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REVIEWS

5751 **Tomato Glycoalkaloids: Role in the Plant and in the Diet**
Mendel Friedman

ANALYTICAL METHODS

5781 **Amylolytic Activity in Fruits: Comparison of Different Substrates and Methods Using Banana as Model**
*Priscila Z. Bassinello, Beatriz R. Cordenunsi, and Franco M. Lajolo**

5787 **Analysis of Xanthophylls in Corn by HPLC**
E. E. Moros, D. Darnoko, M. Cheryan, E. G. Perkins, and J. Jerrell*

5791 **Analysis of Fenbendazole Residues in Bovine Milk by ELISA**
David L. Brandon, Anne H. Bates, Ronald G. Binder, William C. Montague, Jr., Linda C. Whitehand, and Steven A. Barker*

5797 **Analysis of Ethyl Carbamate in Wines Using Solid-Phase Extraction and Multidimensional Gas Chromatography/Mass Spectrometry**
Eshwar Jagerdeo, Sumer Dugar, Gregory D. Foster, and Hilary Schenck*

5803 **Method for Determining Nitrogenous Heterocycle Compounds in Wine**
Harald Keim, Gilles de Revel, Stéphanie Marchand, and Alain Bertrand*

5808 **Detection of Potentially Allergenic Hazelnut (*Corylus avellana*) Residues in Food: A Comparative Study with DNA PCR-ELISA and Protein Sandwich-ELISA**
Thomas Holzhauser, Oliver Stephan, and Stefan Vieths*

5816 **Determination of Isoxaflutole (Balance) and Its Metabolites in Water Using Solid Phase Extraction Followed by High-Performance Liquid Chromatography with Ultraviolet or Mass Spectrometry**
Chung-Ho Lin, Robert N. Lerch, E. Michael Thurman, Harold E. Garrett, and Milon F. George*

5825 **Oil Stability Prediction by High-Resolution ^{13}C Nuclear Magnetic Resonance Spectroscopy**
*Francisco J. Hidalgo, Gemma Gómez, José L. Navarro, and Rosario Zamora**

5832 **Determination of Antioxidant Activity of Wine Byproducts and Its Correlation with Polyphenolic Content**
Ángeles M. Alonso, Dominico A. Guillén, Carmelo G. Barroso, Belén Puertas, and Alberto García*

Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

BIOACTIVE CONSTITUENTS

5837 **Antiproliferative Activities of Citrus Flavonoids against Six Human Cancer Cell Lines**
John A. Manthey and Najla Guthrie*

5844 **Antioxidant Activities and Antitumor Screening of Extracts from Cranberry Fruit (*Vaccinium macrocarpon*)**
*Xiaojun Yan, Brian T. Murphy, Gerald B. Hammond, Joe A. Vinson, and Catherine C. Neto**

5850 **2,2′-Diphenyl-1-picrylhydrazyl Radical-Scavenging Active Components from Adlay (*Coix lachryma-jobi* L. Var. *ma-yuen* Stapf) Hulls**
*Ching-Chuan Kuo, Wenchang Chiang, Guey-Ping Liu, Ya-Lin Chien, Jang-Yang Chang, Ching-Kuo Lee, Jir-Mehng Lo, Shou-Ling Huang, Ming-Chih Shih, and Yueh-Hsiung Kuo**

5856 **Simplified Extraction of Ergovaline and Peramine for Analysis of Tissue Distribution in Endophyte-Infected Grass Tillers**
*Martin J. Spiering, Elizabeth Davies, Brian A. Tapper, Jan Schmid, and Geoffrey A. Lane**

5863 **Recovery Mechanism of the Antioxidant Activity from Carnosic Acid Quinone, an Oxidized Sage and Rosemary Antioxidant**
Toshiya Masuda, Yuzuru Inaba, Tomomi Maekawa, Yoshio Takeda, Hirotoshi Tamura, and Hidemasa Yamaguchi*

5870 **Identification and Quantification of Antioxidant Components of Honeys from Various Floral Sources**
*Nele Gheldof, Xiao-Hong Wang, and Nicki J. Engeseth**

5878 **Chemical Characterization of Basil (*Ocimum basilicum* L.) Found in Local Accessions and Used in Traditional Medicines in Iran**
*Jamal Javanmardi, A. Khalighi, A. Kashi, H. P. Bais, and J. M. Vivanco**

5884 **Peroxynitrite Scavenging Activity of Sinapic Acid (3,5-Dimethoxy-4-hydroxycinnamic Acid) Isolated from *Brassica juncea***
*Yani Zou, Ae Ra Kim, Jung Eun Kim, Jae Sue Choi, and Hae Young Chung**

