, as reported below:

$$130 \quad D = D_{\text{ref}} * 10^{(T_{\text{ref}}-T)/z}$$

131 where for the target microorganism,  $D_{\text{ref}} = 1.5$  min,  $T_{\text{ref}} = 95$  °C and  $z = 7$ °C (Bevilacqua & Corvo, 2011).

132 The 1/D values were then plotted as a function of time and the resulting curves were then integrated to  
133 evaluate the total decimal reductions (Silva & Gibbs, 2004). Microwave conditions were then chosen in  
134 order to achieve 6D for the target microorganism.

135 Another set of bottled fortified purees was submitted to autoclave treatment (100 °C, 30 min).

#### 136 *2.5. Moisture, fibre, protein, carbohydrates, fat and ash contents*

137 Moisture content was determined by drying in a vacuum oven at 70 °C and 50 Torr for 18 h. Protein, fat, and  
138 ash contents were measured according to AOAC official methods of analysis (Tseng & Zhao, 2013).  
139 Glucose and fructose were determined as described in Lavelli, Pagliarini, Ambrosoli, Minati, & Zanoni  
140 (2006). Fibre contents were determined by the Megazyme total dietary fibre assay procedure (based on  
141 AOAC 991.43).

#### 142 *2.6. Sample extraction*

143 For grape skin powder extraction, an aliquot of 1 g was weighed in duplicate, added with 20 mL  
144 methanol:water:formic acid (70:29.9:0.1, v/v/v) and extracted for 2 h at 60 °C with continuous stirring. The  
145 mixture was centrifuged at 10000g for 10 min, the supernatant recovered and the solid residue was re-  
146 extracted using 10 mL of the same solvent. The supernatants were pooled.

147 For tomato puree extraction, 3.75 g was weighed in duplicate and added to 1.9 mL of water, 7 mL of  
148 methanol and 0.3 mL of formic acid (in order to use the same medium as for the grape skin fractions, taking  
149 into account the amount of water present in the puree). Extraction was performed as that of grape skin  
150 fractions. Extracts were stored at -20°C until analytical characterization.

### 151 2.7. Polyphenol analysis by HPLC-DAD

152 The HPLC equipment consisted of a model 600 HPLC pump coupled with a Waters model 2996 photodiode  
153 array detector, operated by Empower software (Waters, Vimodrone, Italy). A 2.6 µm Kinetex C<sub>18</sub> column  
154 (150 x 4.6 mm) equipped with a C<sub>18</sub> precolumn (Phenomenex, Castel Maggiore, Italy) was used for the  
155 separation at a flow-rate of 1.8 mL/min. The injection volume was 50 µL. The column was maintained at  
156 60°C and the separation was performed by means of a gradient elution using (A): 0.1% formic acid and (B):  
157 acetonitrile. The gradient was as follows: from 5% B to 15% B in 15 min, from 15% B to 20% B in 2 min,  
158 from 20% B to 90% B in 4 min; 90% B for 5 min and 5% B for 3 min. DAD analysis was carried out in the  
159 range of 200-600 nm. Standard compounds were used to identify peaks by retention times and UV-vis  
160 spectra. Calibration curves were built with catechin (280 nm), quercetin 3-O glucoside (reference compound  
161 for all flavonols, at 353 nm) and naringenin (at 288 nm). Concentrations of phenolic compounds were  
162 expressed as milligrams per kilogram of dry product.

### 163 2.8. Proanthocyanidin content

164 Proanthocyanidin content was analysed as described previously ([Porter, Hrstich, & Chan, 1986](#)[Sri Harsha et](#)  
165 [al., 2013](#)). Briefly, for evaluation of soluble proanthocyanidins 1 mL of the sample extract (opportunedly  
166 diluted with methanol:water:formic acid (70:29.9:0.1, v/v/v) was added to 6 mL of *n*-butanol:HCl (95:5, v/v)  
167 and 0.2 mL of 2% NH<sub>4</sub>Fe(SO<sub>4</sub>)<sub>2</sub>.12 H<sub>2</sub>O in 2M HCl. For evaluation of insoluble proanthocyanidins, 10 mg of  
168 the extraction residue was weighted in quadruplicate and added to 20 mL methanol, 120 mL *n*-butanol:HCl  
169 (95:5, v/v) and 4 mL of 2% NH<sub>4</sub>Fe(SO<sub>4</sub>)<sub>2</sub>.12 H<sub>2</sub>O in 2M HCl. Hydrolysis was carried out at 95 °C for 40



170 min. The reaction mixtures were cooled and the absorbance was recorded at 550 nm on a Jasco UVDEC-610  
171 spectrophotometer (Jasco Europe, Cremella, Italy) against a blank made as for the sample but incubated at  
172 room temperature. For each sample extract, 2 - 4 dilutions were assessed in duplicate. Proanthocyanidin  
173 amount was determined using 0.1736 (mg/mL) as conversion factor [\(Sri Harsha, Gardana, Simonetti,  
174 Spigno, & Lavelli, 2013\)](#) and expressed as grams per kilogram of dry product.

#### 175 *2.9. Ferric ion reducing antioxidant power (FRAP) assay*

176 The FRAP assay was performed as described previously (Sri Harsha et al., 2013). Briefly, FRAP reagent was  
177 prepared by adding 25 mL of 300 mM acetate buffer, pH 3.6; 2.5 mL of 10 mM 2,4,6-Tripyridyl-*s*-Triazine  
178 in 40 mM HCl and 2.5 mL of 20 mM FeCl<sub>3</sub>. The reaction mixture contained 0.4 mL of sample extracts  
179 opportunely diluted with methanol:water:formic acid (70:29.9:0.1, v/v/v) and 3 mL of FRAP reagent. The  
180 absorbance at 593 nm was evaluated on a Jasco UVDEC-610 spectrophotometer (Jasco Europe, Cremella,  
181 Italy) after 4 min of incubation at 37 °C against a blank with no extract addition. For each sample extract, 2 -  
182 4 dilutions were assessed in duplicate. A methanolic solution of FeSO<sub>4</sub>·7H<sub>2</sub>O was used for calibration.  
183 Results were expressed as millimoles of Fe(II) sulfate equivalents per kilogram of dry product.

