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An Assay To Estimate Tannins Added to Postmortem Turkey Meat

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A method for quantification of tannins in wine was adapted to determine tannins added to turkey meat. Standard curves containing varying amounts of GSE [0, 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0%, (w/w)] as a source of tannins were developed. The R^2 value of the mean standard curve was 0.9992. The overall percent recovery of GSE in meat was determined to be 54.78%. Results showed that estimation of GSE in four out of five of the spiked samples was less than or equal to 10%. It is unclear as to why spiked samples at 0.048 mg of GSE were always underestimated (25.0%). Overall, the method seems applicable for estimation of tannins in poultry meat and is probably applicable to estimation of tannins in other meat products.

KEYWORDS: Grape seed extract; assay for tannins in turkey meat

INTRODUCTION

The "French paradox" hypothesis attributed the French population's low risk of cardiovascular disease to high wine consumption (*1*, *2*). Further research showed that phenolic components in the nonalcoholic fraction of red wine retarded human low-density lipoprotein (LDL) oxidation (*3*). The phenolic compounds of interest are flavonoids, which can be found in plant sources such as grapes, cabbage, and apples (*4*).

In grape processing for wine, an estimated 3.6 million tons of byproducts is produced worldwide (*5*). The byproducts from wine/grape juice processing consist of flavonoid-rich seeds, skins, and/or stems (*6*−*11*). Grape seeds can be extracted and purified into GSE. GSE are flavonoids, known as concentrated oligomeric catechins or tannins, which are antioxidants (*12*−*14*) and are the most abundant group of phenolics (*15*).

Tannins from GSE are sold in Europe as pharmaceuticals for their reported ability to increase the strength of vascular systems, reduce heart disease, and control allergies (*6*, *16*). Also, tannins have traditionally been used for their antidiarrheal, antiinflammatory, and antiseptic effects as well as to heal minor wounds and burns (*17*).

When fed to animals, GSE is proposed to have antioxidant activity (*18*−*22*). Other studies, conducted with rats, suggest that GSE is potentially an antiinflammatory agent (*23*). Dietary GSE in rats was shown to have a preventative effect against some cancers (*24*) and to have antiulcer properties (*25*). In in vitro studies of antioxidant properties of grape extracts, the extracts were concluded to inhibit conjugated diene and hexanal formation in lecithin liposomes (*26*) and human LDL oxidation (*27*).

The antioxidative ability of GSE suggests it might be added to food to inhibit oxidation. Grape skin was added to dehydrated chicken meat to evaluate its antioxidative effect and was found to decrease lipid oxidation as compared to the control sample (no antioxidants) (*28*). Previous research conducted by the present authors showed that postmortem addition of 1.0% GSE to poultry meat decreased lipid oxidation as measured by thiobarbituric acid reactive substances values nearly 10-fold as compared to a control (*29*). If GSE becomes an acceptable antioxidant in poultry meat, it would be valuable to know the amount of GSE remaining in meat after various processing conditions that affect lipid oxidation. The amount of GSE remaining in a sample could then be correlated with the level of lipid oxidation in meat.

Existing assays for quantifying tannins in wine include vanillin measurement (*30*), protein precipitation (*31*−*33*), and oxidation−reduction such as Folin−Ciocalteu, AOAC method 952.03, and Prussian Blue (*34*, *35*). However, these methods were developed for wine and grain samples and do not apply well to the complex matrix including protein in meat. For example, the vanillin assay has specificity for a narrow range of flavonols but does not include procedures for precipitation of proteins. The AOAC method 952.03 results in an erroneous value because both protein and tannins interfere with the reagents used (*36*).