5891 **Antioxidant Capacity and Phenolic Content of Spinach As Affected by Genetics and Growing Season**
L. R. Howard, N. Pandjaitan, T. Morelock, and M. I. Gil*

5897 **3,4-Dihydroxymandelic Acid, a Noradrenalin Metabolite with Powerful Antioxidative Potential**
Jakob Peter Ley, Karin Engelhart, Jürgen Bernhardt, and Heinz-Jürgen Bertram*

5903 **Barley Lunasin Suppresses *ras*-Induced Colony Formation and Inhibits Core Histone Acetylation in Mammalian Cells**
*Hyung J. Jeong, Yi Lam, and Ben O. de Lumen**

5909 **Antioxidant Activities of Grape (*Vitis vinifera*) Pomace Extracts**
*Kotamballi N. Chidambara Murthy, Ravendra P. Singh, and Guddadarangavvanahally K. Jayaprakasha**

BIOTECHNOLOGY

5915 **Molecular Affinity and Permeability of Different Molecular Weight Chitosan Membranes**
*Xi-Guang Chen, Li Zheng, Zhen Wang, Chang-Yong Lee, and Hyun-Jin Park**

Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

CHEMICAL CHANGES DURING PROCESSING/STORAGE

5919 **Kinetics of Oxidative Degradation of White Wines and How They Are Affected by Selected Technological Parameters**
Antonio César Silva Ferreira, Paula Guedes de Pinho, Paula Rodrigues, and Timothy Hogg*

5925 **Induction of Antioxidant Flavonol Biosynthesis in Fresh-Cut Potatoes. Effect of Domestic Cooking**
*Juan A. Tudela, Emma Cantos, Juan C. Espín, Francisco A. Tomás-Barberán, and María I. Gil**

5932 **Controlled-Atmosphere Effects on Postharvest Quality and Antioxidant Activity of Cranberry Fruits**
*Gurbuz Gunes, Rui Hai Liu, and Christopher B. Watkins**

5939 **Extraction of Anthocyanins and Other Phenolics from Black Currants with Sulfured Water**
*J. E. Cacace and G. Mazza**

5947 **Polymerization Kinetics of Wheat Gluten upon Thermosetting. A Mechanistic Model**
Sandra Domenek, Marie-Hélène Morel, Joëlle Bonicel, and Stéphane Guilbert*

5955 **Extractives Content in Cooperage Oak Wood during Natural Seasoning and Toasting; Influence of Tree Species, Geographic Location, and Single-Tree Effects**
*Franck Doussot, Bernard De Jéso, Stéphane Quideau, and Patrick Pardon**

5962 **Influence of Thermal Treatments Simulating Cooking Processes on the Polyphenol Content in Virgin Olive Oil**
Manuel Brenes, Aranzazu García, M. Carmen Dobarganes, Joaquín Velasco, and Concepción Romero*

5968 **Recovery of Anthocyanins from Pulp Wash of Pigmented Oranges by Concentration on Resins**
*Alfio Di Mauro, Elena Arena, Biagio Fallico, Amedeo Passerini, and Emanuele Maccarone**

CHEMISTRY OF CROP AND ANIMAL PROTECTION

5975 **Chlorpyrifos Decline Curves and Residue Levels from Different Commercial Formulations Applied to Oranges**
Nicola Montemurro, Francesco Grieco, Giovanni Lacertosa, and Angelo Visconti*

COMPOSITION OF FOODS/FEEDS/BIOBASED PRODUCTS

5981 **Cholesterol Oxides, Cholesterol, Total Lipid, and Fatty Acid Composition in Turkey Meat**
*Sueli Regina Baggio, Eduardo Vicente, and Neura Bragagnolo**

5987 **Isoflavones in Soy-Based Foods Consumed in Brazil: Levels, Distribution, and Estimated Intake**
*Maria Inés Genovese and Franco M. Lajolo**

5994 **Variation in Diosgenin Levels among 10 Accessions of Fenugreek Seeds Produced in Western Canada**
Wesley G. Taylor, Holly J. Zulyniak, Ken W. Richards, Surya N. Acharya, Shabtai Bittman, and James L. Elder*

Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

5998 **Chemometrical Analysis of 18 Metallic and Nonmetallic Elements Found in Honeys Sold in France**
J. Devillers, J. C. Doré, M. Marenco, F. Poirier-Duchêne, N. Galand, and C. Viel*