#### 184 *2.10. Determination of fructose-induced glycation of bovine serum albumin (BSA)*

185 The inhibition of fructose-induced glycation of BSA was conducted as described in Lavelli & Scarafoni  
186 (2012). The reaction mixture consisted of 100 µL of sample extracts or standard (catechin) opportunely  
187 diluted with methanol:water:formic acid (70:29.9:0.1, v/v/v), 900 µL of phosphate buffer (200 mM  
188 potassium phosphate buffer, pH 7.4 with 0.02% sodium azide), 300 µL of BSA solution (50 mg/mL of BSA  
189 in phosphate buffer), and 300 µL of fructose solution (1.25 M fructose in phosphate buffer). A BSA solution  
190 (blank sample) and control reaction without sample addition were prepared in parallel. The reaction mixtures  
191 were incubated at 37 °C for 72 h. Following incubation, 1.6 mL of 20% trichloroacetic acid was added to the  
192 reaction mixture before centrifugation at 10000g for 10 min. The supernatant was discarded and the  
193 precipitate was re-dissolved in 1.6 mL of phosphate buffer and analyzed for fluorescence on a Perkin-Elmer  
194 LS 55 Luminescence Spectrometer (Perkin-Elmer Italia, Monza, Italy) with an excitation/emission  
195 wavelength pair  $\lambda = 370/440$  nm, 5 nm slit width, against phosphate buffer. For each sample extract, 3 - 4  
196 dilutions were assessed in duplicate. Catechin was analysed at six dilutions to build a calibration curve.

197 Dose-response curves were built reporting % inhibition of fructose-induced glycation of BSA as a function  
198 of sample or catechin concentration. % Inhibition was calculated as:  $100-100*(FL_s-FL_b)/(FL_c-FL_b)$ ,  
199 where  $FL_s$  is the fluorescence intensity of the mixture with the sample extract or with catechin,  $FL_b$  is the  
200 fluorescence intensity of the blank (BSA alone) and  $FL_c$  is the fluorescence intensity of the control mixture.  
201 Results were expressed as millimoles of catechin equivalents (CE) per kilogram of product.

#### 202 *2.11. Liking test*

203 Eighty-six consumers (44 males, 42 females, 19–68 years, mean age 28) participated in the study. They had  
204 seen or received an invitation and volunteered based on their interest and availability. All tests were  
205 conducted individually and social interaction was not permitted. The experimenter verbally introduced the  
206 consumers to the computerised data collection procedure (FIZZ Acquisition software, version 2.46A,  
207 Biosystèmes, Courtenon, France). The consumers' test was organized in two sub-sessions. In the first sub-  
208 session, participants evaluated a set of six fortified tomato purees. In the second sub-session, a set of the  
209 control unfortified purees was tested. Fortified and control purees were analyzed in different sub-sessions to  
210 limit the contrast effect (Meilgaard, Civille, & Carr, 2006).

211 The samples (20 g) were offered to the consumers in completely randomized order within the two sessions,  
212 at  $50 \pm 1$  °C in coded, opaque white plastic cup (38 mL) hermetically sealed with a clear plastic lid. For each  
213 sample, consumers stirred accurately the tomato puree using a plastic teaspoon, observed its appearance and  
214 tasted a full teaspoon of product. Then, consumers rated overall liking, liking for colour and texture on a  
215 nine-point hedonic scale ranging from 'dislike extremely' (1) to 'like extremely' (9). A 30 s gap between  
216 each sample was enforced by the computerised system. Consumers were required to eat unsalted crackers  
217 and rinse their mouth with still water during the gap interval. A 10 min gap was enforced between the two  
218 sub-sessions. Preference tests were performed in individual booths under white light. Consumers took  
219 between 25 and 35 min to complete their evaluation.

#### 220 *2.12. Statistical analysis of data*

221 Experimental data were analyzed by one-way ANOVA using the least significant difference (LSD,  $p \leq 0.05$ )  
222 as a multiple range test, and by linear regression analyses using Statgraphics 5.1 (STCC Inc.; Rockville,  
223 MD). Results are reported as average  $\pm$  SD.

224 Liking data (overall liking, liking for colour and texture) from consumers were independently submitted to a  
225 two-way ANOVA model, assuming sample and subject as main effects, by performing LSD ( $p < 0.05$ ).  
226 Overall liking data expressed by all 86 subjects were analysed by means of an Internal Preference Map for  
227 explorative purposes. A visually oriented approach, based on the inspection of loading plot, was used for  
228 subject clustering and Y-axis was set as limit between consumer segments. Liking data expressed by Cluster  
229 1 and Cluster 2 were independently treated with a two-way ANOVA model, with LDS ( $p \leq 0.05$ ). Liking  
230 data were analyzed using FIZZ Calculations software, version 2.46A (Biosystèmes, Courtenon, France).