A search of the literature reveals no procedure to quantify tannins in meat; thus, this paper describes a procedure that potentially can be used for this purpose. It is derived from the work of Harbertson and Adams (*37*), who modified Hagerman and Butler's analysis of wine tannins (*31*). Essentially, the work

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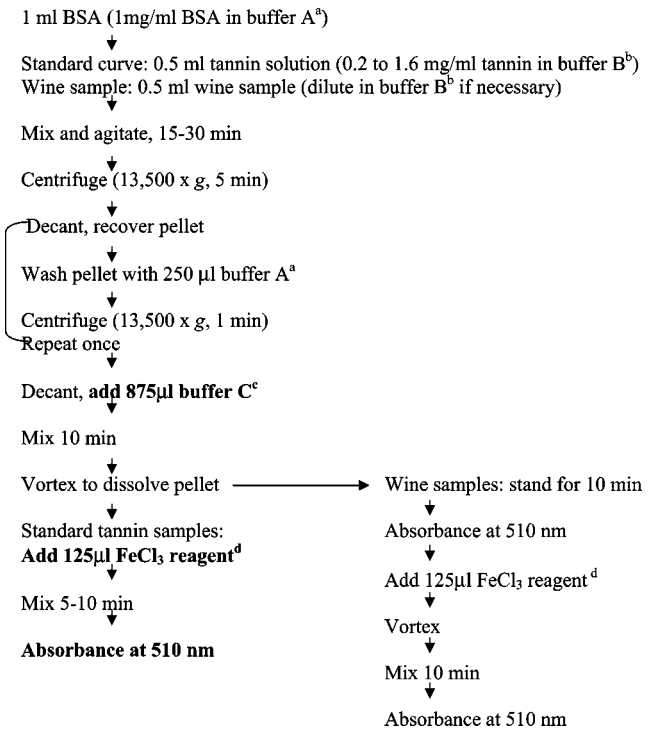

Figure 1. Flow diagram of Harbertson and Adams's assay for determination of tannins in wine. (**a**) Buffer A: 200 mM acetic acid and 170 mM NaCl; pH adjusted with NaOH. (**b**) Buffer B: 12% ethanol and 2.5 g of potassium bitartrate; pH of 3.3 adjusted with HCl. (**c**) Buffer C (SDS buffer): 5% triethanolamine (v/v) and 10% SDS (w/v); pH of 9.4 adjusted with HCl. (**d**) $FeCl_3$ reagent: 0.01 N HCl with 10 mM $FeCl_3$.

of Harbertson and Adams evaluated several conditions for analyzing tannins (mg) in wine. Therefore, at the time the present work began, their method was used to determine how various levels of GSE (mg) in meat affected the linearity of standard curves. Results are expressed as milligrams of tannins in meat.

MATERIAL AND METHODS

Materials/Chemicals. HCl (1 N, VWR Scientific, West Chester, PA), $FeCl_3$ (EM Science, Gibbstown, NJ), SDS (EM Science, >95% purity, and Sigma Chemical Co., >99% purity, St. Louis, MO), TEA (Fisher Scientific, Pittsburgh, PA), and TCA (Fisher Scientific) were used. Ultrapure water, used in all analyses, was obtained from deionized water filtered through a Millipore water filtration system (Milli-Q system, Millipore Corporation, Bedford, MA). GSE, lot 2213-119 [>90 GAE or total phenolic compounds as measured by the Folin method (*35*)], was obtained from Polyphenolics, Inc. (Burlingame, CA).

Procedure of Harbertson and Adams (*37*). The procedure of these investigators is shown in **Figure 1** with applicable parts bolded. The ratio of protein to reagents in their analysis was 1 mg of BSA:875 μL of SDS buffer (buffer C for Harbertson and Adams) and 125 μL of $FeCl_3$ reagent (*37*).

Proteins added to wine samples were used to facilitate the precipitation of the protein−tannin complex. The isolated complex was unbound with SDS buffer so that free tannins could react with $FeCl_3$ to form a colored complex detectable by spectrophotometry.

