JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Cooking on Garlic (*Allium sativum* L.) Antiplatelet Activity and Thiosulfinates Content

PABLO F. CAVAGNARO,† ALEJANDRA CAMARGO,§ CLAUDIO R. GALMARINI,*,†,§ AND PHILIPP W. SIMON‖

INTA - EEA La Consulta and CONICET, INTA, EEA La Consulta CC8, San Carlos, Mendoza (5567), Argentina, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Almirante Brown 500, Chacras de Coria, Mendoza M 5528 AHB, Argentina, and USDA-ARS Vegetable Crops Research Unit and Department of Horticulture, University of Wisconsin, 1575 Linden Drive, Madison, Wisconsin 53706

The raw form of garlic and some of its preparations are widely recognized as antiplatelet agents that may contribute to the prevention of cardiovascular disease. Herein, we examined the in-vitro antiaggregatory activity (IVAA) of human blood platelets induced by extracts of garlic samples that were previously heated (in the form of crushed versus uncrushed cloves) using different cooking methods and intensities. The concentrations of allicin and pyruvate, two predictors of antiplatelet strength, were also monitored. Oven-heating at 200 °C or immersing in boiling water for 3 min or less did not affect the ability of garlic to inhibit platelet aggregation (as compared to raw garlic), whereas heating for 6 min completely suppressed IVAA in uncrushed, but not in previously crushed, samples. The latter samples had reduced, yet significant, antiplatelet activity. Prolonged incubation (more than 10 min) at these temperatures completely suppressed IVAA. Microwaved garlic had no effect on platelet aggregation. However, increasing the concentration of garlic juice in the aggregation reaction had a positive IVAA dose response in crushed, but not in uncrushed, microwaved samples. The addition of raw garlic juice to microwaved uncrushed garlic restored a full complement of antiplatelet activity that was completely lost without the garlic addition. Garlic-induced IVAA was always associated with allicin and pyruvate levels. Our results suggest that (1) allicin and thiosulfinates are responsible for the IVAA response, (2) crushing garlic before moderate cooking can reduce the loss of activity, and (3) the partial loss of antithrombotic effect in crushed-cooked garlic may be compensated by increasing the amount consumed.

KEYWORDS: ***Allium sativum*; antiplatelet activity; thiosulfinates; allicin; pyruvate**

INTRODUCTION

Garlic (*Allium sativum*) and onion (*Allium cepa*) are widely recognized as antiplatelet agents that may contribute to the prevention of cardiovascular disease. The raw forms of both species, and some of their preparations, have demonstrated blood pressure lowering effects and antiplatelet activity (AA) (*1*, *2*). Increased platelet aggregation and atherosclerosis are the principal contributors to the onset and development of cardiovascular disease, by far the leading cause of death in developed countries (*3*).

The AA of *Alliums* have usually been studied with raw, dehydrated, or extracted preparations. Aqueous extracts of raw garlic and onion, garlic oils, and other preparations of garlic inhibited human platelet aggregation in vitro (*4*−*8*). In vivo, chronic intake of raw garlic, garlic powder, garlic oil, and aged garlic extract (AGE) inhibited platelet aggregation in human subjects (*9*−*13*). A single dose of garlic powder (*11*) also demonstrated significant in-vivo AA in humans. Animal feeding studies reported in-vitro (*7*) and in-vivo (*14*) platelet inhibitory activity, induced by raw extracts of garlic and onion. In addition, several isolated compounds from both species have demonstrated in-vitro antiplatelet activity (IVAA) (*15*, *16*).

The flavor and aroma (*17*) as well as the antithrombotic properties of garlic and onion (*1*, *16*) are attributed to a suite of organosulfur compounds formed after the lysis of *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs) by the enzyme alliinase (EC 4.4.1.4) and subsequent reactions (**Figure 1**) (for a comprehensive review on *Allium* biochemistry see ref *18*). The reaction only takes place after tissues are crushed, and alliinase, located in the vacuole, can interact with the ACSOs in the cytoplasm (*19*). Thiosulfinates (TSs) are the major sulfur compounds produced in freshly cut *Allium* species (*18*). Allicin (allyl 2-propenethiosulfinate), formed from the precursor alliin (*S*-2-

* To whom correspondence should be addressed. Phone: +54-2622-470304; fax: +54-2622-470753; e-mail: crgalmarini@laconsulta.inta.gov.ar.
† INTA - EEA La Consulta and CONICET.
§ Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo.
‖ University of Wisconsin.

10.1021/jf062587s CCC: $37.00
Published on Web 01/27/2007

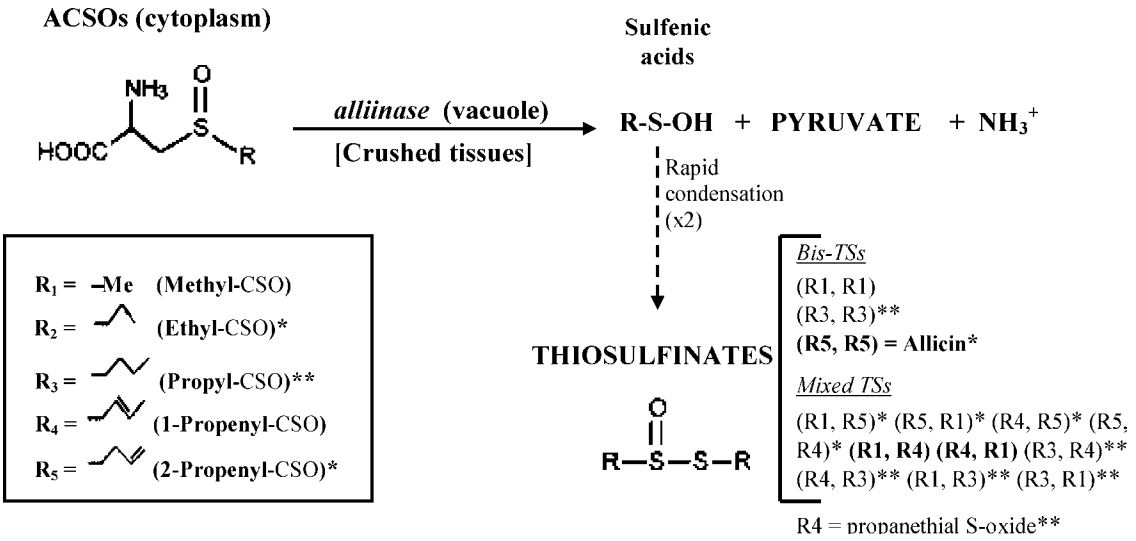

Figure 1. *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs) and thiosulfinates (TSs) formation in garlic and onion (summarized from refs 15 and 20). Upon tissue disruption, alliinase acts on the ACSOs forming unstable sulfenic acids, pyruvate, and ammonia. Sulfenic acids rapidly condense in pairs to form thiosulfinates. Some compounds are either present in garlic (*) or onion (**) whereas others are common to both species (no asterisk). TSs in bold letters are the major TSs of garlic and onions. Alliin (*S*-2-propenyl-CSO) and *S*-1-propenyl-CSO are the predominant ACSOs in garlic and onion, respectively. Ethyl-CSO has only been detected in trace amounts in garlic. Propanethial S-oxide (not a thiosulfinate) is the lacrymatory factor formed in freshly cut onions.