### 231 **3. Results and discussion**

#### 232 *3.1. Product and process design*

233 The increase in fibre content of food generally has a negative impact on texture, which could be greatly  
234 affected by the particle size of the fibrous material. For a fruit puree, particle concentration, size and type  
235 have been found to be key structural parameters controlling the rheological properties (Moelants et al.,  
236 2013). Hence, in this study three granulometric fractions of Ch grape skins (in the range 125 – 500  $\mu\text{m}$ ) and  
237 two tomato purees of different particle sizes (0.5 and 1 mm) were used in combined formulations. In studies  
238 focused on the incorporation of grape skins or pomace into various foods, the selected particle sizes were  
239 less than 1 mm for addition in fish products (Riberio et al, 2012), less than 0.5 in meat products (Sayago-  
240 Ayerdi et al., 2009) less than 0.18 mm for addition in yogurt (Tseng & Zhao, 2013), while in other  
241 incorporation studies the particle size of this ingredient was not specified (Mildner-Szkudlarz et al., 2011).

242 The composition of Ch skins and tomato purees were first characterized in order to choose the level of  
243 addition. In Ch skins, dietary fibre content was 50.5%. Protein, carbohydrate (fructose and glucose), fat, ash  
244 and moisture contents were:  $10.0 \pm 0.6$ ,  $16.2 \pm 0.2$ ,  $5.7 \pm 1.6$ ,  $4.1 \pm 0.7$  and  $4.0 \pm 0.1$  g/100g, respectively.  
245 Insoluble proanthocyanidin contents, analysed after depolymerisation with *n*-butanol/HCl, were  $10.6 \pm 2$  in  
246 the ChL fraction and  $13.9 \pm 1$  in both the ChM and Ch S fractions, respectively. This could be due to a lower  
247 hydrolysis yield in the ChL fraction. The total amount of flavonols, namely: quercetin 3-O glucuronide,  
248 quercetin 3-O glucoside, quercetin, kaempferol 3-O galactoside, kaempferol 3-O glucuronide, kaempferol 3-  
249 O glucoside and kaempferol was about 600 mg/kg (Tables 1, 2). Soluble proanthocyanidin content of the  
250 ChL fraction was  $20700 \pm 42$  mg/kg (Table 3). Higher proanthocyanidin contents were observed in the ChM

251 and ChS fractions. The increased surface/solvent ratio likely increased extraction efficiency of these  
252 compounds, which are strongly associated with the fibre (Perez-Jimenez et al., 2008). FRAP values were >  
253  $170 \pm 26$  mmolFe eq. (II)/kg, which is two order of magnitude higher than that observed in tomato products  
254 (García-Valverde, Navarro-González, García-Alonso, & Jesús Periago, 2013). The highest FRAP value was  
255 observed in the ChS fraction.

256 The ability of the Ch fractions to inhibit protein glycation was analysed by an *in vitro* BSA/fructose model  
257 system (Figure 1). This system was used to simulate protein glycation that occurs at an accelerated rate *in*  
258 *vivo* under non-physiological conditions, accounting for some of the complications of hyperglycaemia and  
259 diabetes (Saraswat, Reddy, Muthenna, & Reddy, 2009). There is a continuous search for novel inhibitors of  
260 protein glycation that could be helpful to prevent advanced-glycation-endproductsAGEs-associated diseases  
261 and with the potential to be used as functional food ingredients (Farrar, Hartle, Hargrove, & Greenspan,  
262 2007; Saraswat et al., 2009; Sri Harsha et al., 2013; Wu et al., 2013).

263 ~~In this study, Grape phenolics have been shown to effectively inhibit protein glycation *in vitro* (Sri Harsha et~~  
264 ~~al., 2013), most likely by acting both as radical scavengers, metal chelators, and carbonyl trapping agents.~~  
265 ~~This process occurs at an accelerated rate *in vivo* under non-physiological conditions, accounting for some of~~  
266 ~~the complications of hyperglycaemia and diabetes (Dearlove, Greenspan, Hartle, Swanson, & Hargrove,~~  
267 ~~2008). In fact, the amino groups of some mammalian proteins react non-enzymatically with both glucose and~~  
268 ~~fructose, *in vivo*. Subsequent reactions may result in the formation of cross-linked, fluorescent, protein~~  
269 ~~derivatives (AGE) which damage their functionality. Hence, in this study the anti-glycation activity of the Ch~~  
270 ~~fractions was analysed (Figure 1). a~~ dose-response effect was observed *in vitro* for the anti-glycation  
271 activity of the Ch fractions. Phenolics are known to ~~can~~ inhibit protein glycation by acting as radical  
272 scavengers, metal chelators and carbonyl trapping agents (Dearlove, Greenspan, Hartle, Swanson, &  
273 Hargrove, 2008; Wu et al., 2013). Hence, i~~n~~ terms of catechin equivalents, the anti-glycation effectiveness  
274 was  $100 \pm 15$  mmol/kg for all the Ch fractions.

275 In PV and PR tomato purees percent contents of major components were:  $4.9 \pm 0.1$  and  $5.7 \pm 0.1$  for  
276 carbohydrates,  $1.5 \pm 0.1$  and  $1.5 \pm 0.1$  for fibres;  $1.2 \pm 0.1$  and  $1.6 \pm 0.1$  for proteins;  $0.1 \pm 0.02$  and  $0.20 \pm$   
277  $0.02$  for fat, respectively. The main flavonoids in tomato purees were rutin and naringenin (Tables 1, 2).

