**Sustainable recovery of grape skins for use in an apple beverage with antiglycation properties**

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## ABSTRACT

1 An apple puree formulated with red grape skins was developed on pilot-scale as a new beverage 2 with antiglycation properties. The addition level of grape skins was selected by a liking test with 70 3 consumers. The selected formulation was a fibre-rich source and delivered grape anthocyanins, 4 flavonols and flavanols resulting in ~ twofold higher antiglycation activity than the apple puree. 5 Pasteurization (3D reduction of the target microorganism *Alicyclobacillus acidoterrestris*) did not 6 affect the antiglycation activity, which decreased by 30% upon sterilization. Storage for 1 month in 7 the temperature range 15° - 35 °C affected the contents of anthocyanins, monomeric, dimeric and 8 oligomeric flavanols, while chlorogenic acid, flavonols and dihydrochalcones were stable. 90% 9 antiglycation activity was retained after one-month storage at 15°C. The use of red grape skin as 10 ingredient could represent an opportunity for the apple processing industry to develop a value- 11 added product.


12


13 **Keywords**: functional foods, sensory, bioactive phytochemicals, dietary fibre, wine and enology


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15

16 **INTRODUCTION**

17 Non-enzymatic protein glycation has a role in the development and progression of different diseases
18 including diabetes, atherosclerosis, and neurological disorders (Engelen *et al.*, 2013). The process
19 can occur *in vivo* both in body fluids, between extracellular proteins and glucose, and within the
20 cells between proteins and dicarbonyl precursors derived from glucose, polyol pathway and lipid
21 peroxidation. Non-enzymatic protein glycation ultimately leads to formation of irreversible protein
22 adducts and cross-links known as advanced glycation end-products (AGEs). AGEs are also present
23 in heat-treated foods and their intake could have a synergistic effect with endogenous AGEs, thus
24 contributing to tissue damage (Yu *et al*., 2016). Some of the biological effects of AGEs are due to
25 the loss of function of the glycated protein. AGEs also exert their damaging and pro-inflammatory
26 effects by activating signalling cascades via specific receptors present on the surface of various cells
27 (Engelen *et al*., 2013).

28 Discovering anti-glycation inhibitors within edible sources would lead to establishment of a dietary
29 strategy to alleviate hyperglycemia-related damage. To this aim, crude phenolic extracts and crude
30 polysaccharide extracts from various plant sources have been identified as effective inhibitors of
31 protein glycation (Daiponmak *et al.*, 2014; Chaouch *et al*., 2015). To make healthier foods while
32 meeting the criteria for a sustainable production, byproducts of plant food processes could be
33 recovered and used as phenolic-rich food ingredients (Galanakis, 2015). It is worth considering that
34 the disposal of food waste is a practice that could not be continued for a long time within the
35 sustainability and bioeconomy framework of the modern food industry. In fact, worldwide
36 legislative requirements for waste disposal have become increasingly restrictive over the last decade
37 and have stimulated industry to reconsider the concept of "byproduct recovery" as an opportunity
38 (Galanakis, 2015). According to the FAO (www.faostat3.fao.org), 77 Mt of grapes were produced
39 throughout the world in 2013, most of which were used in winemaking. On average, 100 kg of
40 processed grapes generates 20 - 25 kg of pomace, a mixture of skins and seeds. Grape pomace
41 poses serious environmental concerns because it is typically produced in a limited time frame, and

42 its high organic matter content prevents direct disposal into the soil, except for limited and regulated
43 amounts. Alternatively, these materials are used for animal feed production, composting and
44 distillation or incineration, which however are not considered remunerative strategies (Lavelli *et al.*,
45 2016c). Grape skin (GS) is a rich source of phenolics, including phenolic acids, flavonols,
46 flavanols, anthocyanins and proanthocyanidins (Kammerer *et al.*, 2004; Saura-Calixto, 2011;
47 Teixeira *et al.*, 2014). *In vitro* studies have demonstrated that these compounds can act as potent
48 radical scavengers and inhibit oxidation of the polyunsaturated fatty acids and DNA, besides acting
49 as inhibitors of various enzymes that produce oxygen radicals (Yu *et al.*, 2013; Teixeira *et al.*,
50 2014). Furthermore, *in vitro* studies have demonstrated that grape skin phenolics prevent structural
51 modification of proteins caused by glycating agents (Sri Harsha *et al.*, 2014).
52 It is worth noticing that an *in vivo* study on humans, carried out by Perez-Jimenez *et al.* (2008) has
53 demonstrated that the intake of whole GS containing fibre and antioxidants significantly reduces the
54 glycoxidative stress and supports the use of GS in the prevention of cardiovascular and diabetes's
55 diseases. Another in *vivo* study by Hokayem *et al.* (2013) has provided evidence that the intake of
56 grape phenolics counteracts the metabolic alterations of high-fructose diet in first-degree relatives
57 of diabetic patients. Beside consumption of high fructose sweetened foods and beverages,
58 consumption of apple juice has also been linked to AGE formation (DeChristopher *et al.*, 2015).
59 Food fortification with GS phenolics and fibre could play a role in disease prevention and address
60 the consumers' demand for functional foods. The development of functional foods with GS has
61 mainly involved bakery, dairy, meat and fish products (Lavelli *et al.*, 2016c). On the other hand, the
62 co-administration of grape phenolics with foods naturally containing fructose, such as fruit-based
63 products, could be a strategy to counteract glycoxidative damage.
64 Food formulation with fibre- and antioxidant-rich ingredients generally results in changes in overall
65 sensorial properties, and hence the fortification level needs to be tailored according to the
66 consumers' liking (Laureati *et al.*, 2016; Tourila, 2007). Furthermore, processing and storage should
67 be optimized to minimize antioxidant degradation, especially for the most heat sensitive compounds

68 such as anthocyanins (Nayak *et al.,* 2015). Hence, in this study, red GS was formulated with an
69 apple puree at two addition levels and processed on pilot-scale. The effects of fortification on
70 consumers' liking, major components and polyphenol contents, reducing capacity and antiglycation
71 activity *in vitro* were investigated, in order to provide criteria for the design of apple-based purees
72 with high antiglycation activity.
73

74 **MATERIAL AND METHODS**

75 ***Chemicals***

76 Standards of catechin, epicatechin, procyanidin B1, procyanidin B2, delphinidin 3-O-glucoside,
77 cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside,
78 quercetin 3-O-glucuronide, quercetin 3-O-glucoside, quercetin, kaempferol, chlorogeinc acid and
79 phloretin, were purchased from Extrasynthese (Lyon, France). The integrated total dietary fibre
80 assay procedure kit was purchased from Megazyme International Ireland Ltd. (Bray, Ireland). All
81 other chemicals were purchased from Sigma–Aldrich Italia (Milan, Italy).