propenyl-L-cysteine sulfoxide), is the predominant garlic TS (*20*) and a potent platelet inhibitor (*15*). Lawson et al. (*16*) found that allicin and other TSs provided nearly all the AA of raw garlic homogenates in whole blood aggregometry. The pyruvate produced by alliinase (**Figure 1**) upon crushing raw garlic (*21*) and onion (*6*, *22*) is significantly correlated with IVAA and is considered a good predictor of antiplatelet strength (*22*). Allicin content demonstrated significant positive correlation with both pyruvate and garlic-induced AA (*21*). Thus, the concentration of pyruvate and allicin can be used as estimators of the antiplatelet potency of raw garlic extracts. The efficacy of garlic as an antithrombotic agent has been mostly demonstrated using raw extracts or different types of preparations (e.g., oils, ethanol-based extracts, dried powder, AGE). Worldwide, however, most of the garlic is not consumed in these forms but, instead, it is usually cooked before consumption.

Only a few reports have investigated the antithrombotic properties of cooked *Alliums*. Chen et al. (*23*) compared the effect of raw versus boiled Welsh onion (*Allium fistulosum*) on several thrombosis-related parameters of the rat, including platelet aggregation, concluding that raw but not boiled Welsh onion inhibited platelet function. In two other studies, whole bulbs of garlic and onion were boiled before homogenization. These extracts had no antiplatelet activity in rabbits (*9*) and rats (*24*), whereas the respective raw extracts significantly reduced TXB_2 and cyclooxygenase activity, two potent inductors of platelet aggregation. In all three previous works, uncrushed *Allium* tissues were extensively boiled before juicing. Alliinase, required for the formation of antiaggregatory organosulfur compounds upon tissue disruption, is thermolabile (*25*). Therefore, it is highly likely that, because of alliinase heat inactivation during the boiling process, no antithrombotic compounds (e.g., TSs) were formed in the homogenates. Presumably, the lack of AA found in uncrushed-boiled *Allium* samples is due to a scarce formation of alliinase-dependent antithrombotic agents.

Allium vegetables are usually crushed to varying extents (e.g., chopped, sliced) before they are cooked, to allow development of flavor and aroma and, consequently, antithrombotic sulfur compounds. We hypothesize that if raw *Allium* tissues are crushed before heating, favoring the formation of active organosulfur compounds, it is possible that they can still provide antithrombotic benefits, since allicin and other TSs seem to be more tolerant to heat than alliinase (e.g., half-life of TSs at 80 °C and pH 4.5 ranged from 50 min to 10 h, depending on the TS (*26*)).

The aim of this study was to evaluate the effect of domestic cooking on garlic-induced antiaggregatory activity of human blood platelets. The degradation of organosulfur antithrombotic compounds with antiplatelet activity during cooking was measured by analysis of allicin and pyruvate levels.

MATERIALS AND METHODS

Plant Materials. The red type garlic clone "Fuego INTA" from the germplasm collection of INTA La Consulta, Mendoza, was used in all the experiments. Fuego INTA is a relatively mild garlic with low pyruvate content (*27*). This released cultivar is extensively cultivated and consumed in Argentina. Garlics were field-grown at the experimental station of INTA La Consulta, Mendoza, in 2005. Mature bulbs were harvested when leaves had senesced and were cured at 35 °C for 2 weeks and then were stored at ambient conditions in sheds for 2 months.

Processing of Samples. Three cooking methods—convection oven, boiling, and microwave oven—were tested on two preparations of garlic tissues, uncrushed (whole cloves) and crushed garlic. For the oven and boiling methods, six different incubation times were tested. For the microwave method, only one combination of intensity and time was used for both crushed and uncrushed garlic. In total, 25 different combinations of cooking method, time, and tissue type were used.

Preparation of garlic samples was as follows. Two kilograms of unsprouted cloves of similar weight (5 ± 0.5 g) from several bulbs were pooled, peeled, and mixed, and samples of 50 g (~10 cloves) were prepared. Three randomly selected 50-g samples (three replicates) were used for each combination of method−time−tissue. For the crushed-tissue treatment, samples were crushed with a garlic press and were incubated at 25 °C for 30 min to allow enzymatic lysis of the ACSOs. The incubation conditions were established on the basis of Lawson and Hughes (*28*), reporting that incubation at 37 °C for 10 min yielded the maximum TS content. Since 37 °C is rarely the temperature at which most people hold garlic during preparation, we used 25 °C and extended the incubation time. After incubation, crushed samples were heat-treated together with uncrushed garlic samples, as described below.

Convection Oven. A convection gas oven (Longvie 2600) was heated to 200 °C; crushed and uncrushed garlic clove samples were placed in the oven in individual dishes and were heated for 1, 3, 6, 10, and 20 min. At each time, samples were removed from the oven and were rapidly cooled in a water−ice mixture. Individual samples were then juiced in three volumes of distilled water using a blender (Braun MR 555 CA Minipimer Control Plus Vario) and were centrifuged twice. The supernatants were aliquoted and stored at − 80 °C until IVAA, pyruvate, and allicin analysis.

Boiling. A standardized volume of water (3 L) was added to an aluminum pot, covered with a glass lid, and was fire-heated until the temperature inside the pot reached 100 °C. Small shallow aluminum containers (~230 mm^3) carrying each of the garlic samples were inmersed in the boiling water and were incubated with the lid on. Three randomly selected samples of crushed and uncrushed garlics were removed after 1, 3, 6, 10, and 20 min of boiling and were handled as described above. Three crushed garlic samples were juiced without boiling (raw controls).

Microwave. Three 50-g samples each of crushed and uncrushed garlic cloves were microwaved, in pairs (one crushed along with one uncrushed garlic sample), in a Whirpool VIP 27 microwave oven, for 3 s/g fresh weight (fw) at 500 W. This power level is relatively moderate considering the power range (160−1000 W) available in the microwave oven. After microwaving, samples were cooled and juiced as described.

Mixtures of Extracts. To investigate whether the loss of AA in microwaved garlic is associated with inactivation of alliinase, lability of the precursors (ACSOs), or the destruction of antiplatelet sulfur compounds (e.g., TS), the following experiment was performed. Extracts of raw, uncrushed microwaved (UnCM) and crushed microwaved (CM) samples were combined, in different ratios, and the resulting mixtures were evaluated for IVAA, pyruvate, and allicin levels. A pool of three extracts (three replicates), for each treatment (raw, UnCM, and CM), was previously prepared and used for the preparation of the mixtures.

The IVAA response to different dosages of raw and microwaved garlic extracts was investigated. Increasing volumes (from 15 to 100 μL) of raw, UnCM, and CM garlic extracts were used in the platelet aggregation reaction, maintaining constant the rest of the reaction parameters.