278 Before heat treatments, flavonol contents (sum of quercetin derivatives) were in the range of 52 - 72 mg/kg  
279 and flavanone contents (naringenin) were in the range of 14 - 51 mg/kg. The PV and PR purees had a  
280 medium-high flavonol and flavanone contents in comparison with previous results obtained on twenty  
281 cultivars of fresh tomatoes extracted with an optimized procedure (Li, Deng, Wuc, Liu, Loewen, & Tsao,  
282 2012). FRAP values of the PR and PV purees were  $1.97 \pm 0.14$  and  $2.68 \pm 0.22$  mmol Fe(II) eq./kg,  
283 respectively (Table 3). Similar values were observed by Garcia-Valverde et al. (2013) in various cultivars of  
284 tomatoes destined to industrial processing. The unfortified tomato purees showed a dose-dependent anti-  
285 glycation activity *in vitro*, with anti-glycation effectiveness of  $2.97 \pm 0.15$  and  $2.82 \pm 0.40$  mmol catechin  
286 eq./kg for PV and PR, respectively. These values were much lower than that of the Ch fractions (Figure 1).  
287 The level of Ch/tomato addition was then chosen to have 3% fibre content in the final products (3.2 g of  
288 grape skins added to 96.8 g of tomato puree). Hence, the purees can be labelled as “fibre-source” according  
289 to the EC Regulation 1924/2006. Furthermore, in a human study, Pérez-Jiménez et al. (2008) have  
290 demonstrated that the intake of grape antioxidant dietary fibre (5.25 g of dietary fibre and 1.06 g of  
291 proanthocyanidins in the supplemented dose) significantly reduces the biomarkers of cardiovascular risk.  
292 Based on Ch fibre and proanthocyanidin contents, a 175 g-dose of the fortified purees (that could be a daily  
293 dose in the Mediterranean diet) can provide 5.25 g of dietary fibres and around 1 g of proanthocyanidins  
294 (soluble + insoluble). Hence, positive *in vivo* effects of these purees can be hypothesised. However, the food  
295 matrix is more complicated than grape skins, therefore an effect of the matrix on food components’  
296 bioavailability cannot be ruled out.

297 The incorporation of grape skin derived fractions into a liquid food, such as tomato puree, requires the design  
298 of an effective heat treatment. The pH values of these products were in the range 4.1 – 4.3. To achieve  
299 pasteurization of low-pH foods, *Alicyclobacillus acidoterrestris* has been proposed as a process target. It is a  
300 thermoacidophilic non-pathogenic and sporeforming bacterium, which has been found in fruit juices,  
301 including tomato puree and white grape juice (Silva & Gibbs, 2004). It is often the most heat resistant  
302 microorganism among the most common spoilage microorganisms found in these foods. The heating  
303 conditions were then selected to achieve 6D-reduction of the target microorganism (Figure 2), which is  
304 considered effective (Silva & Gibbs, 2004). This treatment is representative for an optimized continuous

305 industrial treatment. In parallel, tomato purees were also autoclaved to study the effects of an intensive heat-  
306 treatment on the antioxidant components.

### 307 *3.2. Processing effects on antioxidant components*

308 Flavonols and naringenin were not affected by microwave treatment (not shown). Similarly, Capanoglu,  
309 Beekwilder, Boyacioglu, Hall, & De Vos (2008) found that pasteurization at 98 °C does not change rutin and  
310 naringenin contents of tomato. Upon autoclave treatment, quercetin and kaempferol glycosides and  
311 glucuronides decreased by less than 30% (Tables 2-3). Conversely, the corresponding aglycones increased.  
312 The recovery was ~100% when the sum of quercetin derivatives was considered and ~90% for the sum of  
313 kaempferol derivatives. This means that the prevalent modification occurring during autoclave treatment was  
314 deglycosylation. Interestingly, Stewart, Bozonnet, Mullen, Jenkins, Lean, & Crozier (2000) found that in  
315 contrast to fresh tomatoes, most tomato-based products contained significant amounts of free flavonols and  
316 concluded that the accumulation of quercetin in juices, purees, and paste may be a consequence of enzymatic  
317 hydrolysis of rutin and other quercetin conjugates during pasteurization. Instead, enzymatic activities can be  
318 ruled out in this study, due to the intense heating during autoclave treatment. Rohn, Buchner, Driemel,  
319 Rauser, & Kroh (2007) found that during the roasting process of model flavonols (180°C, 60 min), quercetin  
320 glycosides are degraded and produce quercetin as the major degradation product. Quercetin is not sensitive  
321 to degradation under such conditions and therefore it has to be regarded as a stable end-product. Naringenin  
322 content was above 88%, with lower retention for the unfortified purees than for the fortified purees.

323 After mixing of the purees with the ChL, ChM and ChS skin fractions at room temperature soluble  
324 proanthocyanidin contents were lower in the puree added with the ChL fraction. For all the purees,  
325 proanthocyanidin content was lower than that calculated based on the proanthocyanidin content of grape  
326 skins, with 53-56% recovery percentages (Table 3). These data can be explained with the hypothesis that  
327 proanthocyanidins interacted with tomato components, such as proteins or polysaccharides, to produce high  
328 molecular weight aggregates, through hydrogen bonding or hydrophobic interactions (Pinelo, Arnous, &  
329 Meyer, 2006). These aggregates could not be extracted by the solvents used in this experiment. Similar to  
330 these results, Peng, Maa, Cheng, Jiang, Chen & Wang (2010) found that in a bread added with a  
331 proanthocyanidin-rich grape seed extract, the observed antioxidant activity increases less than what is

332 expected. They did not analyse the unheated samples and concluded that the decreases could be either due to  
333 the interactions of proanthocyanidins with food components to produce insoluble molecules, or due to  
334 thermal degradation.

335 Similarly, FRAP values of the mixtures increased approximately by twofold, probably due to the high  
336 proanthocyanidin contents of the Ch fractions (Table 3). The lowest value was found in the puree added with  
337 the ChL fraction. However, as observed for proanthocyanidins the increase in FRAP values were only 61-  
338 66% of that calculated considering the values of the ChL, ChM and ChS skin fractions.

