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Natural compounds from grape by-products enhance nutritive value and reduce formation of CML in model muffins

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ABSTRACT

This study had the objective of determining the effects of the addition of different ingredients and grape by-products (GP) to muffins on CML content. It was found that ingredients, such as salt, baking powder and protein-rich components, reduced CML from 50% to 86%. The use of all ingredients simultaneously caused the highest reduction in CML, suggesting synergistic effects in the muffin formula. Raw cane sugar produced higher amounts of CML than refined sucrose, probably due to metal-ion mediated degradation of fructoselysine. The CML content was correlated with the level of oleic acid at −0.829 and with the level of linoleic acid at 0.913. Muffins enriched with appropriate levels of GP (20%) showed a lowering of the CML level and no significant changes in the sensory profile. GP added to the model system with protein-rich ingredients resulted in the weakest inhibitory effects, probably due to the polyphenol–protein binding mechanism.

1. Introduction

Numerous epidemiological investigations have established an association between diets rich in phytochemicals and the reduced risk of suffering from many civilization-related diseases (Rice-Evans, Miller, & Paganga, 1996). Grapes are one of the world's largest fruit crops, and approximately 80% of their yield is utilised for winemaking. The winemaking industry thus generates large quantities of waste which, because of its high pollution load, considerably increases chemical and biochemical oxygen demand (Lafka, Sinanoglou, & Lazos, 2007). Grape by-products (GP) have drawn increased attention in recent years for their potential health benefits—not only as an antioxidant agents, but also as antibacterial, antiobesity, antithrombotic, and anticarcinogenic agents (Mildner-Szkudlarz & Bajerska, 2013; Park, Park, & Cha, 2008). These various biological properties are believed to be due to the functions of GP polyphenols (PCs) and dietary fibre (DF): even after contact with the fermenting wine, GP still contains a large amount of such phytochemicals.

Therefore, GP has potential as a bioactive food ingredient which can also increase the profits for grape growers while acting as a value-adding by-product of wine production. The exploration of ways of incorporating these by-products as a health-food ingredient in the human diet could provide many health benefits. Because cereal-based products have been, and still are, a central constituent in the diets of most populations, the use of such products supplemented with various nutritious, protective, and ballast substances may be appropriate. However, utilisation of GP in biscuits is very limited, and no accurate information is available on the effect of GP on the quality of bakery products, such as muffins.

On the other hand, baking processes might have an influence on the stability of PCs (Bajerska, Mildner-Szkudlarz, Jeszka, & Szwengiel, 2010; Wang & Zhou, 2004), and could induce modifications in the chemical composition and properties of food (Michalska, Amigo-Benavent, Zieliński, & del Castillo, 2008). It has been reported that the antioxidant activities of grapeseed extract added to bread were lowered by around 30–40% by thermal processing, probably due to degradation of the PCs (Peng, Ma, & Cheng, 2010). Moreover, free amino groups of lysine (Lys), peptides, and proteins can react with the carbonyl group of reducing sugars or lipid oxidation products during baking to form advanced glycation end products, including N$^{\varepsilon}$-(carboxymethyl)lysine (CML) (Fu et al., 1996; Lima, Assar, & Ames, 2010). Recently, CML has been viewed as potentially toxic in food, and its accumulation *in vivo* has been implicated as a major pathogenic process in diabetic

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complications, atherosclerosis, Alzheimer's disease and normal ageing (Nerlich & Schleicher, 1999). Obviously, the concentration of CML in food is affected by many factors, including temperature, length of the period of heating, pH, concentrations and reactivity of the components present, water content, and the presence of inhibitory compounds like antioxidants (Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; Srey et al., 2010). Natural extracts of beans, cinnamon bark, grapeseed, and peanut skins, along with catechol compounds, have been demonstrated to possess strong inhibitory effects on AGE formation (Peng et al., 2008, 2010). So far, there have been no reports on the addition of GP to cereal-based products, which are consumed daily, or proving their protective effect against CML formation.

The present study was designed to investigate the effects of various food ingredients—protein-rich components, salt, baking powder, different types of sugar, and plant oil—on CML content. Furthermore, the associated effects of the addition of GP, as well as of food ingredients, on CML formation in model muffins was also assessed as the main objective of this study.

2. Materials and methods

2.1. GP preparation

A sample of winemaking by-products of the Pinot Noir red grape (*Vitis vinifera*) variety was provided by a winery in Poland from their 2012 crop. The material was lyophilized to a moisture content of approximately 2–4%, and the skins were separated from the seeds with the aid of a sieve and milled to a fine powder (i.d. $\leqslant$150 μm).

2.2. Preparation of muffins

The muffin formulation contained the ingredients typically used for muffin preparation: 34.05% wheat flour; 32.13% water; 15.42% sugar; 13.88% fat; 2.57% nonfat dry milk powder; 1.29% baking powder; 0.53% dry egg white; and 0.13% salt (weight basis).

To determine the maximum dose of GP that could be included in the muffin formula without altering consumer acceptability, GP was incorporated into muffins, replacing 10%, 20%, and 30% of the wheat flour in the muffin recipe.

The dough (60 g) was placed into paper muffin cups and baked in a preheated oven at 180 °C for 20 min. After baking, the muffins were cooled to room temperature and packed in polypropylene pouches. They were then sealed until sensory and texture analysis. Other muffins intended for chemical analysis were frozen, freeze-dried, ground into a fine powder, and stored at −18 °C in airtight vials.