Measurement of Platelet Aggregation. For determinations of IVAA, garlic juice samples were thawed to room temperature. A freeze−thaw cycle had no effect on AA of *Allium* extracts (*22*). Platelet aggregation was measured using electrical impedance aggregometry (Chrono-Log, Havertown, PA) of whole blood (*29*). Blood was drawn from two healthy, nonsmokers, human donors, one female and one male of ages 25 and 33, respectively, who had abstained from eating *Alliums* or other known platelet-inhibitory foods for at least 1 week. Also, the donors had not taken drugs known to affect platelet aggregation (e.g., aspirin) or other types of drugs for at least 1 week prior to venipuncture. In all cases, blood was drawn from fasting donors between 7 and 8 a.m. Venipuncture was performed by the hematology service at the Hospital Central, Mendoza, Argentina. Blood was drawn into citric acid anticoagulant and was later diluted 1:1 with TRIS-buffered saline (pH 7.4). Platelet aggregation was measured as described previously (*6*) with one minor modification. The standard aggregation reaction included 1 mL of blood, 2.5 μL of the agonist collagen (1 μg/μL), and 30 μL of garlic juice (instead of 200 μL, used for onions in the original protocol). This concentration of garlic extract was chosen on the basis of near-zero aggregations (or ~100% inhibition of aggregation) obtained for its raw form (control). AA was expressed as percent inhibition of platelet aggregation, compared to control samples prepared in the same way but without garlic juice. For each blood sample, reference garlic juice samples (of known antiplatelet activity) were tested for their IVAA, relative to a control without the addition of garlic juice. If the IVAA of the reference samples was significantly different from the expected IVAA values, the blood sample was discarded. By these means, we checked and controlled day-to-day variations in the blood samples.

Pyruvate Analysis. Pyruvate content was determined according to the method of Schwimmer and Weston (*30*). Color development was measured at 420 nm on a spectrophotometer, and the pyruvate concentration in the juice was determined on the basis of calibration curves obtained by measuring pyruvate standards. Values were expressed as μmoles pyruvate/g fw of garlic. Background pyruvate levels were not estimated.

Measurement of Allicin Content. Extracts of garlic, prepared as described, were centrifuged and the supernatants were filtered and mixed with one volume of methanol. Ten microliters of the resulting mixtures were injected onto the high-performance liquid chromatography (HPLC) under isocratic elution conditions. Allicin was measured by reversed-phase HPLC analysis, using the same procedure and equipment described previously (*31*). Alliin was obtained from Extrasynthese S.A., Lyon, France. Each sample was measured at least twice. Values were expressed as mg allicin/g fw.

Statistical Analysis. All data were expressed as mean ± standard deviation (SD). The data were analyzed by ANOVA procedure using the software STATGRAPHICS Plus for Windows 4.0. Means of each treatment group were compared by least significant difference (LSD) test. *P*-values < 0.05 were considered to be significant. Regression analysis among IVAA, pyruvate, and allicin was performed using the same software.

RESULTS

Convection Oven. Aqueous extracts of oven-heated garlic varied significantly ($P < 0.001$) in their ability to inhibit platelet aggregation in vitro (**Figure 2A**). Incubation at 200 °C for 3 min had no significant effect on the IVAA of the extracts, as compared to raw garlic, although crushed oven-heated (CO) samples had lower IVAA than uncrushed oven-heated (UnCO) samples. The AAs of CO and UnCO samples were significantly, although differentially, reduced after 6 min of incubation. Extracts of UnCO were totally ineffective in inhibiting platelet aggregation, whereas CO still had 23% of AA (**Figure 2A**). No effect on aggregation was found in extracts of crushed and uncrushed garlics that were oven-heated for 10 or more minutes under our experimental conditions.

Pyruvate content varied significantly ($P < 0.001$) among oven-heated garlic samples (**Figure 2B**). Uncrushed garlic cloves heated for 3 min or less had pyruvate levels similar to raw garlic, whereas incubation for 6 or more minutes significantly reduced pyruvate levels to less than 10% of raw. Contrary to uncrushed garlic, the pyruvate content of crushed samples varied within a narrow range (from 20.6 to 26.4 μmol/g fw), with values being significantly different from the raw control only for 10- and 20-min samples.

Variation for allicin content was highly significant ($P < 0.001$) among oven-heated samples (**Figure 2C**). Compared to raw garlic, the allicin levels of UnCO were not affected during the first 3 min at 200 °C but decreased rapidly to less than 6% after 6 min of incubation. Crushed garlic, instead, gradually decreased in allicin concentration, reaching levels comparable to those found in 6-min-UnCO after 20 min of incubation. Under our standard platelet-aggregation conditions, samples with less than 3 mg of allicin/g fw had no IVAA.

Boiling. Significant variation ($P < 0.001$) in AA was found among boiled garlic samples (**Figure 3A**). Boiling for 3 min or less did not affect garlic ability to inhibit platelet aggregation, regardless of whether cloves were crushed (CB) or not (UnCB) before the boiling treatment. Whole cloves of garlic boiled for 6 min had no IVAA, whereas garlic samples that were crushed before boiling for 6 min had partial (~20%), yet significant, AA (**Figure 3A**). After 10 or more minutes of boiling, both CB and UnCB samples were equally ineffective in inhibiting aggregation under our experimental conditions.

Significant variation ($P < 0.001$) was also found for pyruvate levels among boiled samples (**Figure 3B**). Heating did not affect