339 Microwave treatment had no effect on the proanthocyanidin contents and FRAP values of any of the  
340 mixtures considered. On the contrary, upon autoclave treatment, proanthocyanidin contents increased in the  
341 fortified puree with respect to the raw mixtures. The parallel increased FRAP values in the fortified purees  
342 can be related to the rise in the content of proanthocyanidins. The intense thermal treatment could have  
343 weakened the binding between proanthocyanins and other food components (Pinelo et al., 2006), or it could  
344 have promoted proanthocyanidin depolymerisation (Chamorro, Goni, Viveros, Hervert-Hernandez, &  
345 Brenes, 2012) and thus increased proanthocyanidins' solubility.

346 | The dose-dependent anti-glycation activity *in vitro* of the fortified purees showed much higher effectiveness  
347 | than the controls, corresponding to  $8.1 \pm 0.1$  and  $7.2 \pm 0.1$  mmol catechin eq./kg for PV and PR, respectively  
348 | (Figure 1). These new purees have the potential ability to act ~~could therefore play a role~~ as dietary factors in  
349 | the prevention of hyperglycaemia's complications.

### 350 3.3. Consumers' preferences

351 The prospective use of fibrous fractions in developing new functional tomato purees needs to be evaluated  
352 not only from an analytical point of view but also exploring the sensory acceptability of the formulations.  
353 Several works have shown that functional benefits may provide added value to consumers but cannot  
354 outweigh the sensory properties of foods. In fact, consumers base their choices more on pleasantness than  
355 perceived healthiness (Lähteenmäki, 2006). For this reason, a liking test was performed in order to  
356 estimate the consumer overall acceptability of the fortified purees. Since variations in particle sizes of fruit  
357 puree influences the texture (Moelants et al., 2013) and processing of fruit puree can affect colour (Lavelli  
358 & Torresani, 2011), liking ratings for texture and colour were also investigated.

359 The average liking ratings expressed by all 86 consumers for overall acceptability, colour and texture of the  
360 analysed tomato purees are reported in Table 4. Consumers highly rated the unfortified purees in terms of  
361 overall acceptability ( $6.9 \pm 1.8$  for PR;  $6.7 \pm 1.9$  for PV), liking for colour ( $7.4 \pm 1.7$  for PR;  $7.2 \pm 1.7$  for  
362 PV) and texture ( $7.0 \pm 1.8$  for PR;  $6.8 \pm 1.7$  for PV). The addition of the Ch fractions to the tomato purees  
363 decreased the ratings for all the sensory parameters ( $p < 0.05$ ). This effect could be explained taking into  
364 account that consumers were familiar with the unfortified samples (commercially available regular tomato  
365 purees), but they had not been previously exposed to the fortified samples. As it is known, the level of  
366 familiarity for a food influences powerfully its acceptability by the consumer and repeated exposure to the  
367 taste of a food can increase liking for it (Wardle & Cooke, 2008).

368 Regarding the overall liking, average ratings of the fortified samples corresponded approximately to the  
369 central value of the scale (5 = neither like nor dislike). PVChL, PVChM and PVChS were significantly  
370 preferred ( $5.3 \pm 1.9$ ) than PRChL ( $4.6 \pm 2.1$ ) ( $p < 0.05$ ). Concerning the texture, as the particle size  
371 decreased, liking increased. This tendency was more evident for the PV formulations. Average ratings of  
372 liking for colour were all above the central value (5). The only significant difference in colour was observed  
373 for PVChS, which was rated higher than the PR formulations.

374 The overall liking data expressed by all 86 subjects for the fortified samples were then submitted to the  
375 principal component analysis in order to obtain an internal preference map (data not shown). The first two  
376 principal components of the model explained the 48% of the total variance, 28% and 21% the first and the  
377 second dimensions, respectively. A visually oriented approach, based on the inspection of loading plot, was  
378 used for subject clustering and segmentation was performed according to whether consumer loadings lie on  
379 the left or right side of the Y-axis set as limit (Næs et al., 2010). Two groups of consumers were obtained:  
380 the first consisting of 46 subjects (53.5%) positioned on the left side of the map (Cluster 1); the second  
381 consisting of 40 subjects (46.5%) positioned on the right side of the map (Cluster 2). Liking data expressed  
382 by subjects belonging to Cluster 1 and Cluster 2 for all samples were independently treated with a two-way  
383 ANOVA model (samples and subjects as factors), with Fisher's LDS post hoc test considered significant for  
384  $p \leq 0.05$  (Table 4). As expected, both clusters provided similar average ratings of the three sensory  
385 parameters evaluated for the unfortified PR and PV purees, confirming the results obtained by the total of



386 subjects (Table 4). Focusing on the fortified purees, different results were obtained by the two clusters. In  
387 terms of overall acceptability, Cluster 1 preferred the purees fortified with the ChM and ChS fibrous  
388 fractions both for the PR and PV formulations. The highest rating was observed for PVChS ( $6.4 \pm 1.5$ ),  
389 which was not significantly different to that of the PV puree ( $7.0 \pm 1.8$ ). For Cluster 1, liking for texture  
390 decreased as the particle size of the added fibrous fraction increased, as noticed by the preference of all  
391 consumers. Again, in terms of texture PVChS reached the highest average value among the fortified purees,  
392 which was the same as that observed for PV. The good ratings given for the ChS fraction were confirmed  
393 also in terms of liking for colour.

394 Cluster 2 did not discriminate among the three PR formulations in terms of overall acceptability, while  
395 among the PV formulations PVChL was preferred. This cluster did not discriminate among the fortified  
396 samples for both texture and colour, but ratings were higher for the control purees than those of the fortified  
397 purees.