Adapted Method. The maximum amount of protein in raw, dark, turkey meat reported was 20.46% (*38*) and that for chicken meat was 20.9% (*39*). Even though turkey was used in the study, the method was developed for use in both types of meat; therefore, the procedure was used for the greater amount of protein. A homogeneous 2.0 g meat sample contained 0.418 g of protein. On the basis of procedures of Harbertson and Adams (*37*), the amounts of 5% TEA/10% SDS buffer and 10 mM $FeCl_3$ reagent needed for a 2.0 g of meat sample were

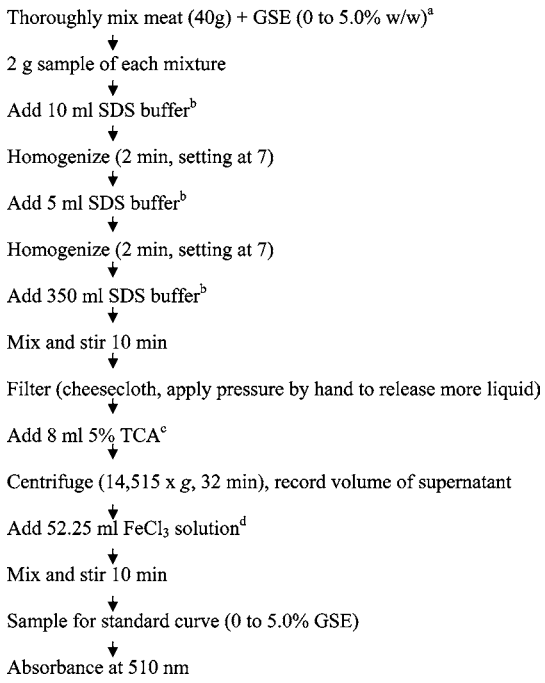

Figure 2. Flow diagram of adapted method for analysis of tannins in poultry meat. (**a**) GSE, w/w (weight of GSE/weight of meat). (**b**) SDS buffer: 5% triethanolamine (v/v) and 10% SDS (w/v); pH of 9.4 adjusted with HCl. (**c**) TCA: 5% TCA (w/v) in ultrapure water. (**d**) $FeCl_3$ reagent: 0.01 N HCl with 10 mM $FeCl_3$.

365.75 and 52.25 mL, respectively. In the current method, 365 mL of SDS was used for ease of measurement (**Figure 2**).

Experimental Design. Several treatments of GSE [0, 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0%, (w/w, weight of GSE/weight of meat)] were used to develop curves to determine recovery. Amounts of GSE to develop the curves (0.5−5.0% GSE) ranged from 0 to 0.034, 0.000−0.066, 0.000−0.099, 0.000−0.132, 0.000−0.165, and 0.000−0.324 mg, respectively. Ten curves were produced for each treatment. Analyses were conducted throughout the day so that four runs per day [(0% and three other treatments (of ones listed previously)] were performed. Therefore, within a 2 day period, one of the 10 repetitions of each treatment was completed.

Preparation of Sample. Ten trays of the same brand of whole, fresh, bone-in turkey thighs ("Naturally Lean", USDA Grade A), were purchased from a local grocery store. Each tray of meat was designated as one replicate with six treatments.

Immediately after the meat was purchased and brought to the laboratory, it was placed into a −80 °C freezer or processed. For ease of analysis, meat was defrosted (~23 °C) on the counter and protected from light and other contaminants (for measuring lipid oxidation, all precautions were taken to eliminate oxidation, i.e., protection from light and air and thawing at 4 °C and further manipulation in the cold room at 2−4 °C). After the meat was deboned and cut into approximately 2.5 cm^2 cubes for single layering in plastic Ziploc freezer bags, the meat was returned immediately to the −80 °C freezer. Samples were defrosted (~23 °C), protected from light on the counter or in a refrigerator (overnight), and ground before mixing with GSE.

Several experimental factors are discussed below. These include initial grinding of meat with SDS buffer, filtration, TCA addition, centrifugation, SDS purity and $FeCl_3$ reagent, and the appropriate blank.

Determination of Experimental Factors. Several experimental factors were determined before a final procedure was established. These factors included duration and speed for grinding of meat; homogenizer types; volume of SDS buffer; filtration materials and methods; addition of TCA to promote protein precipitation (*40*); and method of centrifugation using various centrifuges, rotors, bottles/tubes, forces, and time periods.