2.2.1. Effect of added ingredients—recipe 1

Recipe 1 (R1) consisted of only wheat flour, water, white beet sugar, and margarine with 80% fat content. The additional ingredients—namely, nonfat dry milk powder (in recipe R1M), baking powder (R1B), dry egg white powder (R1E), salt (R1S), and all ingredients together (R1A)—were added to the R1 recipe in the ratio used for muffin preparation (Section 2.2).

2.2.2. Effect of types of sugar and oil—recipe 2

Recipe 2 (R2) contained all the ingredients listed above (Section 2.2). However, the effects of the following different types of sugar were examined: glucose (in recipe R2G), fructose (R2F), white (refined) beet sugar (R2Bs), and raw (unrefined) cane sugar (R2Cs). In these recipes, margarine (80% fat content) was the fat source. The effects of different types of fat were determined by replacing the margarine with olive oil (in recipe R2OO), rapeseed oil (R2RS), rice bran oil (R2RB), and grapeseed oil (R2GS), with white beet sugar as the sugar source.

2.2.3. Effect of added GP to recipe 1 and 2

Model samples of recipes R1 and R2 were prepared with a 20% addition of GP to determine the associated effect of food ingredients with phenolic compounds from the GP on CML concentration.

2.3. Determination of CML in model muffins

The CML measuring method employed here is adapted from Peng et al. (2010). Following defatting, protein reduction, hydrolysis, and derivatization using *o*-phthaldialdehyde, CML determination was performed using a Waters Alliance high-performance liquid chromatography (HPLC System 600, Milord, MA, USA) with a fluorescence detector (Waters 474). The HPLC system was equipped with a Waters Sun Fire C18 column (150 × 4.6 mm, 5 μm; Milord, MA, USA). The flow rate was 1.0 ml/min and the injection volume was 10 μl. The mobile phases were acetate buffer and acetonitrile (9:1, v/v) (solvent A) and 50% acetonitrile (solvent B). Detection was at 340 nm (excitation) and 455 nm (emission). The peaks for CML-derivatives in the muffin samples were confirmed by comparison with an authentic sample of CML provided by PolyPeptide Laboratories France SAS (Strasbourg, France). Identified compounds were quantified using the external standard calibration procedure. The limit of detection (LOD) was 0.42 ng, the limit of quantification (LOQ) was 1.29 ng, the recovery of the analyte compared to the internal standard was ~100% (SD = 10.03%), and the repeatability (method precision) was 3.65% (coefficient of variations).

2.4. Phenolic compound analysis

Phenolic compounds were extracted from muffins and GP with methanol/water/formic acid solution (70:29.7:0.3 v/v/v), using the procedure described by Wang and Zhou (2004). Reversed-phase (C18 column) ultra high-performance liquid chromatography electrospray ionisation mass spectrometry (RP–UHPLC–ESI-MS) analysis was performed using a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Sunnyvale, CA, USA) coupled to a Bruker maXis impact ultrahigh resolution orthogonal quadrupole-time-of-flight accelerator (qTOF) equipped with an ESI source and operated in positive-ion mode (Bruker Daltonik, Bremen, Germany). The RP chromatographic separation was achieved with a Kinetex™ 1.7 μm C18 100 Å, LC column 100 × 2.1 mm (phenomenex, Torrance, CA, USA). The ESI-MS settings were as follows: capillary voltage 4500 V, nebulizing gas 1.8 bar, and dry gas 9 l/min at 200 °C. The scan range was from mass-to-charge ratio (*m*/*z*) 80–1200. The mobile phase was composed of water containing 1% formic acid (A) and acetonitrile containing 5% water and 1% formic acid (B). The flow rate was 0.2 ml/min with a gradient elution of 5–95% B over 35 min, and standing at 95% B for 20 min. The sample injection volume was 2 μl. The column temperature was set at 40 °C. The ESI-MS system was calibrated using sodium formate clusters introduced by loop-injection at the beginning of the LC–MS run. The LC–MS data was processed using Data Analysis 4.1 software (Bruker Daltonik, Bremen, Germany). Molecular ions $[M+H]^+$ were extracted from full scan chromatograms and peak areas were integrated. The extraction window of individual ion chromatograms was ±0.05 *m*/*z* units. The compounds present in each sample were identified by comparing their retention times with those of standards, and based on molecular mass and structural information from the MS detector.

2.5. Protein determination

The protein content was determined by the Kjeldahl method using a conversion factor of 6.25 for cereal foods (AOAC method 920.87, 1995).

2.6. Analysis of the oils used in muffin preparation

The analysis of the fatty acid methyl esters of the oils used in the muffin preparation was carried out using a Hewlett Packard HP 5890 gas chromatograph equipped with a flame ionisation detector and fitted with a HP-Innowax capillary column (30 m × 0.25 mm i.d. × 0.25 μm df, Hewlett–Packard, Waldbronn, Germany), according to the method described previously (Mildner-Szkudlarz, Zawirska-Wojtasiak, Obuchowski, & Gośliński, 2009).

The tocochromanols of oils were analysed by direct injection of the oil samples dissolved in HPLC-grade n-hexane using a Waters Alliance HPLC System 600 (Milord, MA, USA) with a fluorescence detector (Waters 474), according to the previously published method (Górnaś, Siger, & Seglin, 2013).