#### 398 **4. Conclusions**

399 Tomato purees fortified with Ch fractions could be positioned noticeably above with respect to the  
400 conventional purees in terms of potential health benefits. Indeed, tomato is rich in lycopene but it does not  
401 contain proanthocyanidins and hence the addition of grape pomace ingredients could overall improve its  
402 antioxidant and anti-glycation properties *in vitro*. -Upon heat-stabilization, phenolic contents and reducing  
403 capacity remained much higher -in all the fortified purees than in the controls. Increase in anti-glycation  
404 activity was also observed in the fortified formulations, leading to the potential use of these food products in  
405 prevention of hyperglycaemia's complications.

406 The varying particle sizes of puree formulations had a moderate effect on proanthocyanidins' solubility and a  
407 marked influence on consumers' preference. PVChS, having the smallest particle sizes, had the maximum  
408 appreciation by a cluster of consumers, with similar liking ratings to those of the control puree. Thus, this  
409 innovative functional puree can have a positive feedback by a relevant segment of consumers.

410 The overall results indicate that grape skins could be used as ingredients for the development of new tomato  
411 purees, contributing to a sustainable process innovation.

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**Table 1.** Contents of Quercetin Derivatives and Quercetin Aglycone (mg quercetin 3-O glucoside eq./kg) in the ChL, ChM and ChS Fractions, PV and PR Tomato Purees and their Combined Formulations, after Autoclave Treatment.

Sample	Quercetin derivatives					
	Q-ud	Q-rut	Q-gln	Q-glc	Q	tot Q-der
<b>ChL</b>			111 <sup>c</sup> ± 2	98 <sup>b</sup> ± 5	13.8 <sup>e</sup> ± 0.6	223 <sup>c</sup> ± 8
<b>ChM</b>			114 <sup>c</sup> ± 4	92 <sup>b</sup> ± 1	13.6 <sup>e</sup> ± 0.6	220 <sup>c</sup> ± 5
<b>ChS</b>			115 <sup>e</sup> ± 1	97 <sup>b</sup> ± 1	12.8 <sup>e</sup> ± 0.8	225 <sup>c</sup> ± 3
<b>PR</b>	3.28 <sup>a</sup> ± 0.01 (72)	42.10 <sup>b</sup> ± 0.09 (91)			0.35 <sup>a</sup> ± 0.01	45.73 <sup>a</sup> ± 0.12 (88)
<b>PRChL</b>	3.10 <sup>a</sup> ± 0.03 (76)	36.30 <sup>a</sup> ± 1.52 (87)	2.50 <sup>ab</sup> ± 0.03 (73)	2.50 <sup>a</sup> ± 0.01 (87)	4.52 <sup>b</sup> ± 0.16 (1139)	49.12 <sup>a</sup> ± 1.76 (100)
<b>PRChM</b>	2.92 <sup>a</sup> ± 0.08 (71)	36.10 <sup>a</sup> ± 0.05 (86)	2.27 <sup>a</sup> ± 0.03 (67)	2.78 <sup>a</sup> ± 0.03 (97)	5.41 <sup>bc</sup> ± 0.42 (1364)	49.48 <sup>a</sup> ± 0.61 (103)
<b>PRChS</b>	3.80 <sup>a</sup> ± 0.28 (91)	39.00 <sup>a</sup> ± 0.00 (93)	2.64 <sup>bc</sup> ± 0.31 (78)	2.81 <sup>a</sup> ± 0.08 (98)	4.40 <sup>b</sup> ± 0.78 (1109)	52.65 <sup>a</sup> ± 1.45 (102)
<b>PV</b>	10.71 <sup>b</sup> ± 0.44 (81)	55.89 <sup>d</sup> ± 0.34 (95)			0.85 <sup>a</sup> ± 0.01	67.45 <sup>b</sup> ± 0.79 (93)
<b>PVChL</b>	10.92 <sup>b</sup> ± 1.91 (85)	53.59 <sup>c</sup> ± 0.05 (94)	2.93 <sup>cd</sup> ± 0.18 (80)	2.97 <sup>a</sup> ± 0.96 (97)	6.77 <sup>cd</sup> ± 0.04 (1590)	77.17 <sup>b</sup> ± 3.14 (100)
<b>PVChM</b>	10.59 <sup>b</sup> ± 0.62 (82)	52.42 <sup>c</sup> ± 1.07 (92)	3.05 <sup>d</sup> ± 0.29 (84)	2.88 <sup>a</sup> ± 0.74 (94)	6.67 <sup>cd</sup> ± 0.85 (1567)	75.60 <sup>b</sup> ± 3.57 (98)
<b>PVChS</b>	10.49 <sup>b</sup> ± 0.96 (82)	53.61 <sup>c</sup> ± 0.98 (94)	3.05 <sup>d</sup> ± 0.03 (84)	3.03 <sup>a</sup> ± 0.18 (99)	7.10 <sup>d</sup> ± 0.99 (1669)	77.28 <sup>b</sup> ± 3.15 (100)

Data are average ± SD. Percent recovery after autoclave treatment is indicated in parenthesis. *Q-ud*, unidentified quercetin derivative; *Q-rut*, rutin; *Q-gln*, quercetin 3-O glucuronide; *Q-glc*, quercetin 3-O glucoside; *Q*, quercetin; *tot Q-der*, sum of quercetin derivatives. Values in the same column with differing superscripts are significantly different (LSD,  $p < 0.05$ ).

**Table 2.** Contents of Kaempferol Derivatives, Kaempferol Aglycone (mg Kaempferol 3-O glucoside eq./kg) and Naringenin (mg/kg) in the ChL, ChM and ChS Fractions, PV and PR Tomato Purees and their Combined Formulations, after Autoclave Treatment.