$FeCl_3$ Reagent. The reagent was made and held for 4 h (~23 °C) to observe changes in color.

Blank Test. It was important to know whether an all reagent blank and a water blank yielded the same absorbance values. After all factors for the procedure were tested, curves for 0% GSE were obtained where water or all reagents (SDS, TCA, and $FeCl_3$) were used as blanks.

Standard Curve and Percent Recovery. Samples for standard curves and for percent recovery were read in a Shimadzu UV 160U spectrophotometer (Kyoto, Japan) at 510 nm. Six curves used to determine percent recovery were developed from each of the 10 repetitions of 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0% GSE.

Applicability of Procedure. To ascertain the applicability of the adapted procedure for determination of GSE in meat, several spiked samples were analyzed. Thirteen samples containing 0.032 mg of GSE, 26 samples containing 0.048 mg of GSE, and three samples each containing either 0.064, 0.128, or 0.160 mg of GSE were analyzed using the adapted procedure.

Statistical Analysis. A power analysis (*41*) was conducted, and it was determined that 10 repetitions using various quantities were needed to determine percent recovery (SAS Institute, version 8.1, Cary, NC).

RESULTS AND DISCUSSION

Determination of Experimental Factors. Large volumes (200 mL) of SDS buffer caused foaming when the homogenizer was activated. Twenty milliliters of SDS buffer prevented effective grinding of meat, while 5 mL was not sufficient for homogenization. An intermediate volume, 15 mL, was chosen. The hand-held Omni 1000 homogenizer was eliminated because meat became trapped between the blades, so the Omni International's Macro Homogenizer (Warrenton, VA) with star-shaped blades (Omni part 17260) was used. Grinding for 2 min at setting 7 was determined to be effective.

Filter paper (Whatman 2V and 3, Clifton, NJ) and micrometer filters could not be used as they would continually clog with SDS and meat fibers and/or finally break under the weight of the solutions. Vacuum was attempted briefly to expedite the filter paper method; however, SDS caused excessive foaming. Both cheesecloth (catalog 600, Pyrm-Dritz Corp., Spartanburg, SC) and tulle netting (purchased locally) allowed moisture flow-through quicker than filter paper. Cheesecloth was selected as the filter of choice due to ease of use.

In the procedure of Harbertson and Adams (*37*), the 1 mL wine sample was centrifuged for 1 min at 13500*g*. On the basis of several preliminary experiments, the final conditions for centrifugation were determined. These conditions included centrifugation in 50 mL tubes at 14515*g* for 32 min at 4 °C in the Sorvall SA-600 rotor in the DuPont Sorvall RC-5B centrifuge (Kendro Laboratory Products, Newtown, CT).

It was observed that $FeCl_3$ reagent became darker in yellow color after approximately 2 h at room temperature (22 °C). Therefore, as a precaution, a fresh reagent was made every 2 h. The ultrapure water blank and the all reagent blank resulted in similar absorbance readings; therefore, the water blank was chosen for ease of use. Experimental factors tested resulted in the adapted procedure outlined in **Figure 2**.

Analysis of Standard Curves and Percent Recovery. **Figure 3** shows the mean standard curve for points at 0.0 (0.000 mg), 0.5 (0.024 mg), 1.0 (0.048 mg), 1.5 (0.072 mg), 2.0 (0.096 mg), 2.5 (0.120 mg), and 5.0% (0.240 mg) GSE. **Figure 4** shows the 10 replications for 0.5−5.0% GSE with 2.0 g of meat. Percent recovery was determined by subtracting the mean absorbance value for 0.0% GSE from those at 0.5−5.0% GSE. Then, the equation of the resulting line for the mean standard curve was used to determine milligrams of GSE for each percent GSE in curves containing GSE and meat as [(calculated mg GSE in curves with meat)/(known mg GSE in standard curves)] × 100. The overall mean percent recovery value for the 60 analysis was 54.78 ± 14.30%.