2.7. Analysis of the sugars used in muffin preparation

The analysis of the glucose content of the white beet sugar and the raw cane sugar used in muffin preparation was performed as in Trinder (1969).

The analysis of the elemental content of white (refined) beet sugar and raw (unrefined) cane sugar was carried out using an inductively coupled plasma optical emission spectrometer (ICP-OES) Vista MPX (Australia) after digestion of samples in a microwave oven (CEM MARS 5), according to the method described by Chojnacka, Michalak, Zielińska, Górecka, and Górecki (2010).

2.8. Sensory analysis

A ten-member panel performed odour profiling of samples in three sessions. Samples from the same lots were presented to panel members 3 times within 8 h. All assessors had passed the basic odour test and been trained in sensory analysis at numerous sessions over several years (Mildner-Szkudlarz, Bajerska, Zawirska-Wojtasiak, & Górecka, 2013; Mildner-Szkudlarz, Zawirska-Wojtasiak, Szwengiel, & Pacyński, 2011; Zawirska-Wojtasiak, Gośliński, Szwacka, Gajc-Wolska, & Mildner-Szkudlarz, 2009). Their evaluation ability was checked using a control card. The panellists were asked to evaluate the products for colour, appearance, texture, taste, flavour, and overall acceptance. The ratings were made on a 9-point hedonic scale, ranging from 9 (like extremely) to 1 (dislike extremely), for each attribute (Hooda & Jood, 2005). Mean, variance, and standard deviation (SD) were calculated for all attributes of each sample, for each session separately and across all three sessions.

2.9. Statistical analysis

All analytical values represent the mean of three analyses performed in at least two different experiments. Data was analysed using one-way analysis of variance ($P < 0.05$) to determine the differences between the values of the tested compounds. For significant results, Tukey's Honestly Significant Difference test was used. Prior to building the classifying model functions, an exploratory analysis (cluster analysis) was carried out to observe data trends. Statistica 10.0 software (StatSoft, Krakow, Poland) was used for the analysis.

3. Results and discussion

3.1. Effect of added ingredients—recipe 1

The concentrations of CML in the model muffins made according to R1 are shown in Fig. 1. R1 is simply a mixture of wheat flour, water, sugar, and fat in the ratio usually used for preparing muffins (Rupasinghe, Wang, Huber, & Pitts, 2008), to which an individual ingredient was added with the aim of determining its effect on CML formation or elimination. It was found that R1 provided a relatively inert environment for CML that had the precursors necessary for CML formation in the model cereal-based products produced from it. After baking, these R1 samples contained the highest levels of CML (26.55 mg/kg muffins). The addition of the individual ingredients caused significant reductions in CML content (Fig. 1). The most dramatic levels of elimination were achieved with nonfat dry milk powder (R1M; about 82% reduction) and with dry egg white powder (R1E; about 86% reduction). Comparing the recipes with the added protein-rich ingredients to the plain R1

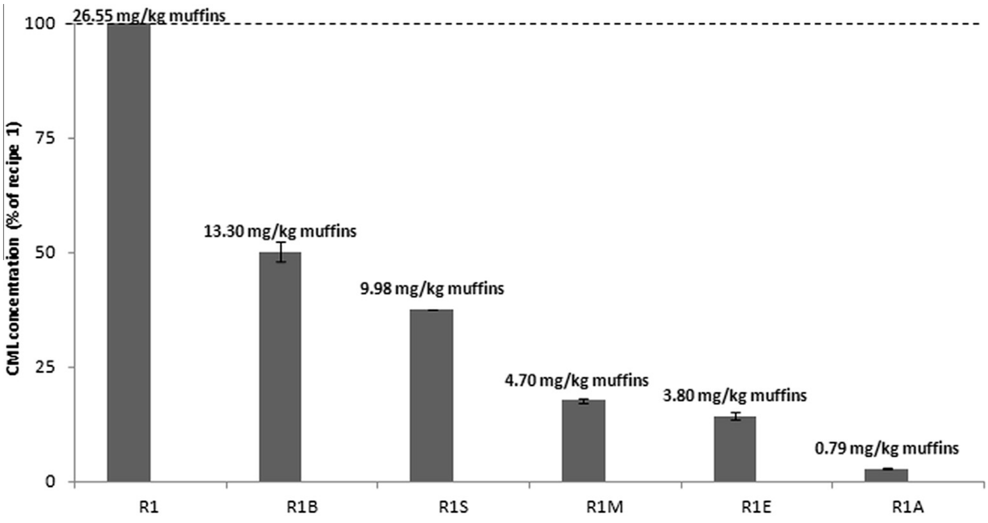

Fig. 1. CML concentration in muffins made according to recipe 1 with added ingredients. Values are mean ± SD of three replicates. Sample codes: R1, recipe 1 (wheat flour, water, sugar, margarine); R1B, recipe 1 + baking powder; R1S, recipe 1 + salt; R1 M, recipe 1 + nonfat dry milk powder; R1E, recipe 1 + dry egg white powder; R1A, recipe 1 + all ingredients together.