82 ***Grape skins (GS)***

83 Fermented grape pomace samples (red variety Barbera) were kindly provided by a winery located in
84 Northern Italy. At the winery, the pomace was sieved (with a 5 mm sieve) to separate GS from the
85 seeds. The seeds were removed since they easily undergo oxidation since they are rich in
86 polyunsaturated fatty acids (Lavelli et al., 2015a). The skins are a vegetable material rich in sugars
87 that rapidly undergo spontaneous fermentation, hence they were frozen, transferred to the lab and
88 dried at 50 °C for 8 h to reach a water activity level below 0.3 to prevent microbial growth and to
89 stabilize phenolic compounds (Lavelli *et al*., 2013). The powders obtained were sieved by using the
90 Octagon Digital sieve shaker (Endecotts Ltd., United Kingdom), with a certified sieve (openings:
91 125 µm) for 10 min at amplitude 8. The sieved GS was stored under vacuum, in the dark, at 4 °C.

92 ***Formulation and processing of the fortified apple purees***

93 Apple puree (AP) was provided by a fruit processing company. Fortified apple puree was prepared
94 by addition of 1.5 % or 5% of GS (indicated as AP-GS 3.0 and AP-GS 4.8, respectively, with
95 reference to the final fibre content). Heat treatment was performed as described previously (Lavelli
96 *et al.,* 2015b). In brief, the fortified and control purees were filled into various 250 mL glass bottles
97 and then submitted to microwave heating at 900 W for 10, 15 or 30 minutes with continuous
98 temperature monitoring using a thermosensor. Upon attainment of a given pasteurization or
99 sterilization effect, the bottles were cooled by immersion in ice. To model the
100 pasteurization/sterilization effectiveness during heat treatment, *Alicylobacillus acidoterrestris* was
101 used as a target microorganism. Decimal reductions (D) values for the target microorganism were
102 calculated as a function of temperature using the Bigelow's model:

103 $$D = D_{ref}*10^{(Tref-T)/z} \quad (1)$$

104 where for the target microorganism, $D_{ref}$ = 1.5 min, $T_{ref}$ = 95℃ and z = 7 ℃.
105 The efficacy of each non-isothermal heat treatment was then calculated considering the variation of
106 D with time, as:
107
108 $$\text{number of decimal reduction achieved} = \int_o^t (dt/D) \quad (2)$$
109

110 where t is the processing time.
111 3 D, 6 D (pasteurization) and 14 D (sterilization) treatments were applied in parallel. Samples
112 submitted to the 14 D heat treatment were stored in triplicate in thermostatic heating cabinets for
113 one month at 15, 25 and 35 ℃.

114 ***Moisture, fibre, protein, carbohydrates, fat and ash contents***

115 Moisture content of AP, GS, AP-GS 3.0 and AP GS 4.8 was determined by drying in a
116 vacuum oven at 70 °C and 50 Torr for 18 h. Protein, fat, fibre, glucose, fructose and ash contents of
117 apple puree, GS, AP-GS 3.0 and AP GS 4.8 were determined as described previously (Lavelli *et al*.,
118 2016a).

119 ***Phenolic extraction***

120 For phenolic extraction from GS, an amount of 100 mg was added with 8 mL
121 methanol:water:HCl (80:20:0.1, v/v/v), for 2 h at room temperature with continuous stirring. The
122 mixture was centrifuged at 10,000*g* for 10 min, the supernatant was recovered and the solid residue
123 was re-extracted using 6 mL of the same solvent twice (Sri Harsha *et al*., 2013).
124 For phenolic extraction from AP, AP-GS 3.0 and AP-GS 4.8 an amount of 1.6 g was extracted in
125 20 mL of methanol:water:HCl (80:20:0.1, v/v/v) by following the same 3-step procedure as
126 described for GS. Duplicate determinations were made for each sample. Extracts were stored in the
127 dark, at -20°C, until performing characterization studies.

128 ***Total phenolics***

129 The Folin–Ciocalteu assay was performed on phenolic extracts as described previously (Sri
130 Harsha *et al*. 2013). The reaction mixture contained 0.5 mL of the extracts diluted with
131 methanol:water:HCl (80:20:0.1, v/v/v), 6.0 mL of distilled water, 0.5 mL of Folin–Ciocalteu
132 reagent and 3 mL of 10% $Na_2CO_3$. The mixtures were incubated for 90 min at room temperature
133 and the absorbance was recorded at 760 nm against a blank with no extract addition. For each
134 extract, 2 - 4 dilutions were assessed in duplicate. A calibration curve was built using gallic acid.
135 Total phenolics were expressed as milligram of gallic acid equivalents (GAE) per kilogram of
136 product.

137 ***Soluble proanthocyanindins***

138 Proanthocyanidin content was analysed as described previously (Sri Harsha *et al*. 2013).
139 Briefly, for evaluation of soluble proanthocyanidins, 1 mL of the sample extract diluted with
140 methanol:water:HCl (80:20:0.1, v/v/v) was added to 6 mL of *n*-butanol:HCl (95:5, v/v) and 0.2 mL
141 of 2% $NH_4Fe(SO_4)_2.12\ H_2O$ in 2 M HCl. Hydrolysis was carried out at 95 °C for 40 min. The
142 reaction mixtures were cooled and the absorbance was recorded at 550 nm on a Jasco UVDEC-610
143 spectrophotometer (Jasco Europe, Cremella, Italy) against a blank made as for the sample but
144 incubated at room temperature. For each sample extract, 2 - 4 dilutions were assessed in duplicate.