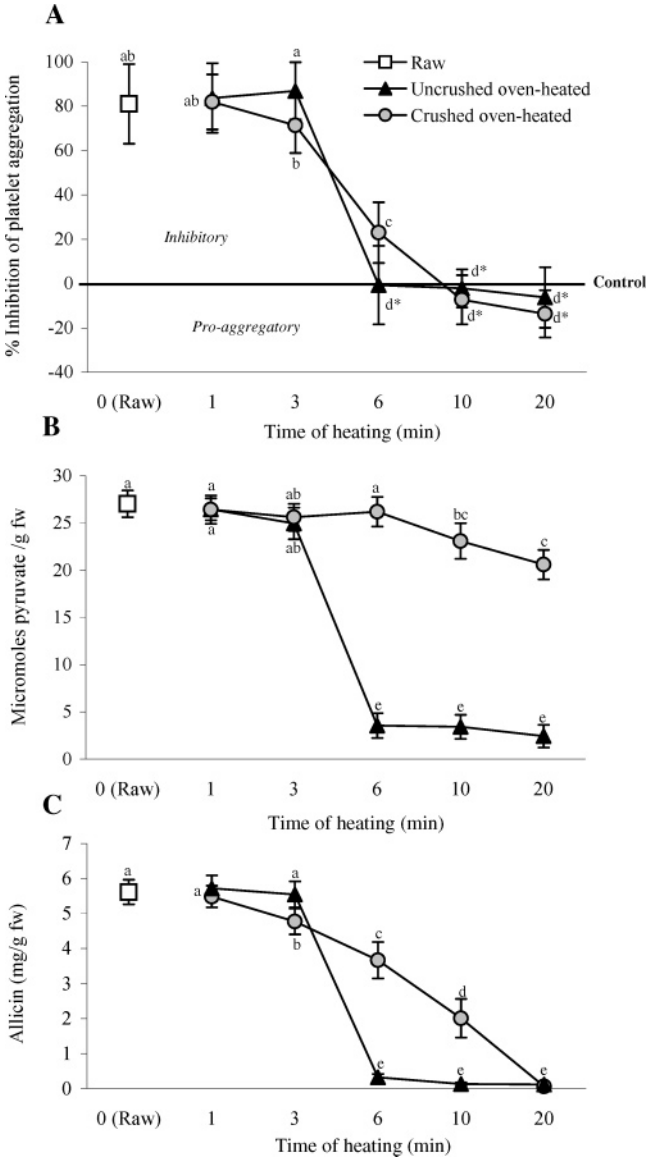

Figure 2. Antiplatelet activity (**A**), pyruvate (**B**), and allicin (**C**) concentration of aqueous extracts of raw (white square) and oven-heated garlic. Uncrushed (black triangle) and crushed (gray circles) garlic samples were oven-heated at 200 °C during 1–20 min. Values for antiplatelet activity (A) are % inhibition of platelet aggregation ± SD, ($n = 6$), as compared with a control without adition of garlic extract. Asterisks indicate samples that are not significantly different from the control, $P < 0.05$. For pyruvate content (**B**), values are the means ($n = 3$) of μmoles pyruvate/g fw ± SD. Allicin contents (**C**) correspond to mean values ($n = 3$) of mg allicin per g of fresh weight. Data points with no common letters differ, $P < 0.05$.

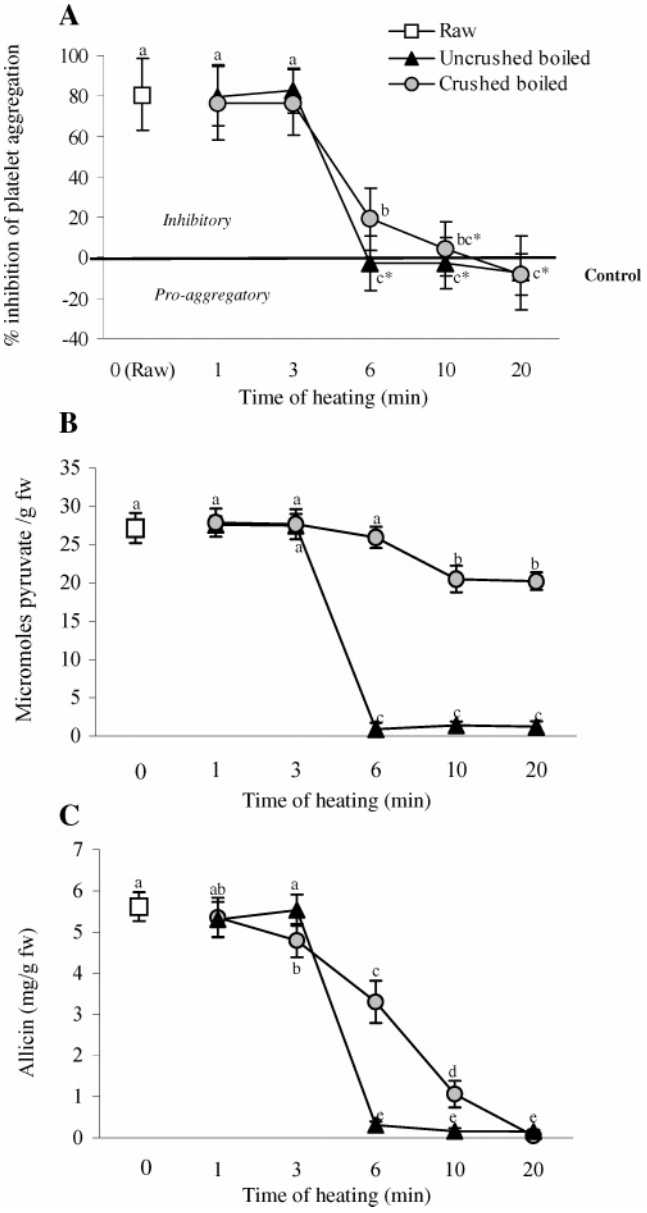

Figure 3. Antiplatelet activity (**A**), pyruvate (**B**), and allicin (**C**) concentration in aqueous extracts of raw (white square) and boiled garlic samples. Uncrushed (black triangle) and crushed (gray circles) garlic samples were heated in boiling water for 1–20 min. Values for antiplatelet activity (**A**) are % inhibition of platelet aggregation ± SD, ($n = 6$) as compared with a control without adition of garlic extract. Asterisks indicate samples that are not significantly different from the control, $P < 0.05$. For pyruvate (**B**), values are the means ($n = 3$) of μmoles pyruvate/g fw ± SD. Allicin contents (**C**) correspond to mean values ($n = 3$) of mg allicin per g of fresh weight. Data points with no common letters differ, $P < 0.05$.

the pyruvate content of crushed and uncrushed garlic boiled for 3 min or less, as compared to the raw control. Longer incubations (6−20 min) significantly reduced pyruvate levels to less than 4% in uncrushed samples, whereas crushing decreased garlic pyruvate concentration, from 27.9 to 20.2 μM/g fw.

Allicin levels varied ($P < 0.001$) in a time-dependent fashion during boiling (**Figure 3C**). Allicin levels in uncrushed garlic were comparable to raw garlic during the first 3 min of boiling and then rapidly decreased to nearly undetectable levels after 6 min. Allicin decreased gradually for CB samples during the boiling treatment. Only samples with allicin levels ≥3 mg/g fw had significant IVAA.

The time-course variation of IVAA in the two previous experiments was gradual for crushed garlic but markedly abrupt for uncrushed garlic cloves. The latter either had the same inhibitory activity as the raw extracts (3 min or less) or no effect on aggregation at all (6−20 min). The pyruvate and allicin content of these samples varied concomitantly with IVAA (**Figure 2A−C**; **Figure 3A−C**). Significant and strong positive correlations were found among the three variables for uncrushed-cooked garlic (**Table 1**). In the case of previously crushed cooked garlic, IVAA was strongly associated ($r > 0.80$) with allicin but not with pyruvate levels. Nonetheless, the correlation between IVAA and pyruvate was also significant (**Table 1**).

Table 1. Correlation (r) among IVAA, Allicin, and Pyruvate Content in Uncrushed (UnCr) and Crushed (Cr) Cooked Garlic Samples from Four Independent Experiments[a]

		IVAA		allicin	
experiment		UnCr	Cr	UnCr	Cr
oven	**pyruvate**	**0.96*****	0.57**	**0.99*****	**0.82*****
	allicin	**0.97*****	**0.88*****		
boiling	**pyruvate**	**0.95*****	**0.70*****	**0.92*****	**0.87*****
	allicin	**0.86*****	**0.89*****		
microwave	**pyruvate**	0.52**		**0.86*****	
	allicin	**0.80*****			
mixtures of extracts	pyruvate	0.56**		**0.83*****	
	allicin	**0.90*****			

[a] Numbers in bold indicate strong correlation ($r \geq 0.80$). The asterisks indicate significance at $P < 0.01$ (**) and $P < 0.001$ (***) for correlation coefficients.