formula, the concentration of CML decreased from the R1 level of 26.55 mg/kg muffin to 4.70 mg/kg muffin (in R1 with nonfat dry milk powder, R1 M) and 3.80 mg/kg muffin (in R1 with dry egg white powder, R1E). This observation might reflect the protective action of proteins through competing and/or covalently bonding reaction of Maillard products with nucleophilic groups (–SH or –NH_2) to amino acid side chains. This finding is supported by Levine and Smith (2005) and Rydberg et al. (2003) for acrylamide elimination. The amount of CML formed was also affected by the addition of baking powder (R1B) and salt (R1S) (Fig. 1). Adding either baking powder or salt to R1 reduced the CML formation by about 50% and 62%, respectively. The mechanisms of elimination in the cases of baking powder and salt are not clearly understood. Presumably, the increase in pH might influence the concentration of CML. The reaction of amino acids with glucose did not occur when the amino residue was in its positive ion form. The extent of protonation of an amino acid is determined by the pK_a value of this group, where the N terminal pK values of Lys is 9.06 (Yamaguchi et al., 2009). At higher pH, the α-amino group of Lys is protonated to a greater degree, and thus is less likely to react with carbonyl groups in carbohydrates. This observation is also supported by Yamaguchi et al. (2009), who found that sodium chloride retarded the browning reaction rate of proteins, as measured by polymerisation degree or by the loss of Lys. Also, Levine and Smith (2005) reported that adding salt or sodium bicarbonate to crackers reduced acrylamide formation. On the other hand, the same authors stated that it was only when pH was raised to 9.6 and 10.5 by the addition of higher levels of NaOH that the effect of acrylamide elimination became significant. Thus, the elimination mechanism of salt or sodium bicarbonate appears to be more than a simple pH effect. The addition of all extra ingredients to recipe 1, giving recipe R1A, produced the highest reduction in CML, which suggests a synergistic effects of all the ingredients in the muffin formula. These samples were characterised by about 97% lower levels of CML, compared to the model muffins made with R1 (Fig. 1).

3.2. Effect of types of sugar and oil—recipe 2

The concentrations of CML detected in the muffins prepared according to R2, using different types of sugar and oils, are shown in Table 1. The amount of CML formed was significantly affected by the type of both sugar and oil used, and ranged from 0.79 to 25.33 mg/kg muffin. The muffins made with glucose (R2G) had the highest levels of CML (at 25.33 mg/kg muffin)—an approximately 3.5-fold greater content than in the case of the second monosaccharide, fructose (R2F) (Table 1). This is confirmed by previous reports that the oxidation of glucose generates a greater yield of glyoxal (the precursor of CML) than the oxidation of fructose (Charissou et al., 2007; Srey et al., 2010). According to Srey et al. (2010), cakes baked using glucose contain about 1.2 times greater levels of CML than do fructose-formulated cakes. The study of Charissou et al. (2007) also demonstrated that high oven temperatures, and the use of fructose as the sugar source, are associated with the lowest levels of Lys damage and CML formation. The muffins made with raw cane sugar (R2Cs) produced about 11.5-fold higher concentrations of CML than the white beet sugar-formulated muffins (R2Bs) (Table 1). This observation is contrary to the results of Srey et al. (2010), who found about 1.4 times greater levels of CML in samples with refined sugar, compared to unrefined. The main differences between white beet sugar and raw cane sugar lie in the minerals, invert sugar, starch, and dextran contents, which are high in raw cane sugar and very low or absent in white beet sugar (Asadi, 2007). The presence of CML in raw cane sugar-formulated muffins (R2Cs) might not derive from starch hydrolysis, due to its stability below 250 °C (Charissou et al., 2007). This could be explained by the presence of glucose (1 mg/g) only in unrefined samples (data not shown). On the other hand, metal ions are known to activate the Maillard reaction, particularly in the formation of CML (Ahmed, Thorpe, & Baynes, 1986). The raw cane sugar were characterised by about 20.4-fold higher levels of metal ions than white (refined) beet sugar (Table 2). When metal concentrations are low, a large number of the metal ions are incorporated into complexes, while an increase in their number in the system can lead to the presence of free metal ions, which are not bound by Maillard reaction products and are more reactive (Ramonaitytė, Keršienė, Adams, Tehrani, & De Kimpe, 2009). Thus,

Table 1
Effect of types of sugar and oil used in R2 on CML formation.

	CML (mg/kg protein)	CML (mg/kg muffin)
Types of sugar		
R2G (glucose-formulated muffins)	274.40 ± 13.82^{d}	25.33 ± 1.28^{d}
R2F (fructose-formulated muffins)	77.59 ± 3.37^{b}	7.16 ± 0.31^{b}
R2Bs (white beet sugar-formulated muffins)	7.86 ± 0.61^{a}	0.79 ± 0.13^{a}
R2Cs (raw cane sugar-formulated muffins)	98.21 ± 7.47^{c}	9.06 ± 0.69^{c}
Types of oil		
R2OO (olive oil-formulated muffins)	19.68 ± 1.37^{a}	1.82 ± 0.13^{a}
R2RS (rapeseed oil-formulated muffins)	32.82 ± 0.52^{b}	3.03 ± 0.07^{b}
R2RB (rice bran oil-formulated muffins)	21.31 ± 0.03^{a}	1.97 ± 0.005^{a}
R2GS (grapeseed oil-formulated muffins)	123.77 ± 3.32^{c}	11.42 ± 0.31^{c}

Values are mean ± SD of three replicates.
$^{a-d}$ Separately for type of sugar and type of oil averages followed by the same superscript letters are not significantly different ($P < 0.05$).

Table 2
Fatty acid, tocopherol (T), tocotrienol (Tt_3) contents of oils and mineral content of sugars used in R2.