Sample	Kaempferol derivatives				Naringenin	
	K-gal	K-gln+K-glc	K	tot K-der		
<b>ChL</b>	77 <sup>b</sup> ± 7	313 <sup>b</sup> ± 6	16.9 <sup>b</sup> ± 1.5	407 <sup>b</sup> ± 12		
<b>ChM</b>	70 <sup>b</sup> ± 2	304 <sup>b</sup> ± 5	16.7 <sup>b</sup> ± 0.4	391 <sup>b</sup> ± 8		
<b>ChS</b>	67 <sup>b</sup> ± 7	297 <sup>b</sup> ± 20	18.2 <sup>b</sup> ± 1.3	382 <sup>b</sup> ± 28		
<b>PR</b>					11.37 <sup>a</sup> ± 0.64	(81)
<b>PRChL</b>	1.58 <sup>a</sup> ± 0.03 (77)	6.93 <sup>a</sup> ± 0.16 (76)	2.15 <sup>a</sup> ± 0.08 (418)	10.66 <sup>a</sup> ± 0.07 (91)	11.13 <sup>a</sup> ± 0.03	(88)
<b>PRChM</b>	1.74 <sup>a</sup> ± 0.02 (84)	6.64 <sup>a</sup> ± 0.21 (73)	2.04 <sup>a</sup> ± 0.14 (397)	10.41 <sup>a</sup> ± 0.10 (89)	10.61 <sup>a</sup> ± 0.70	(84)
<b>PRChS</b>	1.66 <sup>a</sup> ± 0.03 (81)	6.38 <sup>a</sup> ± 0.02 (70)	1.81 <sup>a</sup> ± 0.01 (352)	9.84 <sup>a</sup> ± 0.01 (85)	11.72 <sup>a</sup> ± 0.23	(93)
<b>PV</b>					45.53 <sup>b</sup> ± 0.72	(90)
<b>PVChL</b>	2.10 <sup>a</sup> ± 0.49 (95)	6.81 <sup>a</sup> ± 1.45 (70)	1.79 <sup>a</sup> ± 0.05 (325)	10.70 <sup>a</sup> ± 0.71 (86)	45.99 <sup>b</sup> ± 0.89	(94)
<b>PVChM</b>	2.02 <sup>a</sup> ± 0.27 (91)	7.22 <sup>a</sup> ± 0.46 (74)	2.23 <sup>a</sup> ± 0.02 (404)	11.46 <sup>a</sup> ± 0.22 (92)	44.60 <sup>b</sup> ± 0.36	(91)
<b>PVChS</b>	1.97 <sup>a</sup> ± 0.12 (89)	7.23 <sup>a</sup> ± 0.36 (74)	1.95 <sup>a</sup> ± 0.04 (354)	11.15 <sup>a</sup> ± 0.17 (89)	44.63 <sup>b</sup> ± 0.01	(91)

Data are average ± SD. Percent recovery after autoclave treatment is indicated in parenthesis. K-gal, kaempferol 3-O galactoside; K-gln, kaempferol 3-O glucuronide; K-glc, kaempferol 3-O glucoside; K, kaempferol, tot K-der, sum of total kaempferol derivatives. Values in the same column with differing superscripts are significantly different (LSD, p < 0.05).

**Table 3.** Soluble Proanthocyanin Contents ( $PCy_{soluble}$ , mg/kg) and FRAP Values (mmolFe(II) eq./kg) of the ChL, ChM and ChS Fractions, PV and PR Tomato Purees and their Combined Formulations, after Mixing (raw), Microwave Treatment and Autoclave Treatment.

Puree	$PCy_{soluble}$			FRAP		
	Raw	Microwaved	Autoclaved	Raw	Microwaved	Autoclaved
<b>ChL</b>	20700 <sup>c</sup> ± 42			170 <sup>d</sup> ± 25		
<b>ChM</b>	25300 <sup>d</sup> ± 28			207 <sup>e</sup> ± 26		
<b>ChS</b>	27000 <sup>e</sup> ± 14			217 <sup>f</sup> ± 24		
<b>PR</b>				1.97 <sup>a x</sup> ± 0.14	2.29 <sup>a x</sup> ± 0.14	2.15 <sup>a x</sup> ± 0.11
<b>PRChL</b>	352 <sup>a x</sup> ± 63 (53)	353 <sup>a x</sup> ± 3 (53)	406 <sup>a y</sup> ± 1 (61)	4.74 <sup>abc x</sup> ± 0.04 (64)	4.55 <sup>c x</sup> ± 0.03 (61)	5.34 <sup>b y</sup> ± 0.27 (72)
<b>PRChM</b>	445 <sup>b x</sup> ± 23 (55)	399 <sup>ab x</sup> ± 4 (49)	506 <sup>bc y</sup> ± 10 (62)	5.25 <sup>bc x</sup> ± 0.55 (61)	5.30 <sup>d x</sup> ± 0.09 (62)	6.25 <sup>c y</sup> ± 0.35 (73)
<b>PRChS</b>	482 <sup>b x</sup> ± 14 (56)	450 <sup>bc x</sup> ± 11 (52)	555 <sup>cd y</sup> ± 3 (64)	5.82 <sup>c x</sup> ± 0.12 (65)	6.04 <sup>e x</sup> ± 0.09 (68)	6.91 <sup>de y</sup> ± 0.10 (78)
<b>PV</b>				2.68 <sup>ab x</sup> ± 0.22	2.60 <sup>b x</sup> ± 0.18	2.75 <sup>a x</sup> ± 0.15
<b>PVChL</b>	355 <sup>a x</sup> ± 6 (54)	348 <sup>a x</sup> ± 1 (53)	455 <sup>ab xy</sup> ± 81 (69)	5.16 <sup>bc x</sup> ± 0.04 (64)	5.35 <sup>d x</sup> ± 0.15 (66)	6.27 <sup>cd y</sup> ± 0.38 (77)
<b>PVChM</b>	446 <sup>b x</sup> ± 17 (55)	411 <sup>abc x</sup> ± 45 (51)	629 <sup>dc y</sup> ± 65 (78)	5.89 <sup>c x</sup> ± 0.07 (63)	5.93 <sup>e x</sup> ± 0.04 (64)	6.95 <sup>e y</sup> ± 0.23 (75)
<b>PVChS</b>	487 <sup>b x</sup> ± 35 (56)	468 <sup>c x</sup> ± 44 (54)	668 <sup>e y</sup> ± 19 (77)	6.35 <sup>c x</sup> ± 0.30 (66)	6.02 <sup>e x</sup> ± 0.18 (63)	7.50 <sup>e y</sup> ± 0.45 (78)