145 Proanthocyanidin amount was determined using 0.1736 (mg/mL) as conversion factor and
146 expressed as milligrams per kilogram of product.

147 ***HPLC analysis of phenolics***

148 The phenolic content of the extracts was analysed as described previously (Lavelli *et al.*,
149 2016b). Apparatus consisted of a Shimadzu LC-20 AD pump coupled to a model Shimadzu SPD-
150 M20A photodiode array detector and an RF-20 AXS operated by Labsolution Software (Shimadzu,
151 Kyoto, Japan). A 2.6 µm Kinetex C18 column (150 x 4.6 mm; Phenomenex, Bologna, Italy) was
152 used for the separation, at a flow-rate of 1.5 mL/min. The column was maintained at 40 °C. The
153 separation was performed by means of a linear gradient elution. Eluents were: (A) 0.1% $H_3PO_4$; (B)
154 acetonitrile. The gradient was as follows: from 6% B to 20% B in 18 min; from 20% B to 60% B in
155 7 min; from 60% B to 90% B in 19 min; 90% B for 10 min and then 6% B for 5 min. DAD analysis
156 was carried out in the range of 200 - 600 nm. Anthocyanins, flavonols, dihydrochalcones,
157 hydroxycinnamic acids and hydroxymethylfurfurol were quantified by calibration curves built with
158 external standards, namely, malvidin-3-O-glucoside at 520 nm for anthocyanins, quercetin-3-O-
159 glucoside at 354 nm for flavonols, phlorizin at 280 for dihydrochalcones, hydroxymethylfurfural at
160 280 nm, chlorogenic acid at 330 nm. Flavanols, were quantified by catechin, epicatechin,
161 procyanidin B1 and procyanidin B2 standards with the fluorimetric detector set at $\lambda_{ex}$ 230 and $\lambda_{em}$
162 320. Results were expressed as milligram per kilogram of product.

163 ***Ferric ion reducing antioxidant power (FRAP) assay***

164 The FRAP reagent was prepared by adding 25 mL of 300 mM acetate buffer, pH 3.6; 2.5 mL of 10
165 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3$. The reaction mixture
166 contained 0.4 mL of sample extracts diluted with methanol:water:HCl (80:20:0.1, v/v/v) and 3 mL
167 of FRAP reagent. The absorbance at 593 nm was evaluated on a Jasco UVDEC-610
168 spectrophotometer (Jasco Europe, Cremella, Italy) after 4 min of incubation at 37 °C against a blank
169 with no extract addition. For each sample extract, 2 - 4 dilutions were assessed in duplicate. A

170 methanolic solution of $FeSO_4 \cdot 7H_2O$ was used for calibration. Results were expressed as millimoles
171 of Fe(II) equivalents per kilogram of product (Sri Harsha et al., 2013).

172 ***Determination of fructose-induced glycation of bovine serum albumin (BSA)***

173 The inhibition of fructose-induced glycation of BSA was conducted as described previously
174 (Sri Harsha *et al.*, 2014). The reaction mixture consisted of 100 µL of sample extracts or various
175 standard compounds diluted with methanol:water:HCl (80:20:0.1, v/v/v), 900 µL of phosphate
176 buffer (200 mM potassium phosphate buffer, pH 7.4 with 0.02% sodium azide), 300 µL of BSA
177 solution (50 mg/ml of BSA in phosphate buffer) and 300 µL of fructose solution (1.25 M fructose
178 in phosphate buffer). A BSA solution (blank sample) and control reaction without sample addition
179 were prepared in parallel. The reaction mixtures were incubated at 37 °C for 72 h and then analysed
180 for fluorescence on a Perkin-Elmer LS 55 Luminescence Spectrometer (Perkin-Elmer Italia, Monza,
181 Italy) with an excitation/emission wavelength pair k = 370/440 nm, 5 nm slit width, against
182 phosphate buffer. For each sample extract, 3 - 4 dilutions were assessed in duplicate. Dose–
183 response curves were built reporting % inhibition of fructose-induced glycation of BSA as a
184 function of sample or standard concentration. Results were expressed as millimoles of
185 aminoguanidine (AG) equivalents per kilogram of product.

186 ***Liking test***

187 AP, AP-GS 3.0 and AP-GS 4.8 were formulated and submitted to the 3-D treatment 30 minutes
188 before sensory evaluation. Samples (15 mL) were served under blind conditions, in randomized and
189 balanced order among participants. Purees were served in opaque white plastic cups (38 mL) sealed
190 with a clear plastic lid and identified by random three-digit codes.

191 Seventy consumers (37% males, 96% aged under 35) were recruited. Evaluations were carried out
192 in individual booths under white light. The experimenters verbally introduced the consumers to the
193 computerised data collection procedure (FIZZ Acquisition software, version 2.46A, Biosystèmes,
194 Courtenon, France). General instructions required subjects to rinse their mouth before the beginning
195 of the test and between samples, and to stir the samples accurately with a plastic teaspoon before

196 tasting. Secondly, participants were instructed to observe the appearance, to smell the sample and to
197 taste it by taking a full spoon in the mouth. Participants were then asked to rate their liking on a 9-
198 point hedonic scale ranging from 'dislike extremely' (1) to 'like extremely' (9) considering the
199 appearance, odour, taste, flavour, texture and the overall liking. A rest of 60 seconds was enforced
200 between samples. At the end of the test, socio-demographic data were collected (age, gender,
201 nationality) together with a questionnaire. The questionnaire included: the frequency of
202 consumption of fruit juices, purees, jams, using a 5-point scale (less than once a month, less than
203 once a week, 1 - 3 times a week, 4 - 6 times a week, at least once a day); the declared liking towards
204 different pureed fruit preserves (apple, apple and apricot, apple and prune, apple and blueberry,
205 apple and peach, apple and banana, apple and kiwi, apple and mixed berries, apple and
206 pomegranate, apple and pear, pear) using a 9-point hedonic scale (1= dislike extremely; 9=like
207 extremely); the importance of some items influencing the choice of a pureed fruit (type of fruit,
208 brand, fruit percentage, low calories, low sugars, high in fibres, presence of antioxidants, low price,
209 pleasant sensory properties, organic product, packaging) using a 7-point scale (1= not at all
210 important; 7=very important); the degree of agreement towards statements concerning the
211 consumption of pureed fruit (I consume pureed fruit because: I like it; It contains vitamins and salts;
212 It contains antioxidants; I use it as substitute to fresh fruit; It is practical to carry) using a 7-point
213 scale (1= completely disagree; 7=completely agree). Evaluations had a total duration of around 15
214 minutes.