	Olive oil	Rapeseed oil	Rice bran oil	Grapeseed oil
Fatty acids content (% w/w)				
C16:0	11.63	6.87	17.81	6.61
C18:0	3.33	2.71	2.14	3.72
C18:1	75.64	50.87	42.55	17.77
C18:2	7.93	29.6	34.77	71.11
C18:3	0.7	8.11	1.31	0.30
C20:0	0.45	0.5	0.84	0.16
C20:1	0.32	1.34	0.58	0.33
Tocopherol and tocotrienol contents (mg/kg)				
α-T	205.8 ± 1.5	218.7 ± 1.2	95.1 ± 1.1	119.6 ± 0.2
α-Tt_3	ND	ND	12.1 ± 0.4	57.3 ± 1.0
β-T	1.3 ± 0.6	ND	2.3 ± 0.8	ND
γ-T	8.8 ± 0.4	233.0 ± 1.4	64.9 ± 2.1	6.3 ± 0.6
β-Tt_3	ND	ND	ND	10.3 ± 0.8
γ-Tt_3	ND	ND	123.3 ± 2.1	56.7 ± 1.6
δ-T	ND	3.6 ± 0.7	1.7 ± 0.6	ND
δ-Tt_3	ND	ND	1.4 ± 0.9	ND

	White beet sugar	Raw cane sugar
Mineral content (mg/kg)		
Ca	7.63 ± 0.21	365.00 ± 5.00
Cu	0.1 ± 0.00	1.63 ± 0.06
Fe	0.83 ± 0.06	1.77 ± 0.15
K	25.00 ± 6.08	400.00 ± 3.61
Mg	0.77 ± 0.06	43.00 ± 0.00
Mn	<0.1	0.40 ± 0.00
Na	5.50 ± 0.10	25.67 ± 0.58
P	1.57 ± 0.15	8.93 ± 0.15
Zn	0.20 ± 0.00	1.03 ± 0.06

Values are mean ± SD of three replicates.
Nd, not detected.

higher CML concentrations in the raw cane sugar-formulated muffins can also be explained by the metal-ion mediated degradation of fructoselysine.

The total amount of CML formed was also dependent on the degree of unsaturation of the oils (Tables 1 and 2), which is in agreement with the study of Lima et al. (2010) and that of Fu et al. (1996). Those muffins made with grapeseed oil (R2GS) contained the highest amounts of CML (11.42 mg/kg muffin), while the samples made with olive oil (R2OO) contained the smallest amounts of CML (1.82 mg/kg muffin). The difference in the yields of CML from the various oils probably reflects differences in their oxidative stability. It is well known that the rate of autoxidation of fatty acids depends on the number of double bonds present. According to Holman and Elmer (1947), methyl linoleate is 40 times more reactive than methyl oleate, while linolenate is 2.4 times more reactive than linoleate. Thus, the ability of oils-formulated muffins to promote CML formation increases in the following order: olive oil-formulated cakes (R2OO; 1.82 mg/kg) <rice bran oil-formulated cakes (R2RB; 1.97 mg/kg) < rapeseed oil-formulated cakes (R2RS; 3.03 mg/kg) <grapeseed oil-formulated cakes (R2GS; 11.42 mg/kg). Moreover, correlations of −0.829 and 0.913 ($P < 0.05$) between the level of oleic acid, linoleic acid, and the amount of CML was found. However, Zambiazi and Przybylski (1998) estimated that only about half of the stability of an oil can be explained by its fatty acid composition. Therefore, some of the other 3% of oil components other than triacylglycerols might influence oil stability and CML formation. The most important natural antioxidants present in vegetable oils are tocochromanols, which protect lipids from oxidation by donating hydrogen from the phenolic group on the chromanol ring to a peroxy radical in the propagation step (Nawar, 1996). The relative order of the antioxidant activities of tocopherols *in vitro* is as follows: δ-T > γ-T > β-T > α-T, while their *in vivo* activity is in the reverse order: α-T > β-T > γ-T > δ-T (Munteanu, Zingg, & Azzi, 2004). The α-tocopherol content decreased in the order: rapeseed oil (218.7 mg/kg oil) > olive oil (205.8 mg/kg oil) > grapeseed oil (119.6 mg/kg oil) > rice bran oil (95.1 mg/kg oil) (Table 2). There were no correlations found between the levels of CML and the concentration of α-T, β-T, γ-T, and δ-T, which suggests that other components of vegetable oils—include a wide range of low-molecular-weight lipophilic and amphiphilic components, such as phenolic compounds, chlorophyll and carotenoid pigments, menadione, oryzanols, and plastochromanol-8—might be involved in lipid protection and glycation processes.

3.3. Effect of added GP to recipe 1 and 2

Considering the presence of high levels of antioxidant PCs in GP (Lafka et al., 2007), it was of interest to study the additional beneficial effects associated with these by-products. To this end, we used model muffins made according to recipes R1 and R2 with the addition of GP to assess the effect of food ingredients and GP on CML formation.