Microwave. Microwaved garlic had no IVAA whereas raw samples significantly inhibited platelet aggregation (**Figure 4A**) under our experimental aggregatory conditions. Under these conditions, no significant differences were found between crushed- and uncrushed-microwaved treatments.

Pyruvate levels varied significantly ($P < 0.001$) among raw, uncrushed-microwaved (UnCM) and crushed-microwaved (CM) samples of garlic (**Figure 4B**). CM samples had pyruvate levels similar to their respective raw forms, whereas UnCM samples only had ~1.5% of the pyruvate concentration found in raw garlic. In agreement with its pyruvate content, UnCM garlic had extremely low allicin levels (less than 2% of the content in raw garlic) (**Figure 4C**). Crushed and then microwaved garlic had ~50% of the allicin levels of raw garlic (mean values decreased from 5.45 to 2.79 mg/g fw). Significant correlations were found among IVAA, allicin, and pyruvate values with the degree of association between IVAA and allicin stronger than between IVAA and pyruvate (**Table 1**).

Mixtures of Garlic Extracts. The effect of mixtures on platelet aggregation with varying ratios of raw, UnCM, and CM garlic extracts is shown in **Figure 4A** (samples 1−9). Mixtures between UnCM and CM extracts (microwaved treatments) had no effect on platelet aggregation and were not different from the original extracts, $P < 0.05$, regardless of the ratio used. However, when raw extract was added to extracts of microwaved garlic, the resulting mixtures behaved differently with regard to their AA, depending on whether UnCM or CM extracts were used in the mixture. When 1 part of raw extract was added to 9 parts of UnCM (with no effect on aggregation), the entire AA was reestablished in the mixture, displaying the same antiaggregatory potency as the raw extracts. Increasing the raw:UnCM ratio, up to 9:1, had no further effect on platelet aggregation. Raw:CM mixtures only had an additive effect on IVAA, which covaried with the proportion of raw extract used in the mixture.

Levels of pyruvate were not reduced by microwaving if garlic was previously crushed, whereas garlic that was microwaved and then crushed had almost no pyruvate (**Figure 4B**). Pyruvate contents of UnCM:CM mixtures were additive, increasing with the proportion of CM extract used in the mixtures. Pyruvate levels of raw:CM mixtures did not differ from either the raw or CM extracts ($P < 0.05$), regardless of the ratio. When 1 part of raw extract was added to 9 parts of the nearly pyruvate-free UnCM extract, the resulting mixture had the same concentration as the raw extracts. Raising the raw:UnCM ratio (up to 9:1) did not further increase the pyruvate content of the mixtures.

Combining garlic extracts had an additive effect on the allicin levels of UnCM−CM and raw−CM mixtures (**Figure 4C**, samples 1−6). Adding 1 vol of raw garlic juice to 9 vol of UnCM extracts increased allicin content more than additively, to the same level found in raw garlic **(Figure 4C**, sample 7). Allicin content did not further increase when the raw:UnCM ratio was raised to 9:1.

A dose-dependent response on IVAA was found for raw and CM samples but not for UnCM. (**Figure 5**). At low dosages (≤ 60 μL/mL whole blood), CM extracts were less effective in inhibiting aggregation than raw extracts. Raw extracts reached the maximum inhibition when 45 μL was used, whereas CM extracts needed 80−100 μL for a comparable inhibitory effect. Extracts of UnCM garlic had no antiaggregatory activity for the dosage range used. Positive IVAA dose responses were also observed for extracts of 6-min-CO and 6-min-CB but not for their respective uncrushed samples (6-min-UnCO and 6-min-UnCB) (data not shown).

DISCUSSION

Previous studies on the antithrombotic effect of cooked versus raw *Alliums* have, in most cases, disregarded the mechanistic basis of the formation of antithrombotic compounds in the design of their experiments and interpretation of the results. Bordia et al. (*9*) reported that feeding rats with 15-min-boiled, uncrushed garlic or onions had no effect on the serum level of TXB_2, whereas their raw forms significantly reduced TXB_2 levels. The authors attributed the lack of activity in boiled samples to a destruction of antithrombotic compounds by high temperature and concluded that the antithrombotic effect of garlic and onion can only be achieved by ingestion of their raw form. Similar results were obtained when uncrushed garlic (*24*) and Welsh onions (*23*) were boiled and were used to evaluate their efficacy as antithrombotic agents. Both studies concluded that boiled garlic and Welsh onions were totally ineffective as antithrombotic agents, whereas their raw forms had significant antithrombotic effects. In all three previous studies, extensively boiled uncrushed *Allium* tissues were used for the cooked treatment. Thus, the possibility remains that alliinase, which is thermolabile (*25*), was heat-inactivated before homogenization and, therefore, the active antithrombotic sulfur compounds (e.g., TSs) were never produced rather than being destroyed by the high temperatures.

Our results indicate that, in general, heating was detrimental for garlic IVAA. However, the extent of this effect varied among the different treatments used, ranging from unaffected AA (equal to raw garlic) to an absolute lack of AA depending mainly on how garlic was prepared (crushed or uncrushed) and the intensity of the heat treatment. The fact that garlic oven-heated or boiled for 3 min or less had similar AA as the raw extracts suggests that brief cooking of garlic may provide most of the antithrombotic benefits found in its raw form. Crushing garlic followed by 6-min oven-heating or boiling resulted in some retention of IVAA that was completely lost if garlic was heated uncrushed. Thus, crushing garlic cloves before cooking may effectively reduce the loss of AA if the cooking is done for moderate periods of time. Prolonged incubation (e.g., more than 10 min) at temperatures ≥ 100 °C completely suppressed garlic AA, under our experimental conditions, regardless of whether crushed or uncrushed tissues were used.

Our measurements of pyruvate and allicin levels provided an explanation for the loss of IVAA because of cooking. While heating for more than 3 min reduced garlic AA in both uncrushed and crushed samples, the underlying cause of this loss is likely different between these two treatments. Heat