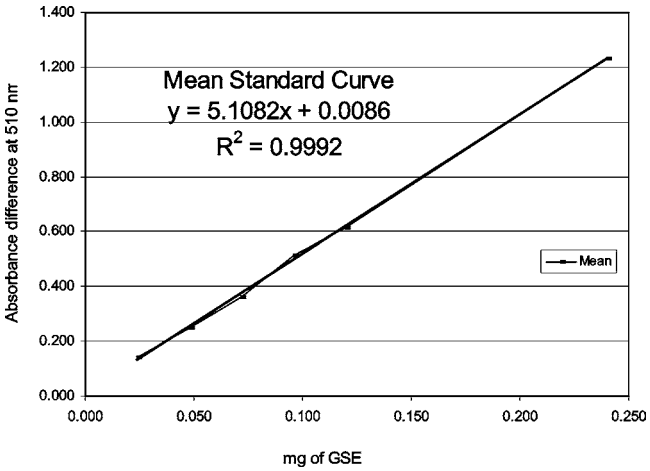

Figure 3. Standard curve (0.5–5.0% GSE) with average milligrams of GSE contents ranging from 0 to 0.240 mg.

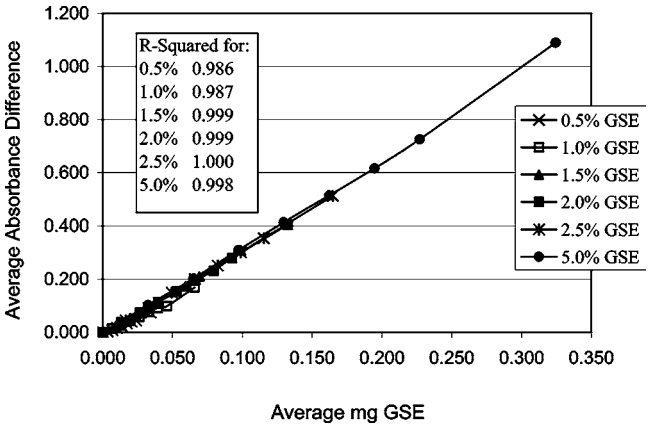

Figure 4. Mean curves for 10 replications of six treatments with GSE (GSE was added on w/w, weight of GSE/weight of meat). Curves for recovery (0.5–5.0% GSE) were developed with milligrams of GSE ranging from 0 to 0.034, 0.000–0.066, 0.000–0.099, 0.000–0.132, 0.000–0.165, and 0.000–0.324 mg, respectively.

Table 1. Determination of GSE in Spiked Samples

experimental standard curves			
mg of GSE			
spiked	determined	adjusted with recovery	% over/ underestimation
0.032[a]	0.016 ± 0.011	0.030 ± 0.019	6.3
0.048[b]	0.020 ± 0.006	0.036 ± 0.012	25.0
0.064[c]	0.036 ± 0.004	0.067 ± 0.007	4.7
0.128[c]	0.074 ± 0.006	0.135 ± 0.010	5.5
0.160[c]	0.096 ± 0.004	0.176 ± 0.008	10.0

[a] $n = 13$. [b] $n = 26$. [c] $n = 3$.

Results for determination of GSE in unknown samples are shown in **Table 1**. The adapted method seems applicable in that the error in the estimation of GSE in four out of five of the spiked samples was less than or equal to 10%. Reasons for underestimating samples with 0.048 mg of GSE are being investigated. Perhaps the release of tannins from protein in the meat was not completed causing underestimation of GSE. Moreover, a mean recovery value was used to estimate GSE in all spiked samples. More accurate estimations of GSE in spiked samples may possibly be obtained by determining the percent recovery with each analysis. Overall, the adapted method seems useful for obtaining an estimate of GSE as an antioxidant in

poultry meat. The method can possibly be used for determination of GSE in other types of meat as well.

Results reported in this paper indicate the potential for Harbertson and Adams' modified tannin assay to be used to quantify GSE in products such as turkey meat. This procedure would be of particular value in quality control of GSE in commercial poultry products.