However, it is known that high levels of added phytochemicals in food products can be significantly involved in the taste sensation and odour of cereal-based products. Therefore, the attractiveness of control and GP-enriched muffins was first investigated with respect to sensory properties, in order to determine the maximum acceptable dose. Fig. 2 presents the radar plots of sensory data of muffins made with all typically used ingredients and GP at three different levels: 10%, 20%, and 30%. The sensory evaluation of the muffin samples showed that, as the levels of GP increased, the scores for colour, appearance, taste, flavour, and overall acceptance decreased. However, no significant differences were observed up to 20% GP. Samples with the addition of 30% GP were described as having stronger fruity-acidic and sharp notes, and too brown a colour, making them unacceptable. The sharp note, which was perceived significantly only at the highest GP level, probably originates from the presence of PCs, and especially of catechins (Scharbert & Hofmann, 2005). In contrast, the score for texture exhibited an opposite trend, and samples with the addition of 10% GP had significantly higher texture scores than the control muffins. Based on these results, it seems that 20% GP could be added to muffin formulations without altering consumer acceptability. This level was selected for CML analysis.

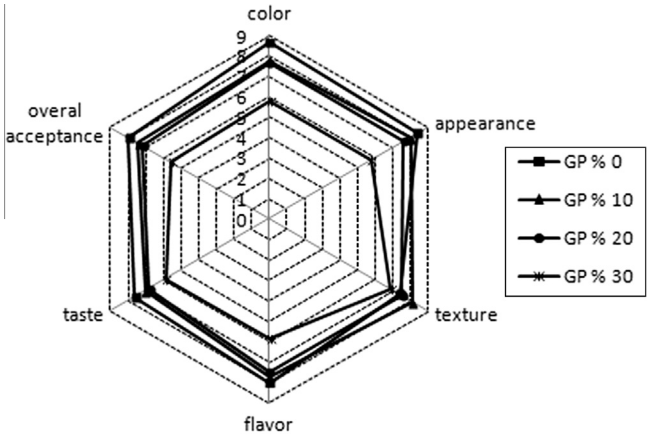

Fig. 2. Radar plots of sensory data of muffins with the addition of 0%, 10%, 20%, and 30% GP. Sample codes: GP% 0; GP% 10; GP% 20; GP% 30; muffins formulation with addition of 0%, 10%, 20% and 30% of grape by-products, respectively.

As shown in Table 3, the addition of 20% GP to muffins made according to recipes R1 and R2 exhibited a strong inhibitory effect, in some cases even below the limit of detection (LOD = 0.42 ng). Presumably, such strong inhibitory effects are due to the incorporation of PCs—mainly gallic acid, catechin, epicatechin, procyanidins, and quercetin 3-beta-D-glucoside—which have been shown to display strong antioxidant activity (scavenging free radicals and/or chelating metal ions) (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1999), and are present only in the supplemented samples. The ability of components possessing antioxidant properties to inhibit AGE formation depends not only on the free-radical scavenging activity of the samples, but also on other factors, such as the type and concentration of ingredients, heating time, and heating temperature (Charissou et al., 2007; Michalska et al., 2008; Srey et al., 2010). GP added to recipe 1 with the addition of protein-rich ingredients displayed the weakest inhibitory effects (Table 3). In this case, the CML content was reduced from 4.70 and 3.80 mg/kg muffin in recipes 1 with nonfat dry milk powder (R1M) and dry egg white powder (R1E) produced without addition of GP to 3.94 and 2.37 mg/kg muffin in those with addition of GP (R1 M + GP and R1E + GP). In fact, it is possible that interaction among the phenolic compounds and ingredients added to these samples might promote a negative synergism, minimising the inhibitory effect of GP. It is known that polyphenols are able to bind certain kinds of nutrients, such as proteins. The main mechanism behind polyphenol–protein binding is considered to be non-covalent interaction of the amino, hydroxyl, and carboxyl groups of protein with the gallate and hydroxylate benzol groups of polyphenols (Huang, Kwok, & Liang, 2004). Moreover, the polyphenols have a preference for proteins with a high level of the amino acid proline—such as caseins and the alpha-lactalbumin and beta-lactoglobulin found in dairy products. Although both baking powder and salt increase the pH of the system, PCs from GP were more stable than in samples with protein-rich ingredients, which resulted in significantly higher reductions in CML content, from 13.30 and 9.98 mg/kg muffin (recipe 1 with baking powder (R1B) and salt

Table 3

Effect of the addition of GP to R1 and R2 on phenolic compound profile and CML concentrations in model muffins.