215 ***Statistical analysis of data***

216 Experimental data were analysed by one-way ANOVA using the least significant difference (LSD)
217 as a multiple range test, and by linear regression analysis using Statgraphics 5.1 (STCC Inc.;
218 Rockville, MD). Results are reported as average ± standard error (SE). Liking data (appearance,
219 odour, taste, flavour, texture, overall liking) were separately submitted to two-way mixed ANOVA
220 models (fixed factor: sample; random factor: subject) by performing Fisher's Least Significance
221 Difference (LSD; $p < 0.05$). In order to better explore consumer's preference for prototypes, a

222 subject segmentation was performed conducting K-Means Cluster Analysis on liking data using the
223 software XLStat 2012.6 (Addinsoft), obtaining two clusters. Liking data of each obtained cluster
224 were separately submitted to two-way ANOVA models (fixed factor: sample; random factor:
225 subject) by performing Fisher's LSD ($p < 0.05$). Unpaired t-tests were conducted to compare the
226 mean hedonic scores between clusters. Differences between the two clusters considering age,
227 gender, nationality and frequency of consumption of fruit conserves were analysed by Pearson chi-
228 square distribution. Mean values and SE were calculated for the declared liking towards different
229 fruit preserves, the importance of some items influencing the choice, and the agreement with
230 statements concerning the consumption of pureed fruit. All data analyses were conducted using the
231 software SYSTAT version 13.1 (Systat Software Inc, San José, USA).
232

233 **RESULTS AND DISCUSSION**

234 ***Formulation of apple puree fortified with GS***

235 Major components of GS, AP and its formulations with GS at two addition levels are shown in
236 Table 1. In all the purees, the content of carbohydrates was ~ 119 g/kg, including fructose (50%),
237 glucose (30%) and sucrose (20%). Fibre content of AP was 22 g/kg and increased to 30 and 48
238 g/kg, in AP-GS 3.0 and AP-GS 4.8, respectively. Hence the fortified purees could be labelled as a
239 ''fibre-source'' according to the EC Regulation 1924/2006.
240 Total soluble phenolic content of GS and apple purees is shown in Table 2. The main antioxidants
241 in AP extract were catechin, epicatechin, procyanidin B1, procyanidin B2, soluble
242 proanthocyanidins, chlorogenic acid, phloridzin (phloretin 2-O-glucoside) and phloretin
243 xyloglucoside (Table 2), as already observed (Lavelli & Vantaggi, 2009). Upon addition of GS to
244 AP the main anthocyanins of red GS, namely the 3-O glucosides of five common anthocyanidins:
245 cyanidin, peonidin, petunidin, delphinidin and malvidin and a malvidin derivative, along with the
246 flavonol compounds, namely: 3-O glucoside and 3-O glucuronide of quercetin, quercetin and
247 kaempferol were observed (Table 2). Total phenolic content of AP was 1000 mg/kg, consistent with

values reported previously (Landl *et al.*, 2010; Sun *et al.*, 2015) and increased by 30% and 130% (to 1300 mg/kg and 2300) in AP-GS 3.0 and AP-GS 4.8, respectively. The amounts of soluble phenolics found in the fortified purees were exactly as expected based on the initial levels in apple and GS and on the addition, suggesting that these compounds were not strongly bounded to apple matrix. On the contrary, upon addition to GS to a tomato puree and a wheat dough, which do not contain proanthocyanidins, the amount of soluble proanthocyanidins, especially the higher mass oligomers, decreased. This decrease was due to the formation of insoluble protein-proanthocyanidin complexes (Lavelli *et al.*, 2016a). The different behaviour of apple matrix could be attributed to the natural presence of a high amount proanthocyanidins in apple, which could form complexes with apple protein prior to the addition of GS. Lack of strong interaction between added grape skin phenolics and apple matrix can be considered as a positive result, since these interactions could negatively affect phenolic bioavailability.

The FRAP value of apple puree, which depend on the redox potential of phenolic compounds (Pulido *et al.*, 2000) was similar to that reported previously for apple juice treated by an optimized pulsed electric field process (Shilling *et al.*, 2008), indicating that the quality of the food matrix chosen for fortification was good. FRAP value increased upon GS addition, by 36 and 130% in AP-GS 3.0 and AP-GS 4.8, respectively, consistent with the increase in phenolic compounds.

The antiglycation activity of apple and GS fortified apple purees was studied *in vitro* by evaluation of their ability to inhibit a model reaction between fructose and BSA. Phenolics can inhibit protein glycation by carbonyl trapping activity, radical scavenging activity and metal chelation (Matsuda *et al.*, 2003). In the current study, the efficacy of inhibition was expressed with reference to aminoguanidine, which is a synthetic antiglycation agent (Engelen *et al.*, 2013). Phloridzin had a similar antiglycation activity as aminoguanidine ($I_{50}$ 870 ± 10 and 730 ± 20 nmol, respectively), while other apple and GS phenolics were far more efficient. In fact, the efficacy ranking for standard phenolics was quercetin 3-O-glucoside ($I_{50}$ 27 ± 2 nmol) > malvidin 3-O-glucoside ($I_{50}$ 80 ± 4 nmol) > catechin, epicatechin ($I_{50}$ 110 ± 5 mol) > chlorogenic acid ($I_{50}$ 126 ± 4 nmol) >>>

phloridzin. It is worth noting that GS phenolics (flavonols, anthocyanins) were the most efficient antiglycation agents. Hence, addition of GS to apple puree increased the antiglycation activity by 100 and 200% in AP-GS 3.0 and AP-GS 4.8, respectively and this increase was higher compare to the increase in phenolic content.

