Food Chemistry 119 (2010) 49–53

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# The effects of grape seed extract fortification on the antioxidant activity and quality attributes of bread

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ARTICLE INFO

*Article history:*
Received 9 February 2009
Received in revised form 20 May 2009
Accepted 27 May 2009

*Keywords:*
Antioxidant activity
Grape seed extract
Bread
Advanced glycation endproducts

ABSTRACT

The antioxidant activity change of breads added with grape seed extract (GSE) was investigated. The results showed that bread with the addition of GSE had stronger antioxidant activity than that of blank bread, and increasing the level of GSE addition further enhanced the antioxidant capacity of the bread. However, thermal processing caused antioxidant activity of GSE added to bread to decrease by around 30–40%. We also studied the effect of GSE on the formation of detrimental $N^{\varepsilon}$-(carboxymethyl)lysine (CML), a famous advanced glycation endproduct in bread. According to the results, GSE could reduce CML in bread and acted in a dose-dependent manner. Meanwhile, except for an acceptable colour change, adding GSE to bread had only little effect on the quality attributes of the bread. Altogether, our findings indicate that GSE-fortified bread is promising to be developed as a functional food with relatively lower CML-related health risks, yet a high antioxidant activity.



## 1. Introduction

Phenolic compounds are widely distributed in foods, such as fruits (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999), vegetables (Bonoli, Gallina Toschi, & Lercker, 2005) and cereals (Dykes & Rooney, 2007; Liyana-Pathirana & Shahidi, 2006). As naturally occurring antioxidants, phenolic compounds have been reported to possess diverse beneficial bioactivities, including anti-allergic, antiviral, anti-inflammatory and anti-mutagenic properties (Yao et al., 2004). Meanwhile, a large number of *in vitro* and animal studies have also suggested that phenolic compounds may be effective in protecting against cancer, and cardiovascular diseases. The protective effects might be mediated through their action as antioxidants to prevent oxidative damage induced by reactive oxygen species to some important biomolecules (like DNA, lipids and proteins) under pathological conditions (Hollman, 2001; Yao et al., 2004). Recently, phenolic antioxidants have been viewed as an important class of food ingredients either as food additives or as novel ingredients to introduce extra health benefits to various food products. Considering the fact that heat treatment is a widespread processing method in the food industry, a salient question is whether these thermal processes would lead to significant alterations in the antioxidant capacities of phenolic additives. It has been reported that total antioxidant activities of tomatoes and carrots were enhanced with thermal processing (Dewanto, Wu, Adom, & Liu, 2002; Patras, Brunton, Da Pieve, Butler, & Downey, 2009) while antioxidant capacities of soybeans were lowered with similar processing (Xu & Chang, 2008).

So far, little work has been focused on evaluating the relationship between thermal processing of food and changes in antioxidant capacities of phenolic additives. For this consideration, we investigated the influence of thermal processing on antioxidant capacity of grape seed extract (GSE) in the present work. As a well-known nutraceutical product, GSE is an abundant source of catechins and proanthocyanidins with a strong antioxidant and free radical scavenging activity (Liang, Wang, Simon, & Ho, 2004; Wu, Wang, & Simon, 2005). Moreover, it shows other biological effects as well, such as inhibition of platelet aggregation, anti-inflammation and anti-ulcer activity (Saito, Hosoyama, Ariga, Kataoka, & Yamaji, 1998; Vitseva, Varghese, Chakrabarti, Folts, & Freedman, 2005). In this study, different amounts of GES were mixed with bread ingredients before starting the bread-making program on bread makers. Comparing the antioxidant activities of the GSE-fortified bread with those of standard GSE solutions would enable estimation of the extent to which the thermal process might affect the antioxidant of GSE. Textural analysis, colour measurement and sensory evaluation were also conducted to investigate whether addition of GSE will affect quality attributes of bread or not.