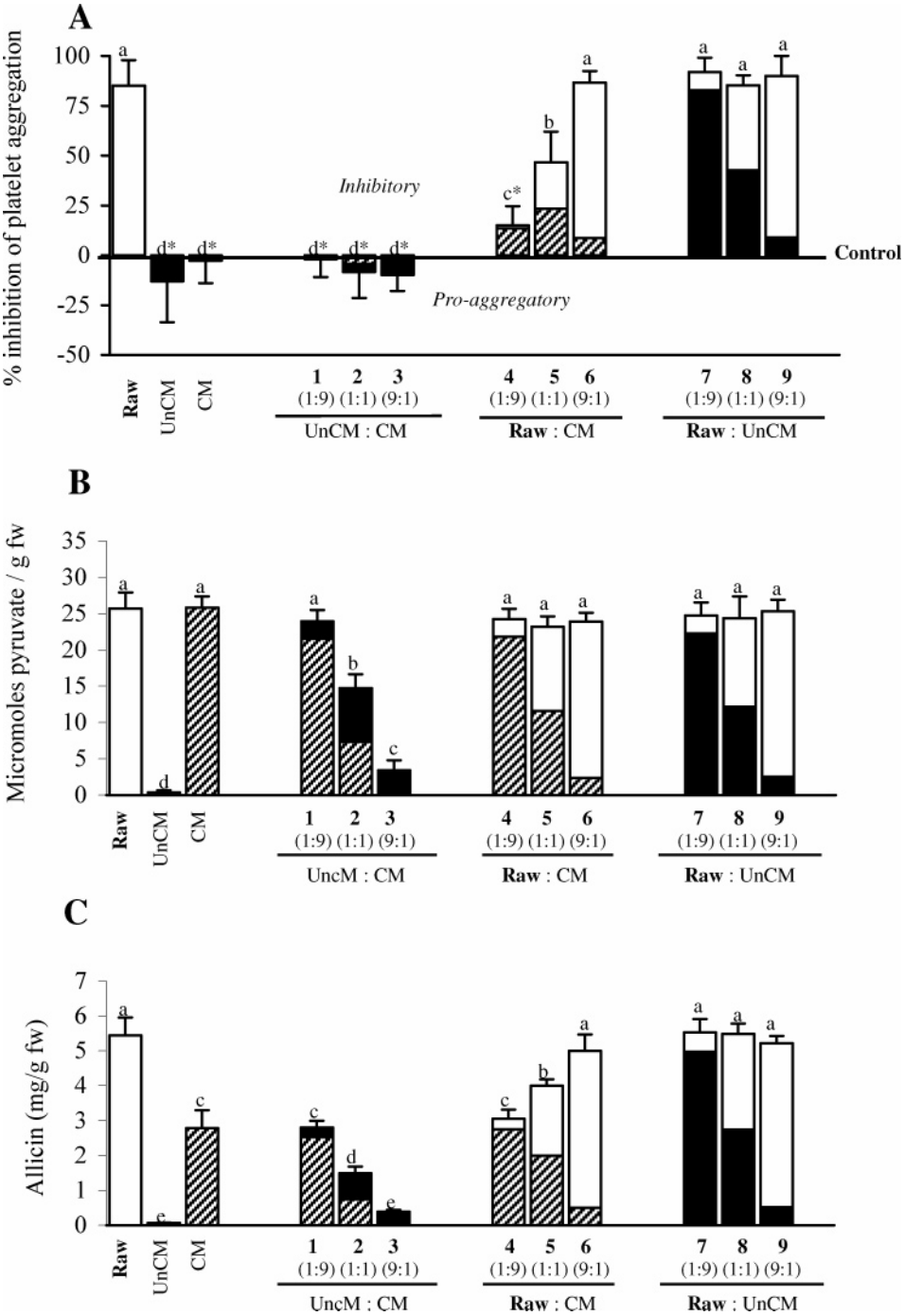

Figure 4. Antiplatelet activity (**A**), pyruvic acid (**B**), and allicin content (**C**) in raw (white), uncrushed-microwaved (UnCM) (black), and crushed-microwaved (CM) (striped) garlic samples and of mixtures with different ratios (in parentheses) of their extracts. The relative areas of two-color bars represent the juice ratio of the mixture. Values for antiplatelet activity (**A**) are % inhibition of platelet aggregation ± SD, as compared with a control without adition of garlic extract. Asterisks indicate samples that are not significantly different from the control, $P < 0.05$. For pyruvate (**B**) and allicin (**C**) content, values are means of μmoles pyruvate/g fw ± SD and mg allicin/g fw ± SD, respectively. Sample sizes were $n = 6$ for raw, UnCM, and CM and were $n = 3$ for mixtures of extracts. Bars with no common letters differ, $P < 0.05$.

treatment of uncrushed garlic cloves would inactivate the enzyme alliinase before homogenization, not allowing the formation of sulfur compounds with AA. This is consistent with the abrupt decrease in pyruvate and allicin levels, observed between the third and sixth minutes of oven-heating and boiling (**Figure 3**). Presumably, during the first 3 min of heating, alliinase inactivation was incomplete and the remaining active enzyme was enough to convert the ACSOs to TSs, thus reestablishing the entire allicin and pyruvate levels, as well as the full IVAA. The extremely low allicin and pyruvate formed in uncrushed 6-min-cooked garlic (associated with an absolute lack of AA) is consistent with a complete alliinase inactivation, which would also explain the low pyruvate and allicin content found in uncrushed microwaved samples. When a minimal amount of fresh garlic juice (containing active alliinase) was added to these samples, the allicin and pyruvate levels were reestablished, in full, concomitantly with a raise in IVAA (sample 7 in **Figure 4A**−**C**), indicating that inactivity of alliinase was responsible for the low formation of TSs and consequently the lack of AA.

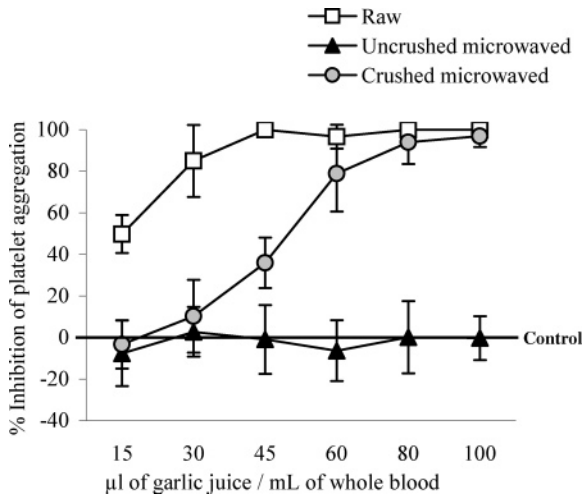

Figure 5. Dosage inhibition of in vitro platelet aggregation by aqueous extracts of raw, uncrushed microwaved, and crushed microwaved garlic. Increasing volumes of garlic extracts were added to the standard platelet aggregation reaction, containing 1 mL of whole blood and using 2.5 μL of collagen as agonist. Values are % inhibition of platelet aggregation ± SD ($n = 4$).

The fact that small measurable amounts of pyruvate and allicin were detected in uncrushed-heated samples (as opposed to the expected absence of both compounds in hypothetically alliinase-free garlic) could be due to the lysis of a few cells during the heat treatment, with the subsequent formation of small amounts of TSs and pyruvate. Also, pyruvate from other sources (e.g., from glycolysis) can accumulate in relatively low quantities in *Allium* tissues, contributing to levels detected.

Heat treatment of crushed garlic cloves would drive off or destroy products of the allinase reaction that are responsible for IVAA. In crushed-then-cooked garlic, the loss of AA is consistent with a gradual destruction of previously formed antithrombotic compounds, as indicated by the gradual decay in activity associated with the concomitant gradual and correlated reduction in allicin and pyruvate levels. The addition of fresh garlic juice providing active alliinase to these samples only had an additive effect on IVAA and allicin levels, indicating that no organosulfur antiplatelet compounds were newly formed. These results demonstrate the lack of ACSOs for alliinase to act upon in these samples since they were cleaved by alliinase before the heat treatment.