ABBREVIATIONS USED

GSE, grape seed extract; AOAC, Association of Official Analytical Chemists; HCl, hydrochloric acid; $FeCl_3$, ferric chloride; SDS, sodium dodecyl sulfate; TEA, triethanolamine; TCA, trichloroacetic acid; GAE, gallic acid equivalents; NaCl, sodium chloride; BSA, bovine serum albumin.

LITERATURE CITED

(1) St. Leger, A. S.; Cochrane, A. L.; Moore, F. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet* **1979**, *1*, 1017−1020.

(2) Renaud, S.; De Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(3) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

(4) Shahidi, F.; Naczk, M. *Food Phenolics: Sources, Chemistry, Effects, Applications*; Technomic Publishing Co.: Lancaster, PA, 1995.

(5) El Boushy, A. R. Y.; Poel, A. F. B. v. d. Fruit, vegetable and brewers' waste. *Handbook of Poultry Feed from Waste: Processing and Use*; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000.

(6) Waterhouse, A. L.; Walzem, R. L. Nutrition of grape phenolics. In *Flavonoids in Health and Disease*; Rice-Evans, C., and Packer, L., Eds.; Marcel Dekker: New York, 1998; pp 359−385.

(7) El Boushy, A. R. Y.; van der Poel, A. F. B. Fruit, vegetable and brewers' waste. *Poultry Feed from Waste: Processing and Use*; Chapman & Hall: London, 1994; pp 187−340.

(8) Lu, Y.; Yeap Foo, L. The polyphenol constituents of grape pomace. *Food Chem.* **1999**, *65*, 1−8.

(9) Ricardo da Silva, J. M.; Rigaud, J.; Cheynier, V.; Cheminat, A.; Moutounet, M. Procyanidin dimers and trimers from grape seeds. *Phytochemistry* **1991**, *30*, 1259−1264.

(10) Yang, Y.; Chien, M.; Yang, Y.; Chien, M. J. Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Agric. Food Chem.* **2000**, *48*, 3990−3996.

(11) Souquet, J.-M.; Labarbe, B.; Le Guerneve, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.

(12) Richardson, G. A.; El-Rafey, M. S.; Long, M. L. Flavones and flavone derivatives as antioxidants. *J. Dairy Sci.* **1947**, *30*, 397−413.

(13) Mehta, A. C.; Seshadri, T. R. Flavonoids as antioxidants. *J. Sci. Ind. Res.* **1958**, *18B*, 24−28.

(14) Rice-Evans, C. A.; Miller, N. J. Antioxidant activities of flavonoids as bioactive components of food. *Biochem. Soc. Trans.* **1996**, *24*, 790−795.

(15) Thomson, R. H. Structure and reactivity of phenolic compounds. In *Biochemistry of Phenolic Compounds*; Harborne, J. B., Ed.; Academic Press: London, 1964.

(16) Kandaswami, C.; Clouatre, D. *The Health Benefits of Grapeseed*; Keats Publishing: Connecticut, 1998.

(17) Bruneton, J. *Pharmacognosy, Phytochemistry, Medicinal Plants*, 2nd ed.; Technique & Documentation; Lavoisier: Paris, Secaucus, NJ, 1999.

(18) Tebib, K.; Rouanet, J. M.; Besancon, P. Antioxidant effects of dietary polymeric grape seed tannins in tissues of rats fed a high cholesterol-vitamin E-deficient diet. *Food Chem.* **1997**, *59*, 135−141.

(19) Tebib, K.; Bitri, L.; Besancon, P.; Rouanet, J. M. Polymeric grape seed tannins prevent plasma cholesterol changes in high-cholesterol-fed rats. *Food Chem.* **1994**, *49*, 403−406.

(20) Yamakoshi, J.; Kataoka, S.; Koga, T.; Ariga, T. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **1999**, *142*, 139−149.

(21) Koga, T.; Moro, K.; Nakamori, K.; Yamakoshi, J.; Hosoyama, H.; Kataoka, S.; Ariga, T. Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. *J. Agric. Food Chem.* **1999**, *47*, 1892−1897.