In addition, the effect of GSE on the formation of $N^{\varepsilon}$-(carboxymethyl)lysine (CML) in bread was studied. As a well-characterised detrimental advanced glycation endproduct (AGE) (Sebekova & Somoza, 2007; Tessier & Niquet, 2007), CML was widely found in a range of foods including bread (Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; Hartkopf & Erbersdobler, 1994) and its content in crust was reported to be much higher than in crumb (Assar,

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doi:10.1016/j.foodchem.2009.05.083

Moloney, Lima, Magee, & Ames, 2009). Currently it is viewed as a potential toxicant in food. Moreover, it has become a biomarker associated with oxidative stress, atherosclerosis and diabetes in humans (Nerlich & Schleicher, 1999; Schleicher, Wagner, & Nerlich, 1997). Natural antioxidants like bean extracts (Madhujith, Amarowicz, & Shahidi, 2004), peanut skin and some flavonoids have been proven to possess strong inhibitory effects on the formation of AGEs *in vitro*, which are mainly attributed to their potent antioxidant activities (Lou, Yuan, Yamazaki, Sasaki, & Oka, 2001; Peng et al., 2007; Wu & Yen, 2005). In addition, capability of certain phenolics such as tea catechins and proanthocyanidins to scavenge reactive carbonyl species (such as glyoxal, methylglyoxal) in glycation process has also been proposed to contribute to inhibition of AGE formation (Lo et al., 2006; Peng et al., 2008). Therefore, it is of great interest to investigate whether GSE fortification could reduce CML content in bread. In this regard, influence of different levels of GSE addition on CML content of bread was examined.

## 2. Materials and methods

### 2.1. Chemicals

Ingredients for bread-making were bought from a local supermarket in Hong Kong. Grape seed extract with 95% proanthocyandins including catechin and epicatechin was a gift from Shenzhen BannerBio Inc. (Shenzhen, PR China). The compound $N^{\varepsilon}$-(carboxymethyl)lysine (CML) was purchased from NeoMPS (Strasbourg, France). Ortho-phthalaldehyde (OPA), 2-mercaptoethanol, sodium borohydride, boric acid, sodium tetraborate decahydrate, sodium hydroxide, hydrochloric acid, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium peroxodisulphate and trolox were all obtained from Sigma–Aldrich Company (St. Louis, MO, USA). All analytical and HPLC grade solvents used were obtained from BDH Laboratory Supplies (Poole, UK).

### 2.2. Preparation of bread

Bread was made using a bread maker (Moulinex, Ecully Cedex, France) bought from a local supermarket. The program 2 for fast basic white bread (500 g/each) was chosen for bread-making. Briefly, this program adopts the following sequential process: first kneading (5 min), rest (5 min), second kneading (20 min), first rising (15 min), third kneading (10 s), second rising (8 min and 50 s), fourth kneading (10 s), third rising (29 min and 50 s) and baking (43 min). The recipe for bread includes canola oil (3.5 tea spoon), water (190 mL), salt (1 tea spoon, about 5.7 g), sugar (2.5 tea spoon, about 8.88 g), milk powder (1.5 table spoon, about 10.28 g), white bread flour (350 g) and flaked dried yeast (1 tea spoon, about 2.85 g). GSE with different levels (300 mg, 600 mg and 1 g) was added into breads (500 g/each), respectively. All breads were made in three batches. Bread crusts were carefully sliced off and ground for later determination of CML.

### 2.3. Antioxidant activity measurement

Ground bread powders were vortex-dispersed (5 s), respectively, into water, 30%, 50% and 70% ethanol with a final concentration of 100 mg/mL. The samples were extracted by sonication for 30 min and then centrifuged (3233*g*) for 20 min. The supernatants were used for subsequent determination of antioxidant capacity. Meanwhile, 0.06, 0.12 and 0.2 mg/mL GSE solutions were prepared as controls corresponding to different concentrations of GSE added to bread. Total antioxidant capacity was determined using the trolox equivalent antioxidant capacity (TEAC) assay according to the literature (Cheng, Chen, & Wang, 2007) with minor modifications. In brief, 7 mM ABTS salt solution was reacted with 2.45 mM potassium peroxodisulphate solution and the reaction mixture was allowed to stand in the dark for 16 h at room temperature (25 °C) and was used in 2 days. The resultant radical solution was diluted with deionized water to an absorbance of 0.7 ± 0.05 at 734 nm. Fifty microlitres of bread sample solution or standard (different concentrations of trolox) and 50 μL of water were added to 1.8 mL of diluted $ABTS^{\cdot+}$ solution and absorbance was taken at 734 nm on a UV-1206 Spectrophotometer (Shimadzu, Kyoto, Japan) after 6-min incubation. Results were expressed as TEAC values (nmol trolox/mg bread sample). Triplicate analyses were performed.

### 2.4. Textural analysis

After cooling for 1 h, breads were cut into slices of 25 mm thickness with a bread knife. The central two slices were used to perform textural analysis on a TA-XT2 texture analyzer (Stable Micro System, Surrey, UK) equipped with a cylindrical probe of 20 mm in diameter. Based on texture profile analysis (TPA), hardness was calculated. Textural analysis was performed in the mode of "Measure Force in Compression", while trigger type was set as auto-5 g at the speed of 2 mm/s followed by a 10 mm compression distance.