***Sensory evaluation***

Results from the 2-way mixed ANOVA model applied to hedonic ratings of 70 consumers showed a significant effect of product formulation on appearance, odour, taste, flavour, texture and overall liking ($p < 0.001$) (Table 3). The control sample resulted as the most liked product for all the sensory attributes. AP-GS 3.0 and AP-GS 4.8 were not significantly discriminated for liking of appearance and odour. On the contrary, AP GS 3.0 had a better hedonic performance over AP-GS 4.8 considering the acceptability of taste, flavour, texture and overall liking. Interestingly, the liking for texture of AP-GS 3.0 was comparable to that of the reference sample. Overall, these consumers' ratings could be considered to be good. In fact, in general addition of winemaking derived ingredients results in a marked decrease of appreciation of sensory attributes and overall liking (Soto *et al*., 2011).

Consumers are increasingly segmented on the basis of their attitudes towards food, particularly towards health and hedonic characteristics of food. In fact, identifying segments of consumers with different attitudes towards food and nutrition might allow targeting different types of products for each segment (Laureati *et al*., 2012; Laureati *et al*., 2013). Consumers' segmentation by means of K-mean Cluster Analysis on liking data provided two clusters of subjects: Cluster 1 (Cl 1; n=24; males=4) and Cluster 2 (Cl 2; n=46; males=22). Cl 1 generally disliked new fortified prototypes, as showed by the hedonic ratings which never reached the acceptability (the central point of the scale, i.e. 5) for any attribute (Table 3). By Cl 1, even the reference standard was not considered acceptable. Results clearly discourage to consider this cluster as suitable consumer target for the fortified prototypes.

299 Cl 2, the most numerous one (66%), showed very satisfying results. Reference sample AP and AP-
300 GS 3.0 reached the acceptability for all the attributes. Ratings for appearance and odour of samples
301 AP-GS 3.0 and AP-GS 4.8 were not significantly different and both samples resulted slightly
302 pleasant. On the contrary, AP-GS 3.0 was significantly preferred over AP-GS 4.8 considering taste,
303 flavour, texture and overall liking. In particular, an extremely positive result was reached by AP-GS
304 3.0 whose liking was comparable to those of reference sample for taste, texture and overall liking.
305 Un-paired t-tests applied to mean data of the two clusters considering items of the questionnaire
306 showed some interesting differences. Regarding the motivations behind the consumption of pureed
307 fruits, Cl 2 declared to agree with the statement "I consume pureed fruit because it is a substitute of
308 fresh fruit" (3.39 ± 0.27) more than Cl 1 (2.29 ± 0.36). No significant differences between clusters
309 were observed in terms of reason of consumption related to the presence of antioxidants and fibres.
310 Considering the importance of some items in the choice of a pureed fruit, for Cl 2 the hedonic
311 pleasantness was a significantly ($p < 0.01$) more important issue (6.33 ± 0.11) in respect to what
312 declared by Cl 1 (5.54 ± 0.25). From the t-test performed on mean data of clusters considering
313 declared liking for some fruit-based products, no difference was found between the two clusters for
314 the pureed apple as such. On the contrary, a significantly higher ($p < 0.05$) mean score was given by
315 Cl 2 for apple paired with many other fruits, in particular with apricot (Cl 1: 4.71 ± 0.42, Cl 2: 6.24
316 ± 0.22), prune (Cl 1: 3.96 ± 0.33, Cl 2: 5.63 ± 0.25), blueberry (Cl 1: 5.08 ± 0.44, Cl 2: 6.41 ± 0.27),
317 peach (Cl 1:5.21 ± 0.39, Cl 2: 6.4 4± 0.26), banana (Cl 1: 3.79 ± 0.46, Cl 2: 6.00 ± 0.30), kiwi (Cl
318 1: 4.25 ± 0.44, Cl 2: 5.35 ± 0.29), mixed berries (Cl 1: 4.79 ± 0.35, Cl 2: 6.07 ± 0.28) and
319 pomegranate (Cl 1: 4.92 ± 0.43, Cl 2: 6.09 ± 0.25).
320 In summary, Cl 2 could be representative of a segment of consumers potentially interested in the
321 future consumption of the developed enriched pureed apple. In fact, Cl 2, who declared to pay more
322 attention to the sensory properties of food, not only liked the prototypes but also appreciate the
323 pureed apple as substitute of the fresh fruit, even when in pairing with other fruits.
324 ***Processing and storage of GS fortified apple puree***

325 The effects of heat treatment and storage on the phenolic fraction of the preferred GS apple puree
326 (AP-GS 3.0) and of control apple puree were studied. *A. acidoterrestris* was proposed as the target
327 microorganism to set up the heat treatment conditions for low pH foods, including apple purees and
328 juices. A pasteurization value resulting in 2- and 3-D in the target microorganism is recommended,
329 but higher D values have also been proposed (Silva & Gibbs, 2004). In the present study, the
330 fortified and control apple purees were submitted to 3-D and 6-D treatments, which simulate a
331 continuous heat process and 14- D treatment, which simulates an autoclave treatment.
332 The 3-D treatment, did not cause any change in phenolic content, FRAP values and antiglycation
333 activity (Table 4). Upon the 6-D and 14-D treatments, in both AP and AP-GS 3.0, percent retention
334 of monomeric and dimeric flavanols and dihydrochalcones were higher than 80%, while
335 proanthocyanidin and chlorogenic acid contents did not vary. In AP-GS 3.0, percent retentions of
336 monomeric anthocyanins were 92 and 86% upon the 6-D and 14-D, respectively. The amount of
337 flavonols was found to be stable. Variation of total phenolic content was only significant after the
338 14-D treatment. Reducing capacity (FRAP values) and antiglycation activity decreased with
339 increasing heat intensity. The most intensive conditions (14-D) resulted in a 70% retention of
340 antiglycaton activity, while the efficacy remained significantly higher for AP-GS 3.0 than for AP.
341 Hydroxymethylfurfural content in AP and AP-GS 3.0 gradually increased with increasing
342 the intensity of heat treatment up to 4.1 and 5.4 mg/kg, respectively, indicating the occurrence of
343 Maillard reaction. These values are higher than those found in other fruit beverages (Lavelli *et al.*,
344 2009), but they are far below the threshold defined by the AIJN Code of practice for apple juice, i.e.
345 20 mg/L (Shilling *et al*., 2008). Considering the effects of the 3-D, 6-D and 14-D treatments on
346 phenolic components and antiglycaton activity, it may be suggested that a continuous mild heat
347 treatment (3-D) is more appropriate than an intensive treatment for the fortified puree.
348 Storage for one month in the temperature range 15 - 35 °C caused further decrease in phenolic
349 compounds (Table 5). In both AP and AP-GS 3.0, monomeric and dimeric flavanols and
350 proanthocyanidins were mostly affected by storage, with percent retention ~ 50% at 35 °C. In