	Phenolic compounds (mg/100 g DM)						CML	
	Gallic acid [M+H]$^{+}$ 171.0288 ± 0.005	Catechin [M+H]$^{+}$ 291.0863 ± 0.005	Epicatechin [M + H]$^{+}$ (291.0863; 273.0758) ± 0.005	Procyanidin B1[M+H$^{]+}$ 579.1497 ± 0.005	Procyanidin B2 [M+H]$^{+}$ 579.1497 ± 0.005	Quercetin 3-beta-D-glucoside [M+H]$^{+}$ 465.1028 ± 0.005	(mg/kg protein)	(mg/kg muffins)
Recipe 1 – effect of added ingredients								
R1 + GP	0.60 ± 0.04^{d}	3.06 ± 0.06^{c}	1.57 ± 0.04^{d}	1.62 ± 0.04^{c}	1.60 ± 0.08	18.56 ± 0.45^{d}	$64.6 \pm 6\ 1.85^{a}$	5.04 ± 0.14^{a}
R1B + GP	0.80 ± 0.05^{b}	3.98 ± 0.20^{b}	1.97 ± 0.09^{b}	1.65 ± 0.00^{c}	1.63 ± 0.02	18.67 ± 0.46^{d}	11.38 ± 1.08^{e}	0.89 ± 0.08^{e}
R1S + GP	$0.71 \pm 0.07^{b,c}$	3.81 ± 0.23^{b}	$1.78 \pm 0.18^{b,c}$	1.77 ± 0.04^{b}	1.70 ± 0.29	21.70 ± 0.39^{b}	22.66 ± 0.27^{d}	1.77 ± 0.02^{d}
R1 M + GP	$0.71 \pm 0.05^{b,c}$	3.24 ± 0.03^{c}	$1.64 \pm 0.02^{c,d}$	1.58 ± 0.03^{c}	1.61 ± 0.03	$18.79 \pm 0.11^{c,d}$	45.77 ± 1.83^{b}	3.94 ± 0.16^{b}
R1E + GP	0.64 ± 0.06^{c}	3.25 ± 0.21^{c}	$1.72 \pm 0.09^{c,d}$	$1.68 \pm 0.04^{b,c}$	1.67 ± 0.05	19.65 ± 0.56^{c}	28.02 ± 0.70^{c}	2.37 ± 0.06^{c}
R1A + GP	0.96 ± 0.06^{a}	4.66 ± 0.14^{a}	2.64 ± 0.13^{a}	1.99 ± 0.14^{a}	1.72 ± 0.05	35.52 ± 0.85^{a}	<LOD	<LOD^{f}
Recipe 2 – effect of types of sugar								
R2G + GP	0.97 ± 0.03	4.65 ± 0.15	2.61 ± 0.18	2.01 ± 0.08	1.73 ± 0.04	35.98 ± 0.47	<LOD	<LOD
R2F + GP	0.94 ± 0.08	4.69 ± 0.14	2.70 ± 0.10	1.98 ± 0.11	1.75 ± 0.07	36.19 ± 0.71	<LOD	<LOD
R2Bs + GP	0.96 ± 0.06	4.66 ± 0.14	2.64 ± 0.13	1.99 ± 0.13	1.72 ± 0.05	35.52 ± 0.85	<LOD	<LOD
R2Cs + GP	0.92 ± 0.07	4.66 ± 0.14	2.61 ± 0.18	1.97 ± 0.06	1.69 ± 0.06	36.02 ± 0.35	<LOD	<LOD
Recipe 2 – effect of types of oil								
R2OO + GP	0.97 ± 0.06	4.64 ± 0.17	2.67 ± 0.11	2.00 ± 0.12	1.70 ± 0.02	35.39 ± 0.35	<LOD	<LOD
R2RS + GP	0.96 ± 0.05	4.57 ± 0.11	2.71 ± 0.11	1.95 ± 0.10	1.75 ± 0.05	34.72 ± 0.74	<LOD	<LOD
R2RB + GP	0.95 ± 0.06	4.52 ± 0.14	2.64 ± 0.13	1.94 ± 0.09	1.72 ± 0.08	35.65 ± 0.66	<LOD	<LOD
R2GS + GP	0.98 ± 0.03	4.73 ± 0.12	2.59 ± 0.13	2.04 ± 0.03	1.77 ± 0.07	35.30 ± 0.52	<LOD	<LOD

Values are mean ± SD of three replicates.

$^{a-d}$ Separately for R1 and R2 averages followed by the same superscript letters are not significantly different ($P < 0.05$).

LOD = 0.42 ng.

Sample codes: recipe 1 – effect of added ingredients: R1 + GP, recipe 1 (wheat flour, water, sugar, margarine) with addition of GP; R1B + GP, recipe 1 + baking powder + GP; R1S + GP, recipe 1 + salt + GP; R1 M + GP, recipe 1 + nonfat dry milk powder + GP; R1E + GP, recipe 1 + dry egg white powder + GP; R1A + GP, recipe 1 + all ingredients together + GP; recipe 2 – effect of types of sugar: R2G + GP, glucose-formulated muffins + GP; R2F + GP, fructose-formulated muffins + GP; R2Bs + GP, white beet sugar-formulated muffins + GP; R2Cs + GP, raw cane sugar-formulated muffins + GP; recipe 2 – effect of types of oil: R2OO + GP, olive oil-formulated muffins + GP; R2RS + GP, rapeseed oil-formulated muffins + GP; R2RB + GP, rice bran oil-formulated muffins + GP; R2GS + GP, grapeseed oil-formulated muffins + GP.