### 2.5. Measurement of colour changes

Colour changes of bread slices with or without the addition of different concentrations of GSE was measured with a tristimulus reflectance colourimeter (Minolta CR-400 Chroma Meter, Konica Minolta, NJ, USA), and readings were expressed as $L^*$, $a^*$ and $b^*$ values, where $L^*$ indicates whiteness (value 100) or blackness (value 0), $a^*$ indicates red (positive value) or green (negative value), and $b^*$ indicates yellow (positive value) or blue (negative value).

### 2.6. Sensory evaluation

Ten untrained panelists engaged in the sensory evaluation based on scoring various bread samples according to five attributes: sweetness, porosity, astringency, stickness and colour. In terms of corresponding intensity, each attribute was ranked as low, medium and high grades gaining 1, 2, 3 score, respectively. Sensory evaluation was conducted on three batches of bread. The mean scores and corresponding standard deviation values for each attribute were calculated for comparison.

### 2.7. Determination of CML in bread crust

HPLC analysis was adopted to determine CML content in bread crust with reference to the reported method (Drusch, Faist, & Erbersdobler, 1999) with some modifications. The sample preparation procedures include defatting, reduction, hydrolysis and chemical derivatization.

#### 2.7.1. Defatting

Bread crusts (1 g) were defatted using two successive extractions in 9 mL of a chloroform/methanol (2:1, v/v) solution followed by centrifugation (3233*g* at room temperature) for 20 min. Then, the defatted bread crusts were dried completely at 50 °C.

#### 2.7.2. Reduction

Defatted bread crusts (100 mg) were reduced with 4 mL sodium borate buffer (0.2 M, pH 9.4) and 2 mL sodium borohydride (1 M in 0.1 M NaOH) and then incubated for 4 h at room temperature.

*2.7.3. Hydrolysis*

Subsequently, hydrochloric acid (HCl) was added to the reduced samples to achieve a final concentration of 6 M HCl. In order to avoid any oxidative process, samples were degassed with a stream of nitrogen for 5 min. Then samples were hydrolysed at 110 °C for 20 h. After hydrolysis, samples were dried by rotary evaporation and then mixed with 10 mL of water before filtration. Two millilitres of the filtrates were concentrated by rotary evaporation and re-dissolved in sodium borate buffer (0.2 M, pH 9.4) for later derivatization.

*2.7.4. Derivatization*

OPA (10 mg) was dissolved in 2 mL of methanol to obtain an OPA stock solution. The derivatization reagent was composed of 1 mL of OPA stock solution, 8 μL of 2-mercaptoethanol and 3.992 mL borate buffer (consisting of 0.2 M boric acid and 0.2 M NaOH, pH 9.9) (Hanczko & Molnar-Perl, 2003). The reagent was prepared at least 90 min before use for derivatization and was disposed after 2 days. Hydrolysates (200 μL) were mixed with 100 μL of derivatization reagent and incubated for 3 min before subjecting to HPLC analysis.

*2.7.5. HPLC analysis*

Analytical HPLC was carried out using a Waters 2695 Separation Module equipped with a Waters 2475 Multi λ fluorescence detector. A pre-packed ODS-A column (150 × 4.6 mm, 5 μm, YMC Co. Ltd., Kyoto, Japan) was selected for HPLC analysis and detection was at 340 nm (excitation) and 455 nm (emission). The flow rate was 1.0 mL/min and the injection volume was 10 μL. The mobile phases were: (solvent A) sodium acetate buffer (pH 6.7, 20 mM)–acetonitrile (90/10, v/v) and (solvent B) acetonitrile. The elution started with 5% B and hold for 9 min, and then it was linear gradient to 70% B in 5 min and kept at 70% B till 17 min. The gradient was subsequently set back to 95% B within 1 min and the post running time was 10 min. Peaks for CML-derivatives in bread samples were confirmed by comparison with an authentic compound. CML contents of the bread samples were calculated based on the peak areas of the corresponding CML derivatives.

*2.8. Statistical analysis*

Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL). Paired samples *T* test was applied to determine whether a particular treatment of the sample would result in a significant difference compared with the corresponding control. $P < 0.05$ was selected as the level decision for significant differences.