Several reports have found a direct or indirect linkage between sulfur compounds and AA in *Alliums*. Galmarini et al. (*6*) reported significant positive phenotypic and genetic correlation among IVAA, pyruvate, solids content, and dry matter in onion F_3 families. In garlic, we have previously found significant correlation among IVAA, allicin, and pyruvate levels (*21*). The IVAA of some TSs has been demonstrated by several authors (*15*, *16*, *32*), being allicin the one TS explaining most of the garlic-induced IVAA (*16*). Together with the above findings, the fact that these three variables were also significantly associated in cooked garlic samples (**Table 1**) strongly suggests that TSs are important contributors to the overall garlic-induced IVAA.

Our results suggest that the lack of antithrombotic activity found previously (*9*, *23*, *24*) in boiled samples was due to inactivity of alliinase. In these studies, uncrushed *Alliums* were boiled for 15−30 min, in full agreement with our results for these particular experimental conditions. These samples (UnCB, 10, 20 min boiling) not only lacked antiaggregatory activity but, consistent with alliinase inactivation, also had extremely low levels of pyruvate and allicin (**Figure 3A**−**C**). Our results suggest that if garlic is previously crushed and moderately cooked, it may still provide antiplatelet benefits.

Ali et al. (*32*) investigated the IVAA of raw garlic aqueous extracts that were boiled for 10 min after preparation of the extract. In agreement with the results reported herein for most of crushed-cooked garlic samples, they found reduced AA in boiled garlic (as compared to raw extracts). The authors reported dose-dependent IVAAs for both raw and boiled garlic using rabbit platelet-rich plasma. The fact that garlic was homogenized before the boiling treatment, thus allowing the formation of antiplatelet sulfur compounds, may account for the partial IVAA found in their boiled samples, as opposed to the lack of activity reported previously for uncrushed-boiled *Alliums* (*9*, *23*, *24*).

The importance of processing garlic before heating has been previously reported with regard to other biological effects. Song and Milner (*33*) found that garlic crushed and then allowed to stand for 10 min before microwaving for 60 s had the same anticarcinogenic activity as raw crushed garlic, whereas uncrushed 60-s-microwaved garlic had no anticarcinogenic properties in rats (not different from the untreated group). Furthermore, they found that the lack of anticarcinogenic activity in uncrushed-microwaved garlic was associated with inactivation of alliinase. Chutani and Bordia (*34*) found that frying previously crushed garlic cloves did not significantly affect garlic's fibrinolytic activity in man, as compared with raw garlic.

The antiplatelet dose−response of crushed-microwaved, but not of uncrushed-microwaved, garlic likely reflects their differences in the content of antiaggregatory sulfur compounds, such as allicin and other TSs, since CM garlic had ∼50% of the allicin content found in the raw form, whereas UnCM had less than 2%. The variation in IVAA, relative to the dosages used, can be fully explained by the allicin concentration of CM extracts, which required nearly twice the dosage of raw garlic extract to achieve a comparable inhibitory effect (**Figure 5**). These results further suggest an important role for allicin in the antiplatelet response of garlic in whole blood aggregometry. Also, they suggest that the partial loss of AA during the cooking of previously crushed garlic can be compensated by increasing its consumption. In this case, the amount of garlic necessary to be consumed for an equivalent effect to that obtained from raw garlic will depend on factors such us the degree of tissue disruption (as more cells are crushed, more TSs are formed) and the intensity of cooking, which will determine the remaining content of active antithrombotic compounds (e.g., TSs). These studies emphasize the effect of preparation methods upon garlic TSs formation but this variation is also built upon genetic and environmental variation in TSs established before the crop reaches the kitchen. Significant variation exists among garlic cultivars for TSs and pyruvate content (*35*). The use of garlic cultivars with high TSs content may provide higher antithrombotic benefits, as more active antiplatelet compounds are expected to remain after the cooking process. Also, cultural practices such as sulfur fertilization may improve *Allium* antiplatelet potency as this can affect their content in sulfur compounds (*22*, *36*).

In summary, the present study provides evidence that processing of garlic and the conditions used for cooking can markedly influence its effectiveness as a platelet inhibitor. Additionally, these studies strongly suggest that allicin and other TSs are important contributors to the garlic-induced antiplatelet activity in whole blood aggregometry. Whether our in vitro results are extrapolable to in vivo conditions needs to be

confirmed. Our results point to the importance of critically examining the manner in which garlic is processed and consumed when evaluating its antithrombotic properties and its efficacy in preventing cardiovascular disease. Most notable, we found that addition of raw garlic to microwaved-uncrushed garlic restored a full complement of antiplatelet activity that was completely lost without the raw garlic addition, suggesting that special attention to garlic preparation for consumption can improve its nutritional value. Our results also suggest that the precursors to sulfur-antithrombotic compounds are not destroyed by heating undamaged tissue and that the level of ACSOs, rather than alliinase, is the limiting factor to the generation of sulfur antiplatelet products in raw garlic.

ACKNOWLEDGMENT

We gratefully acknowledge Carolina Soto for technical assistance with HPLC analysis and the hematological service of the Hospital Central (Mendoza, Argentina) for performing the blood extractions.

LITERATURE CITED

(1) Augusti, K. T. Therapeutic and medicinal values of onions and garlic. In *Onions and allied crops*; Rabinowitch, H. D., Brewster, J. L., Eds.; CRC Press: Boca Raton, FL, 1990; Vol. 3, pp 94−108.

(2) Banerjee, S. K.; Maulik, S. K. Effect of garlic on cardiovascular disorders: a review. *Nutr. J.* **2002**, *1*, 1−14.

(3) Rahman, K. Historical perspective on garlic and cardiovascular disease. *J. Nutr.* **2001**, *131*, 977S-979S.

(4) Apiz-Castro, R.; Cabrera, S.; Cruz, M. R.; Ledesma, E.; Jain, M. K. Effects of garlic extract and of three pure components isolated from it on human platelet aggregation, arachidonate metabolism, release activity and platelet ultrastructure. *Thromb. Res.* **1983**, *32*, 155−169.

(5) Bordia, A. Effect of garlic on human platelet aggregation in vitro. *Atherosclerosis* **1978**, *30*, 355−360.

(6) Galmarini, C. R.; Goldman, I. L.; Havey, M. J. Genetic analysis of correlated solids, flavor, and health-enhancing traits in onion (*Allium cepa* L.). *Mol. Genet. Genomics* **2001**, *265*, 543−551.

(7) Srivastava, K. C. Effects of aqueous extracts of onion, garlic and ginger on platelet aggregation and metabolism of arachidonic acid in the blood vascular system: in vitro study. *Prostaglandins, Leukotrienes Med.* **1984**, *13*, 227−235.

(8) Vanderhock, J. Y.; Makheja, A. N.; Bailey, J. M. Inhibition of fatty acid oxygenases by onion and garlic acts. Evidence for the mechanism by which these oils inhibit platelet aggregation. *Biochem. Pharmacol.* **1980**, *29*, 3169−3173.

(9) Bordia, A.; Mohammed, N.; Thomson, M.; Ali, M. An evaluation of garlic and onion as antithrombotic agents. *Prostaglandins Leukotrienes Essent. Fatty Acids* **1996**, *54*, 183−186.