351 previous studies, low stability of flavanols during storage has already been observed either after
352 conventional processing (Oszmianski *et al*., 2011) or after innovative high pressure processing
353 (Shilling *et al*., 2008). In AP-GS 3.0, monomeric anthocyanin retention was ~ 70% at 15 °C, but it
354 decreased to 15% at 35° C (Table 5). Flavonol content remained unchanged. Total phenolics
355 decreased markedly during storage, especially at 35 °C. Consequently, FRAP values and
356 antiglycation activity decreased. However, 90% antiglycation activity was retained in AP-GS 3.0
357 after one month storage at 15°C.

358 Hydroxymethylfurfurol increased up to 14 mg/kg in both AP and AP-GS 3.0 after one
359 month of storage at the highest storage temperature. This value remained below the threshold
360 reported above (Shilling *et al*., 2008) On the other hand, the loss of phenolic compounds during
361 storage indicates that storage at low temperature is advisable for the both AP and AP-GS 3.0.

362 **CONCLUSION**

363 In conclusion, the necessary issue to deliver health-promoting compounds as food
364 ingredients is to gain consumers' acceptability. Considering the most numerous cluster obtained
365 from the consumers segmentation (Cl 2, 66% of the population), good ratings were obtained for the
366 apple puree added with 1.5% GS for appearance, colour, odour taste, flavour, texture and overall
367 liking. Higher amounts of GS (5%) negatively affected the acceptability for colour, taste and
368 flavour. The apple puree added with 1.5% GS can be labelled as "fibre source" and provide
369 anthocyanins, flavonols and flavanols resulting in ~ twofold higher antiglycation activity than AP.
370 Mild heat treatment (3D reduction of the target microorganism) and storage at temperature below
371 15°C are advisable for the fortified product. The designed functional product could be proposed to
372 pair fructose intake from apple puree with both fibre and efficient antiglycation agents. Hence, use
373 of GS as ingredient could represent a new opportunity for the apple processing industry to develop a
374 value-added product.

375 **ABBREVIATIONS**

376 AGEs, advanced glycation end-products; AG, aminoguanidine; AP, apple puree; AP-GS 3.0, apple
377 puree added with grape skins containing 3.0% of dietary fibre; AP-GS 4.8, apple puree added with
378 grape skin containing 4.8% of dietary fibre; Cl 1, consumers' cluster 1; Cl 2, consumers' cluster 2;
379 D, decimal reductions; FRAP, ferric ion reducing antioxidant power; GAE, gallic acid
380 equivalents; GS, grape skins.
381

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384

385 **CONFLICT OF INTEREST**
386 No conflict of interest is declared.

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504

505 **Table 1.** Major components (g/kg) of grape skins (GS), apple puree (AP) and apple
506 puree formulations with GS (AP-GS 3.0 and AP-GS 4.8).

507

508

| | GS | AP | AP-GS 3.0 | AP-GS 4.8 |
|---|---|---|---|---|
| Fibre | $560^{d} \pm 1$ | $22^{a} \pm 1$ | $30^{b} \pm 1$ | $48^{c} \pm 1$ |
| Protein | $91^{d} \pm 1$ | $3.0^{a} \pm 0.1$ | $4.3^{b} \pm 0.1$ | $7.2^{c} \pm 0.1$ |
| Carbohydrate | $37^{a} \pm 1$ | $119^{c} \pm 1$ | $118^{c} \pm 1$ | $115^{b} \pm 1$ |
| Fat | $77^{d} \pm 2$ | $2.0^{a} \pm 0.1$ | $3.1^{b} \pm 0.1$ | $5.6^{c} \pm 0.1$ |
| Ash | $89^{d} \pm 1$ | $2.0^{a} \pm 0.1$ | $3.3^{b} \pm 0.1$ | $6.1^{c} \pm 0.1$ |
| Moisture | $54^{a} \pm 1$ | $870^{d} \pm 1$ | $856^{c} \pm 1$ | $821^{b} \pm 1$ |

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513 Results are average ± SE. Different letters within the same row indicate significant
514 differences (LSD; $p < 0.05$).

515

516 **Table 2.** Phenolic contents (mg/kg), FRAP values (mmol Fe(II) Eq/kg) and antiglycation
517 activity (mmolAG Eq/kg) of grape skins (GS), apple puree (AP) and apple puree
518 formulations with GS (AP-GS 3.0 and AP-GS 4.8).