Data are average ± SD. Percent recovery is indicated in parenthesis. Values in the same column with differing superscripts (a-f) are significantly different (LSD,  $p < 0.05$ ). Values in the same row with differing superscripts (x-z) are significantly different (LSD,  $p < 0.05$ ).



**Table 4.** Overall Liking and Liking for Texture and Colour of the PV and PR Tomato Purees and their Formulations with ChL, ChM and ChS Fractions Expressed by All Consumers (n=86), Cluster 1 (n=46) and Cluster 2 (n=40).

Puree	Overall			Texture			Colour		
	All	Cluster 1	Cluster 2	All	Cluster 1	Cluster 2	All	Cluster 1	Cluster 2
<b>PR</b>	6.9 <sup>a</sup> ± 1.8	6.9 <sup>a</sup> ± 1.5	7.0 <sup>a</sup> ± 2.1	7.0 <sup>a</sup> ± 1.8	6.8 <sup>a</sup> ± 2.0	7.1 <sup>a</sup> ± 1.6	7.4 <sup>a</sup> ± 1.7	7.4 <sup>a</sup> ± 1.8	7.5 <sup>a</sup> ± 1.6
<b>PRChL</b>	4.6 <sup>d</sup> ± 2.1	3.6 <sup>d</sup> ± 1.7	5.7 <sup>bc</sup> ± 2.0	4.3 <sup>e</sup> ± 2.3	3.5 <sup>d</sup> ± 1.9	5.3 <sup>b</sup> ± 2.4	5.3 <sup>c</sup> ± 1.8	4.7 <sup>d</sup> ± 1.7	6.0 <sup>b</sup> ± 1.7
<b>PRChM</b>	4.8 <sup>cd</sup> ± 2.1	4.7 <sup>c</sup> ± 1.9	5.0 <sup>cd</sup> ± 2.4	4.9 <sup>cd</sup> ± 2.1	4.7 <sup>c</sup> ± 1.9	5.3 <sup>b</sup> ± 2.3	5.3 <sup>c</sup> ± 1.7	5.1 <sup>cd</sup> ± 1.5	5.7 <sup>b</sup> ± 1.8
<b>PRChS</b>	5.0 <sup>bcd</sup> ± 2.1	5.1 <sup>c</sup> ± 1.9	5.0 <sup>cd</sup> ± 2.3	5.0 <sup>cd</sup> ± 2.1	4.9 <sup>bc</sup> ± 1.8	5.1 <sup>b</sup> ± 2.4	5.3 <sup>c</sup> ± 1.7	5.1 <sup>cd</sup> ± 1.6	5.6 <sup>b</sup> ± 1.8
<b>PV</b>	6.7 <sup>a</sup> ± 1.9	7.0 <sup>a</sup> ± 1.8	6.3 <sup>ab</sup> ± 1.9	6.8 <sup>a</sup> ± 1.7	7.0 <sup>a</sup> ± 1.6	6.7 <sup>a</sup> ± 1.7	7.2 <sup>a</sup> ± 1.7	7.4 <sup>a</sup> ± 1.8	7.1 <sup>a</sup> ± 1.7
<b>PVChL</b>	5.3 <sup>b</sup> ± 1.9	5.2 <sup>c</sup> ± 1.9	5.5 <sup>c</sup> ± 2.0	4.7 <sup>de</sup> ± 2.3	4.6 <sup>c</sup> ± 2.3	4.8 <sup>b</sup> ± 2.4	5.6 <sup>bc</sup> ± 1.8	5.4 <sup>c</sup> ± 1.8	5.9 <sup>b</sup> ± 1.7
<b>PVChM</b>	5.3 <sup>bc</sup> ± 2.1	6.0 <sup>b</sup> ± 1.5	4.5 <sup>d</sup> ± 2.5	5.3 <sup>c</sup> ± 2.0	5.4 <sup>b</sup> ± 1.7	5.2 <sup>b</sup> ± 2.3	5.5 <sup>bc</sup> ± 1.8	5.5 <sup>bc</sup> ± 1.6	5.6 <sup>b</sup> ± 2.0
<b>PVChS</b>	5.5 <sup>b</sup> ± 2.1	6.4 <sup>ab</sup> ± 1.5	4.5 <sup>d</sup> ± 2.2	5.9 <sup>b</sup> ± 1.9	6.6 <sup>a</sup> ± 1.3	5.2 <sup>b</sup> ± 2.2	5.8 <sup>b</sup> ± 1.8	6.1 <sup>b</sup> ± 1.7	5.5 <sup>b</sup> ± 1.8

Data are average ± SD. Values in the same column with differing superscripts are significantly different (LSD, p < 0.05).