(10) Gadkari, J. V.; Joshi, V. D. Effect of ingestion of raw garlic on serum cholesterol level, clotting time and fibrinolytic activity in normal subjects. *J. Postgrad. Med.* **1991**, *37*, 128−131.

(11) Legnani, C.; Frascaro, M.; Guazzaloca, G.; Ludovici, S.; Cesarano, G.; Coccheri, S. Effects of a dried garlic preparation on fibrinolysis and platelet aggregation in healthy subjects. *Arzneimittelforschung* **1993**, *43*, 119−122.

(12) Rahman, K.; Billington, D. Dietary supplementation with aged garlic extract inhibits ADP-induced platelet aggregation in humans. *J. Nutr.* **2000**, *130*, 2662−2665.

(13) Bordia, A. K.; Sharma, K. D.; Parmar, V. K.; Varma, S. K. Protective effect of garlic oil on the changes produced by 3 weeks of fatty diet on serum cholesterol, serum triglycerides, fibrinolytic activity and platelet adhesion in man. *Ind. Heart J.* **1982**, *34*, 86−88.

(14) Laurence, W. D.; John, D. F. Garlic extract prevents acute platelet thrombus formation in stenosed canine coronary arteries. *Am. Heart J.* **1989**, *117*, 973−975.

(15) Briggs, W. H.; Xiao, H.; Parkin, K. L.; Shen, C.; Goldman, I. L. Differential inhibition of human platelet aggregation by selected *Allium* thiosulfinates. *J. Agric. Food Chem.* **2000**, *48*, 5731−5735.

(16) Lawson, L. D.; Ramson, D. K.; Hughes, B. G. Inhibition of whole blood platelet aggregation by compounds in garlic clove extracts and commercial garlic preparations. *Thromb. Res.* **1992**, *65*, 141−156.

(17) Lancaster, J. E.; Boland, M. J. Flavor biochemistry. In *Onions and allied crops*; Rabinowitch, H. D., Brewster, J. L., Eds.; CRC Press: Boca Raton, FL, 1990; Vol. 3, pp 33−72.

(18) Block, E. The organosulfur chemistry of the genus *Allium*. Implications for organic sulfur chemistry. *Agnew. Chem., Int. Ed. Engl.* **1992**, *31*, 1135−1178.

(19) Lancaster, J.; Collin, H. Presence of alliinase in isolated vacuoles and of alkyl cysteine sulphoxides in the cytoplasm of bulbs of onion (*Allium cepa*). *Plant Sci. Lett.* **1981**, *22*, 169−176.

(20) Block, E.; Naganathan, S.; Putman, D.; Zhao, S-H. *Allium* chemistry: HPLC analysis of thiosulfinates from onion, garlic, wild garlic (ramsoms), leek, scallion, shallot, elephant (great-headed) garlic, chive, Chinese chive. Uniquely high allyl to methyl ratios in some garlic samples. *J. Agric. Food. Chem.* **1992**, *40*, 2418−2430.

(21) Cavagnaro, P. F.; Senalik, D.; Galmarini, C. R.; Simon, P. W. Correlation of pungency, thiosulfinates, antiplatelet activity and total soluble solids in two garlic families. American Society for Horticultural Sciences Annual Conference, Las Vegas, Nevada, July, 2005. *HortScience* **2005**, *40*, 1019.

(22) Goldman, I. L.; Kopelberg, M.; Debaene, J. P.; Schwartz, B. S. Antiplatelet activity in onion (*Allium cepa* L.) is sulfur dependent. *Thromb. Haemostasis* **1996**, *76*, 450−452.

(23) Chen, J. H.; Chen, H. I.; Tsai, S. J.; Jen, C. J. Chronic consumption of raw but not boiled Welsh onion juice inhibits rat platelet function. *J. Nutr.* **2000**, *130*, 34−37.

(24) Ali, M. Mechanism by which garlic (*Allium sativum*) inhibits cyclooxygenase activity. Effect of raw versus boiled garlic extract on the synthesis of prostanoids. *Prostaglandins Leukotrienes Essent. Fatty Acids* **1995**, *53*, 397−400.

(25) Jansen, H.; Muller, B.; Knobloch, K. Characterization of an alliin lyase preparation from garlic (*Allium sativum*). *Planta Med.* **1989**, *55*, 434−439.

(26) Shen, C.; Xiao, H.; Parkin, K. L. *In vitro* stability and chemical reactivity of thiosulfinates. *J. Agric. Food Chem.* **2002**, *50*, 2644−2651.

(27) Natale, P. J.; Camargo, A.; Galmarini, C. R. Characterization of argentine garlic cultivars by their pungency. *Acta Hortic. (ISHS)* **2005**, *688*, 313−316.

(28) Lawson, L. D.; Hughes, B. G. Characterization of the formation of allicin and other thiosulfinates from garlic. *Planta Med.* **1992**, *58*, 345−350.

(29) Cardinal, D.; Flower, R. J. The electronic aggregometer: a novel device for assessing platelet behavior in blood. *J. Pharm. Methods* **1980**, *3*, 135−158.

(30) Schwimmer, S.; Weston, W. Enzymatic development of pyruvic acid in onion as a measure of pungency. *J. Agric. Food Chem.* **1961**, *9*, 301−304.

(31) Cavagnaro, P. F.; Camargo, A.; Píccolo, R. J.; García, Lampasona, S.; Burba, J. L.; Masuelli, R. W. Resistance to Penicillium hirsutum Dierckx in garlic accessions. *Eur. J. Plant Pathol.* **2005**, *112*, 195−199.

(32) Ali, M.; Bordia, T.; Mustafa, T. Effect of raw versus boiled aqueous extract of garlic and onion on platelet aggregation. *Prostaglandins Leukotrienes Essent. Fatty Acids* **1999**, *60*, 43−47.

(33) Song, K.; Milner, J. A. Heating garlic inhibits its ability to suppress 7,12-dimethylbenz(a)anthracene-induced DNA adduct formation in rat mammary tissue. *J. Nutr.* **1999**, *129*, 657−661.

(34) Chutani, S. K.; Bordia, A. The effect of fried versus raw garlic on fibrinolytic activity in man. *Atherosclerosis* **1981**, *38*, 417−421.

(35) Cavagnaro, P. F.; Galmarini, C. R.; Simon, Evaluación, P. W. de caracteres ligados al sabor y a características nutracéuticas de una colección de 162 clones de ajo [Evaluation of traits associated with flavor and health-enhancing attributes in a collection of 162 garlic clones]. Resúmenes del XII Congreso Latinoamericano, y XXVIII Congreso Argentino, de Horticultura, Rio Negro, Argentina, September 2005. *INTA* **2005**, 267.

(36) Randle, W. M.; Kopsell, D. E.; Kopsell, D. A.; Snyder, R. L. Total sulfur and sulfate accumulation in onion is affected by sulfur fertility. *J. Plant Nutr.* **1999**, *22*, 45−51.

Received for review September 8, 2006. Revised manuscript received December 11, 2006. Accepted December 15, 2006. Funding provided by Project INTA-BID 12908, PICT-O 12902.

JF062587S