(R1S) produced without addition of GP) to 0.89 and 1.77 mg/kg muffin (recipe 1 with baking powder (R1B + GP) and salt (R1S + GP) made using GP) (Table 3). Particular phenolic compounds are well correlated with CML content, indicating that they might influence the glycation process. The highest negative correlations between phenolic compounds and the level of CML of samples made according to R1 with GP was found for catechin ($r = -0.893$, $P < 0.05$), epicatechin ($r = -0.811$, $P < 0.05$), and gallic acid ($r = -0.800$, $P < 0.05$). The data on the phenolic and CML content of these samples were treated as variables in cluster analysis, confirming the differences between the model muffins (Fig. 3). The analysis of hierarchical tree showed that the plain R1 formula (R1 + GP) and recipe 1 with nonfat dry milk powder (R1M + GP), both produced with addition of GP, characterised the similar profiles. These formed one cluster (A). The other samples were scattered and do not tend to be distributed in a homogeneous groups. The most similar to cluster "A" was muffins made according to recipe 1 with egg white powder produced with addition of GP (R1E + GP). Those three samples were characterised by the smallest amounts of phenolic compounds and the highest concentrations of CML. The samples coded as R1A + GP (recipe 1 with all ingredients together produced with addition of GP) formed the most distinct cluster, which was linked to the other cluster at a large distance, indicating a significant difference. These samples were characterised by the highest amount of phenolic compounds, and the smallest (below LOD) levels of CML. The inhibition of free-radical generation derived from the glycation process and the subsequent inhibition of protein modification is considered one of the mechanisms of the antiglycation effect. Wu and Yen (2005) reported that flavonoids suppress fluorescence in the order flavones (luteolin) > flavonol (kaempferol, quercetin, and rutin) > flavanol (catechin, EC, ECG, EGC, and EGCG) > flavanone (naringenin). They suggested that the hydroxyl group at the C-3' position contributed to the inhibitory activity of these compounds on AGE formation. According to Peng et al. (2008), the antiglycation properties of catechin, procyanidin B2, and epicatechin are not only the result of their antioxidant activities, but are also related to ability to trap reactive carbonyl species, such as methylglyoxal (MGO), which is an intermediate reactive carbonyl in AGE formation.

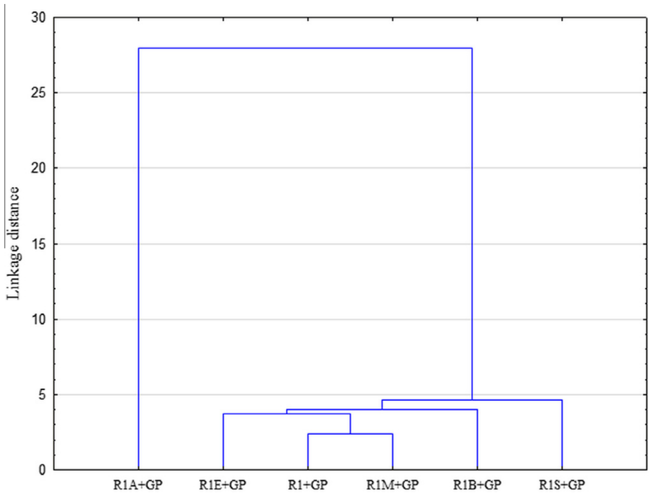

Fig. 3. Dendrogram obtained from cluster analysis of muffins made with recipe R1 with the addition of GP using means of the phenolic compounds and CML. Sample codes: R1 + GP, recipe 1 (wheat flour, water, sugar, margarine) with addition of GP; R1B + GP, recipe 1 + baking powder + GP; R1S + GP, recipe 1 + salt + GP; R1 M + GP, recipe 1 + nonfat dry milk powder + GP; R1E + GP, recipe 1 + dry egg white powder + GP; R1A + GP, recipe 1 + all ingredients together + GP.

GP added to the samples made according to R2 displayed the strongest inhibitory effect, but showed a common PC profile (Table 3). Despite the high level of CML in the R2 samples, the concentration of CML drastically decreased below the limit of detection when GP was added. The estimation of the antioxidant activity of plant phytochemicals added to food cannot be based only on the activities of a particular compound; on account of interactions, accompanying compounds should also be taken into account. For example, additive effects were observed in mixtures containing catechin and ascorbic acid or α-tocopherol, whereas in the presence of sulphur dioxide, a synergistic effect was seen (Saucier & Waterhouse, 1999). In this way might arise the strong inhibitory effect of GP added to the oil-formulated muffins, rich in tocopherols. Clearly, the particular ingredients, as well as PCs from GP, play important roles in the reduction of CML levels; however, the influence of all the ingredients on the viscoelastic properties of the product should also be taken into account. Modifying the pore structure in the crumb by adding ingredients might potentially contribute to variations in CML content, through changes in the migration of water and temperature.

4. Conclusion

In conclusion, ingredients such as protein-rich compounds, baking powder, salt, and various types of sugar and plant oil have a substantial effect on CML content. The individual ingredients added to R1 significantly reduced CML content, while the addition of all the ingredients to R1 led to the highest reduction in CML—suggesting a synergistic effect between all the ingredients in the muffin formula. Muffins made using glucose had the highest CML levels, while those with white beet sugar contained the smallest concentrations of CML. Raw cane sugar produced significantly higher amounts of CML than did refined sucrose, probably due to the metal-ion mediated degradation of fructoselysine. The overall amount of CML formed was also dependent on the degree of unsaturation of the oils. However, other components of vegetable oils—including tocopherols, phenolic compounds, chlorophyll and carotenoid pigments, menadione, oryzanols, and plastochromanol-8—might be involved in glycation. Muffins enriched with appropriate levels of polyphenol-rich GP (20%) did not show significant changes in the sensory profile; such enrichment has the ability to diminish the negative impact of the thermal modification of the proteins, lowering CML levels. Further studies on individual phenolic compounds of GP may be undertaken to elucidate the mechanisms involved in the protein protection, and also to explore the possible synergism, which may potentiate the protective effect against CML formation. Obviously, before these by-products are incorporated as AGE inhibitors, it is necessary to carry out further studies about their toxicity (i.e., possible residual presence of pesticides or heavy metals). Considering the possible presence of hazardous contaminants in the integral grapes, for the preparation of powdered GP in large scale the producer should utilise the ecologically grown raw material where the synthetic pesticides and herbicides are not used.

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