## 3. Results and discussion

Thermal treatment is among the most popular ways of food processing. During heating, a complex array of chemical reactions takes place, which plays a pivotal role in determining the quality attributes (sensory characteristics, nutritional value and safety) of processed foods. While some compounds are destroyed during food processing, many more new compounds might be introduced into the food system. Some of these compounds contribute significantly to the organoleptic properties of foods, such as colour and flavour. On the other hand, compounds with various biological activities could also result from the heat-induced reactions, some of which are shown to be potential antioxidative and chemopreventive agents. In contrast, some are harmful and might pose significant health risks for human beings in the long term. The formation of these compounds involves complex networks of reactions, though many start with fundamental food components such as glucose, amino acids/proteins and lipids. Other ingredients such as phenolic compounds may interact with these reactions and change the quality attributes of the food products. In our current research, it was found the antioxidant activity of bread extract was significantly increased with the addition of three different levels of GSE, which was prepared by extraction with four different types of solvents (water, 30%, 50% and 70% ethanol, respectively). From Fig. 1, it is obvious that a higher level of GSE addition resulted in a higher antioxidant activity of the corresponding bread sample. However, when compared with standard solutions of equivalent GSE concentrations, antioxidant activities of bread samples added with the water or aqueous ethanol GSE were reduced by 30–40%. These data suggest that the thermal processing in bread-making might lower the antioxidant capacity of GSE. This phenomenon could be brought about by heat-induced reactions of GSE proanthocyanidins with food components, such as proteins or starch to produce large molecules, which could not be extracted by the solvents used in this experiment. Alternatively, thermal processing might cause GSE proanthocyanidins to degrade, and thus lead to reduced antioxidant content. Our data suggest that thermal processing exerts a significant effect on the antioxidant activity of cell-free systems. However, whether similar effects will be observed in humans remains unknown (it is possible that thermal processing can increase the bioavailability of phenolics, thus increase the antioxidant activities). Thus, further experimental clarification is needed.

For a novel food additive, it is necessary to study its impact on food quality attributes. Some researchers found that incorporation of green tea extract to bread could affect certain bread quality attributes (Wang, Zhou, & Isabelle, 2007). Therefore, the influence of GSE on bread quality was further investigated. Hardness, namely firmness, is one of the commonly used indices to describe bread quality as the change of hardness normally reflects the loss of resilience (Wang et al., 2007). In textural analysis, no significant difference in hardness was observed between bread samples with and without the addition of GSE (Fig. 2). This indicates that appropriate addition levels of GSE (0–1 g/500 g bread) would not cause any undesirable change in hardness of bread. However, GSE could greatly change the colour of bread as shown in Table 1. With increase in the level of GES addition, $L^*$ values decreased while $a^*$ and $b^*$ values increased, thus suggesting that GSE could affect bread colour obviously. For a comprehensive analysis combining $L^*$, $a^*$ and $b^*$ values together, *E* index calculated by the equation $E = (L^{*2} + a^{*2} + b^{*2})^{1/2}$, which is largely influenced by the brightness (Delgado-Andrade, Seiquer, Navarro, & Morales, 2007), was introduced to describe the colour change. Samples of bread fortified with GSE had relatively lower *E* values than blank bread samples. Moreover, *E* values of the former samples followed a trend which was inversely related to the levels of GSE addition to bread. These observations are indicative of a loss in brightness of bread caused

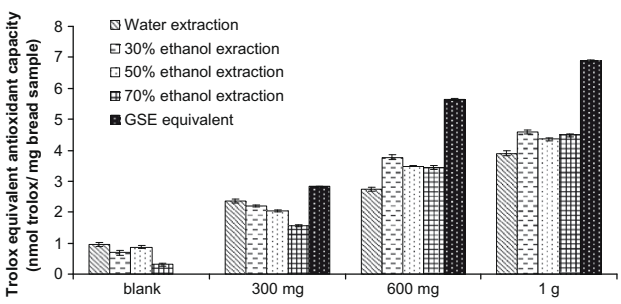


**Fig. 1.** Antioxidant capacities of bread samples with and without the addition of various concentrations of GSE and equivalent GSE solutions. Results are expressed as means ± SD for $n = 3$, $P < 0.05$.