| | GS | AP | AP-GS 3.0 | AP-GS 4.8 |
|---|---|---|---|---|
| Delphinidin 3-O-glucoside | 300[c] ± 44 | nd | 4.6[a] ± 0.5 | 17[b] ± 2 |
| Cyanidin 3-O-glucoside | 136[c] ± 10 | nd | 2.1[a] ± 0.3 | 6.9[b] ± 1 |
| Petunidin 3-O-glucoside | 385[c] ± 44 | nd | 5.9[a] ± 0.8 | 20[b] ± 3 |
| Peonidin 3-O-glucoside | 151[c] ± 24 | nd | 2.2[a] ± 0.2 | 8.6[b] ± 0.6 |
| Malvidin 3-O-glucoside | 880[c] ± 130 | nd | 13[a] ± 3 | 50[b] ± 10 |
| Malvidin derivative | 203[c] ± 23 | nd | 3.0[a] ± 0.8 | 13[b] ± 3 |
| Catechin | 370[d] ± 39 | 7.4[a] ± 0.4 | 12.7[b] ± 0.8 | 25[c] ± 3 |
| Epicatechin | 199[c] ± 31 | 32[a] ± 2 | 34[a] ± 1 | 43[b] ± 4 |
| Procyanidin B2 | 44[b] ± 3 | 24[a] ± 3 | 24[a] ± 1 | 26[a] ± 4 |
| Procyanidin B1 | 227[d] ± 3 | 6.5[a] ± 0.1 | 9.7[b] ± 0.6 | 17[c] ± 2 |
| Soluble proanthocyanidins | 21000[d] ± 2000 | 650[a] ± 10 | 900[b] ± 10 | 1220[c] ± 10 |
| Chlorogenic acid | nd | 132[a] ± 5 | 130[a] ± 10 | 143[a] ± 10 |
| Phoretin-2-O-xyloglucoside | nd | 37[a] ± 2 | 37[a] ± 1 | 33[a] ± 3 |
| Phloridzin | nd | 59[a] ± 2 | 59.2[a] ± 0.7 | 56[a] ± 2 |
| Quercetin 3-O-glucoside and 3-O-glucuronide | 343[c] ± 54 | nd | 5.2[a] ± 0.1 | 20.1[b] ± 0.1 |
| Quercetin | 520[c] ± 15 | nd | 7.7[a] ± 0.3 | 30.2[b] ± 0.9 |
| Kaempferol | 150[c] ± 4 | nd | 2.3[a] ± 0.1 | 5.7[b] ± 0.2 |
| Total phenolics | 30000[d] ± 2000 | 1000[a] ± 10 | 1300[b] ± 100 | 2300[c] ± 40 |
| FRAP values | 271[d] ± 24 | 7.2[a] ± 0.1 | 9.8[b] ± 0.3 | 18[c] ± 1 |
| Antiglycation activity | 2320[d] ± 10 | 35.3[a] ± 0.7 | 69.0[b] ± 0.4 | 130[c] ± 1 |

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520 Results are average ± SE. nd: not detected. Different letters within the same row
521 indicate significant differences (LSD; $p < 0.05$).

22 **Table 3**. Overall liking and liking for appearance, odour, taste, flavour and texture towards apple puree
23 (AP) and apple puree formulations with GS (AP-GS 3.0 and AP-GS 4.8) expressed by all consumers,
24 Cluster1 and Cluster 2.

| Subjects | AP | AP-GS 3.0 | AP-GS 4.8 |
|---|---|---|---|
| **All (n=70)** | | | |
| appearance | $6.37^b \pm 0.19$ | $5.57^a \pm 0.21$ | $5.41^a \pm 0.21$ |
| odour | $6.16^b \pm 0.21$ | $5.10^a \pm 0.24$ | $4.70^a \pm 0.24$ |
| taste | $5.69^c \pm 0.22$ | $4.99^b \pm 0.24$ | $4.04^a \pm 0.24$ |
| flavour | $5.79^c \pm 0.24$ | $4.73^b \pm 0.24$ | $4.11^a \pm 0.25$ |
| texture | $5.86^b \pm 0.26$ | $5.56^b \pm 0.27$ | $3.86^a \pm 0.26$ |
| overall | $5.96^c \pm 0.21$ | $5.09^b \pm 0.24$ | $4.13^a \pm 0.23$ |
| **Cluster 1 (n=24)** | | | |
| appearance | $5.71^b \pm 0.36$ | $4.38^a \pm 0.36$ | $4.63^a \pm 0.36$ |
| odour | $5.21^b \pm 0.36$ | $3.75^a \pm 0.36$ | $3.42^a \pm 0.36$ |
| taste | $4.42^b \pm 0.32$ | $2.83^a \pm 0.32$ | $2.71^a \pm 0.32$ |
| flavour | $4.38^b \pm 0.34$ | $2.63^a \pm 0.34$ | $2.96^a \pm 0.34$ |
| texture | $4.04 \pm 0.37$ | $3.71 \pm 0.49$ | $3.13 \pm 0.42$ |
| overall | $4.54^b \pm 0.29$ | $2.67^a \pm 0.29$ | $2.79^a \pm 0.29$ |
| **Cluster 2 (n=46)** | | | |
| appearance | $6.72^b \pm 0.23$ | $6.20^a \pm 0.23$ | $5.83^a \pm 0.23$ |
| odour | $6.65^b \pm 0.25$ | $5.80^a \pm 0.25$ | $5.37^a \pm 0.25$ |
| taste | $6.35^b \pm 0.24$ | $6.11^b \pm 0.24$ | $4.74^a \pm 0.24$ |
| flavour | $6.52^c \pm 0.25$ | $5.83^b \pm 0.25$ | $4.72^a \pm 0.25$ |
| texture | $6.80^b \pm 0.27$ | $6.52^b \pm 0.27$ | $4.24^a \pm 0.27$ |
| overall | $6.70^b \pm 0.20$ | $6.35^b \pm 0.20$ | $4.83^a \pm 0.20$ |

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26 Results are average ± SE. Different letters within the same row indicate significant differences (LSD; $p <$
27 $0.05$). Hedonic scale from 1 (extremely dislike) to 9 (extremely like)