(22) Bouhamidi, R.; Prevost, V.; Nouvelot, A. High protection by grape seed proanthocyanidins (GSPC) of polyunsaturated fatty acids against UV-C induced peroxidation. *C. R. Acad. Sci., Ser. III* **1998**, *321*, 31−38.

(23) Sen, C. K.; Bagchi, D. Regulation of inducible adhesion molecule expression in human endothelial cells by grape seed proanthocyanidin extract. *Mol. Cell. Biochem.* **2001**, *216*, 1−7.

(24) Singletary, K. W.; Meline, B. Effect of grape seed proanthocyanidins on colon aberrant crypts and breast tumors in a rat dual-organ tumor model. *Nutr. Cancer* **2001**, *39*, 252−258.

(25) Saito, M.; Hosoyama, H.; Ariga, T.; Kataoka, S.; Yamaji, N. Antiulcer activity of grape seed extract and procyanidins. *J. Agric. Food Chem.* **1998**, *46*, 1460−1464.

(26) Yi, O.; Meyer, A. S.; Frankel, E. N.; Yi, O. S. Antioxidant activity of grape extracts in a lecithin liposome system. *J. Am. Oil Chem. Soc.* **1997**, *74*, 1301−1307.

(27) Frankel, E. N.; Meyer, A. S. Antioxidants in grapes and grape juices and their potential health effects. *Pharm. Biol.* **1998**, *36*, 14−20.

(28) Nissen, L. R.; Mansson, L.; Bertelsen, G.; Tuong Huynh, B.; Skibsted, L. H. Protection of dehydrated chicken meat by natural antioxidants as evaluated by electron spin resonance spectrometry. *J. Agric. Food Chem.* **2000**, *48*, 5548−5556.

(29) Lau, D. W.; King, A. J. Pre- and Postmortem Use of Grape Seed Extract in Dark Poultry Meat to Inhibit Development of Thiobarbituric Acid Reactive Substances. *J. Agric. Food Chem.* **2003**, *51*, 1602−1607.

(30) Price, M. L.; Scoyoc, S. V.; Butler, L. G. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem.* **1978**, *26*, 1214−1218.

(31) Hagerman, A. E.; Butler, L. G. Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* **1978**, *26*, 809−812.

(32) Hagerman, A. E.; Butler, L. G. Determination of protein in tannin-protein precipitates. *J. Sci. Food Chem.* **1980**, *28*, 944−947.

(33) Makkar, H. P. S.; Dawra, R. K.; Bhupinder, S. Determination of both tannin and protein in a tannin-protein complex. *J. Agric. Food Chem.* **1988**, *36*, 523−525.

(34) AOAC. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 15th ed.; The Association of Official Analytical Chemists: Arlington, VA, 1990.

(35) Singleton, V. L.; Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

(36) Peterson, G. L. Review of the Folin phenol protein quantification method of Lowery, Rosebrough, Farr and Randall. *Anal. Biochem.* **1979**, *100*, 201−220.

(37) Harbertson, J. F.; Adams, D. O. Personal Correspondence. Department of Viticulture and Enology, University of California, Davis, 2000.

(38) Nutrient Data Laboratory, A. R. S., Beltsville Human Nutrition Research Center. *USDA Nutrient Database for Standard Reference*, Release 14, 2001.

(39) Chan, W.; McCance, R. A. *Meat, Poultry and Game: Fifth Supplement to the Fifth Edition of McCance and Widdowson's The Composition of Foods*; Royal Society of Chemistry, Ministry of Agriculture, Fisheries and Food: Cambridge, 1995.

(40) Raharjo, S.; Sofos, J. N.; Schmidt, G. R. Improved speed, specificity, and limit of determination of an aqueous acid extraction thiobarbituric acid-C18 method for measuring lipid peroxidation in beef. *J. Agric. Food Chem.* **1992**, *40*, 2182−2185.

(41) Cohen, J. *Statistical Power Analysis for the Behavioral Sciences*, 2nd ed.; L. Erlbaum Associates: Hillsdale, NJ, 1988.

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