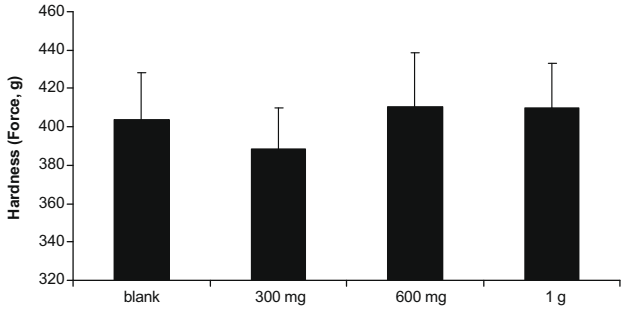


**Fig. 2.** Textural analysis of bread samples with and without the addition of various concentrations of GSE. Hardness was chosen as the main parameter to indicate the quality status. Results are expressed as means ± SD for $n = 6$.

**Table 1**
Colorimetric parameters of different bread samples. Results are expressed as means ± SD for $n = 6$, $P < 0.05$.

| | $L^*$ | $a^*$ | $b^*$ | $E$ |
|---|---|---|---|---|
| Blank | 71.35 ± 0.41 | −0.58 ± 0.01 | 17.19 ± 0.17 | 73.39 ± 0.36 |
| 300 mg | 65.05 ± 0.36 | 0.86 ± 0.09 | 17.70 ± 0.18 | 67.42 ± 0.36 |
| 600 mg | 62.85 ± 0.57 | 3.15 ± 0.08 | 18.26 ± 0.29 | 65.53 ± 0.47 |
| 1 g | 62.13 ± 0.41 | 3.39 ± 0.21 | 20.15 ± 0.31 | 65.41 ± 0.49 |

by GSE. In sensory evaluation, scores given by the 10 panelists did not indicate any significant alteration in quality attributes (sweetness, porosity, astringency and stickiness) of bread added with different levels of GSE. Visual evaluation of colour change was consistent with the results from colorimetric analysis. Nevertheless, 70% of the panelists liked better the colour of GSE-fortified bread than that of the blank bread.

Besides introducing antioxidant activity to bread, GSE also appeared to attenuate CML content in bread crust. In our analysis, CML content determined in blank bread crust (35 mg/kg food) was close to the literature data (37.1 mg/kg food) (Assar et al., 2009). As shown in Fig. 3, addition of GSE dose-dependently reduced CML content in bread crust relative to the control. In particular, adding 600 and 1000 mg of GSE to bread (500 g) led to over 30% and 50% reduction in bread crust CML content, respectively. As a major product of oxidative modification of glycated proteins, CML can be formed through different pathways, such as degradation of fructoselysine (Ahmed, Thorpe, & Baynes, 1986), modification of lysine by glyoxal (Glomb & Monnier, 1995), and direct reaction between lysine and products of autoxidation of ascorbate (Dunn et al., 1990). Strong antioxidant activities of catechins and proanthocyanidins abundant in GSE may contribute to the reduction of CML in GSE-fortified bread. On the other hand, catechins and proanthocyanidins proved to be able to scavenge the intermediate dicarbonyls (such as methylglyoxal, glyoxal) (Lo et al., 2006; Peng et al., 2008) in the glycation process, which may also decrease the CML content of GSE-fortified bread.

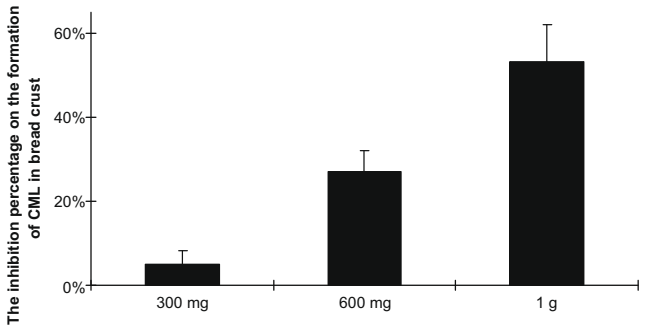


**Fig. 3.** Inhibitory effects of different concentrations of GSE (300 mg, 600 mg and 1 g/bread) on the formation of CML in bread crust. Inhibition percentage was calculated using blank bread (without the addition of GSE) as a control. Results are expressed as means ± SD for $n = 3$, $P < 0.05$.

## 4. Conclusions

Thermal processing decreased the antioxidant activity of GSE additive in bread. However, using GSE as an additive could greatly enhance the total antioxidant capacity of bread, and at the same time, decrease the level of CML. Our results also demonstrated that with appropriate levels of addition, GSE could lead to a favourable change in the colour of bread without causing significant changes in other sensory properties. Altogether, GSE-fortified bread is promising to be developed as a functional food with relatively lower CML-associated health risks, yet a stronger antioxidant activity.

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