**Table 4.** Phenolic contents (mg/kg), FRAP values (mmol Fe(II) Eq/kg), antiglycation activity (mmolAG Eq/kg) and hydroxymethylfurfurol content (mg/kg) of apple puree (AP) and apple puree formulation with GS (AP-GS 3.0) after heat treatments achieving 3, 6 and 14 D of the target microorganism *A. acidoterrestris.*

| | **AP** | | | **AP-GS 3.0** | | |
|---|---|---|---|---|---|---|
| | **3 D** | **6 D** | **14 D** | **3 D** | **6 D** | **14 D** |
| Anthocyanins* | | | | $30.8^{c} \pm 0.3$ (100) | $28.3^{b} \pm 0.1$ (92) | $26.6^{a} \pm 0.1$ (86) |
| Flavanol monomer, dimers* | $69^{b} \pm 9$ (100) | $60^{a} \pm 2$ (87) | $56^{a} \pm 5$ (81) | $81^{c} \pm 1$ (100) | $70^{b} \pm 1$ (86) | $62^{a} \pm 1$ (77) |
| Proanthocyanidins | $660^{a} \pm 10$ (100) | $637^{a} \pm 13$ (97) | $650^{a} \pm 17$ (99) | $900^{b} \pm 15$ (100) | $900^{b} \pm 27$ (100) | $953^{b} \pm 15$ (106) |
| Flavonols* | nd | nd | nd | $15.2^{a} \pm 0.1$ (100) | $15.2^{a} \pm 0.1$ (100) | $14.9^{a} \pm 0.3$ (98) |
| Chlorogenic acid | $132^{a} \pm 3$ (100) | $132^{a} \pm 8$ (100) | $141^{a} \pm 10$ (100) | $130^{a} \pm 1$ (100) | $135^{a} \pm 13$ (100) | $132^{a} \pm 6.0$ (101) |
| Dihydrochalcones* | $96^{c} \pm 6$ (100) | $86^{a} \pm 6$ (90) | $83^{a} \pm 6$ (86) | $97^{c} \pm 3$ (100) | $104^{c} \pm 7$ (107) | $83^{a} \pm 4$ (86) |
| Total phenolics | $1050^{b} \pm 30$ (100) | $978^{ab} \pm 18$ (93) | $936^{a} \pm 14$ (89) | $1300^{d} \pm 20$ (100) | $1250^{d} \pm 34$ (96) | $1140^{c} \pm 50$ (83) |
| FRAP values | $6.9^{c} \pm 0.2$ (100) | $6.0^{b} \pm 0.3$ (86) | $4.9^{a} \pm 0.2$ (70) | $9.8^{f} \pm 0.1$ (100) | $8.9^{e} \pm 0.1$ (91) | $7.6^{d} \pm 0.1$ (78) |
| Antiglycation activity | $35^{c} \pm 2$ (100) | $29^{b} \pm 1$ (83) | $25^{a} \pm 1$ (70) | $69^{f} \pm 1$ (100) | $53^{e} \pm 1$ (78) | $48^{d} \pm 1$ (70) |
| Hydroxymethylfurfural | $1.0^{a} \pm 0.4$ | $1.5^{ab} \pm 0.4$ | $4.1^{c} \pm 0.1$ | $1.2^{a} \pm 0.1$ (100) | $1.7^{b} \pm 0.1$ | $5.4^{c} \pm 0.1$ |

Results are average ± SE. Different letters within the same row indicate significant differences (LSD; $p < 0.05$). Values in brackets indicate percent retention. *Identified phenolic compounds are reported in Table 2. nd: not detected.

**Table 5.** Phenolic contents (mg/kg), FRAP values (mmol Fe(II) Eq/kg), antiglycation activity (mmolAG Eq/kg) and hydroxymethylfurfurol content (mg/kg) of apple puree (AP) and apple puree formulation with GS (AP-GS 3.0) after 1 month storage in the temperature range 15 - 35 °C.

| | **AP** | | | **AP-GS 3.0** | | |
|---|---|---|---|---|---|---|
| | **15 °C** | **25 °C** | **35 °C** | **15 °C** | **25 °C** | **35 °C** |
| Anthocyanins* | | | | $18^{c}$ ± 8 (69) | $13^{b}$ ± 1 (50) | $4^{a}$ ± 1 (15) |
| Flavanol monomers, dimers* | $35^{b}$ ± 1 (62) | $31^{a}$ ± 2 (55) | $27^{a}$ ± 3 (47) | $44^{d}$ ± 5 (70) | $37^{c}$ ± 1 (60) | $30^{a}$ ± 1 (48) |
| Soluble proanthocyanidins | $651^{c}$ ± 13 (100) | $637^{c}$ ± 13 (98) | $429^{a}$ ± 13 (66) | $807^{d}$ ± 31 (85) | $801^{d}$ ± 30 (84) | $543^{b}$ ± 3 (57) |
| Flavonols* | nd | nd | nd | $15^{a}$ ± 2 (100) | $15^{a}$ ± 1 (100) | $14^{a}$ ± 4 (96) |
| Chlorogenic acid | $143^{a}$ ± 8 (100) | $141^{a}$ ± 9 (100) | $140^{a}$ ± 7 (100) | $139^{a}$ ± **7** (100) | $132^{a}$ ± 15 (100) | $132^{a}$ ± 3 100 |
| Dihydrochalcones* | $83^{a}$ ± 2 (100) | $83^{a}$ ± 11 (100) | $83^{a}$ ± 4 (100) | $87^{a}$ ± 6 (100) | $83^{a}$ ± 3 (100) | $78^{a}$ ± 5 (94) |
| Total phenolics | $956^{c}$ ± 39 (100) | $837^{b}$ ± 41 (89) | $642^{a}$ ± 82 (69) | $1075^{d}$ ± 65 (94) | $913^{c}$ ± 60 (80) | $814^{b}$ ± 40 (71) |
| FRAP values | $4.3^{b}$ ± 0.3 (88) | $4.2^{b}$ ± 0.1 (85) | $3.3^{a}$ ± 0.1 (67) | $8.0^{e}$ ± 0.4 (100) | $6.4^{d}$ ± 0.2 (84) | $5.6^{c}$ ± 0.2 (73) |
| Antiglycation activity | $20^{b}$ ± 1 (80) | $19^{b}$ ± 1 (75) | $15^{a}$ ± 1 (61) | $43^{d}$ ± 1 (90) | $37^{d}$ ± 1 (77) | $29^{c}$ ± 1 (60) |
| Hydroxymethylfurfural | $6.5^{a}$ ± 2.6 | $10.5^{b}$ ± 0.6 | $14.1^{c}$ ± 0.7 | $5.4^{a}$ ± 0.4 | $9.7^{b}$ ± 0.9 | $14.3^{c}$ ± 0.4 |

Results are average ± SE. Different letters within the same row indicate significant differences (LSD; $p < 0.05$). Values in brackets indicate percent retention. *Identified phenolic compounds are reported in Table 2. nd: not detected.