6008 **Model Studies of Ferulate−Coniferyl Alcohol Cross-Product Formation in Primary Maize Walls: Implications for Lignification in Grasses**
John H. Grabber, John Ralph, and Ronald D. Hatfield*

ENVIRONMENTAL CHEMISTRY

6017 **Physicochemical Properties and Bacterial Resistance of Biodegradable Milk Protein Films Containing Agar and Pectin**
M. Letendre, G. D'Aprano, M. Lacroix, S. Salmieri, and D. St-Gelais*

FLAVORS AND AROMAS

6023 **Characterization of Volatiles in Costa Rican Guava [*Psidium friedrichsthalianum* (Berg) Niedenzu] Fruit**
Jorge A. Pino, Rolando Marbot, and Carlos Vázquez*

6027 **Reinvestigation of the Chemical Structure of Bitter-Tasting Quinizolate and Homoquinizolate and Studies on Their Maillard-Type Formation Pathways Using Suitable ^{13}C-Labeling Experiments**
*Oliver Frank and Thomas Hofmann**

FOOD CHEMISTRY/BIOCHEMISTRY

6037 **Phenylboronic Acid−a Potent Inhibitor of Lipase from *Oryza sativa***
*M. P. Raghavendra and V. Prakash**

6042 **Principal Component Similarity Analysis of Raman Spectra To Study the Effects of pH, Heating, and κ-Carrageenan on Whey Protein Structure**
*Nooshin Alizadeh-Pasdar, Shuryo Nakai, and Eunice C. Y. Li-Chan**

6053 **Isothermal Calorimetry Study of Calcium Caseinate and Whey Protein Isolate Edible Films Cross-Linked by Heating and γ-Irradiation**
*M. Letendre, G. D'Aprano, G. Delmas-Patterson, and M. Lacroix**

6058 **Warm Temperatures or Drought during Seed Maturation Increase Free α-Tocopherol in Seeds of Soybean (*Glycine max* [L.] Merr.)**
Steven J. Britz and Diane F. Kremer*

6064 **Influence of pH and Ionic Strength on Heat-Induced Formation and Rheological Properties of Soy Protein Gels in Relation to Denaturation and Their Protein Compositions**
*Jacoba M. S. Renkema, Harry Gruppen, and Ton van Vliet**

6072 **Antioxidant Properties of Several Medicinal Mushrooms**
Jeng-Leun Mau, Hsiu-Ching Lin, and Chin-Chu Chen*

6078 **Localization of Puroindoline-a and Lipids in Bread Dough Using Confocal Scanning Laser Microscopy**
Laurence Dubreil, Samares C. Biswas, and Didier Marion*

6086 **Fruit Flesh Betacyanin Pigments in *Hylocereus* Cacti**
Sławomir Wybraniec and Yosef Mizrahi*

Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

6090 **Application of a DNA Analysis Method for the Cultivar Identification of Grape Musts and Experimental and Commercial Wines of *Vitis vinifera* L. Using Microsatellite Markers**
*Eva García-Beneytez, María V. Moreno-Arribas, Joaquín Borrego, María C. Polo, and Javier Ibáñez**

6097 **Characterization of White Wine Mannoproteins**
Fernando Gonçalves, Alain Heyraud, Maria Norberta de Pinho, and Marguerite Rinaudo*

6102 **Olive Oils Improve Lipid Metabolism and Increase Antioxidant Potential in Rats Fed Diets Containing Cholesterol**
Shela Gorinstein, Hanna Leontowicz, Antonin Lojek, Maria Leontowicz, Milan Ciz, Ryszard Krzeminski, Mikolaj Gralak, Jan Czerwinski, Zenon Jastrzebski, Simon Trakhtenberg, Nuria Grigelmo-Miguel, Robert Soliva-Fortuny, and Olga Martin-Belloso*

6109 **Both Dioscorin, the Tuber Storage Protein of Yam (*Dioscorea alata* cv. Tainong No. 1), and Its Peptic Hydrolysates Exhibited Angiotensin Converting Enzyme Inhibitory Activities**
*Feng-Lin Hsu, Yaw-Huei Lin, Mei-Hsien Lee, Chien-Liang Lin, and Wen-Chi Hou**

6114 **Characterization of Free Radicals in Soluble Coffee by Electron Paramagnetic Resonance Spectroscopy**
Ederlinda C. Pascual, Bernard A. Goodman, and Chahan Yeretzian*

6123 **Subcellular Localization and Isoenzyme Pattern of Peroxidase and Polyphenol Oxidase in Beet Root (*Beta vulgaris* L.)**
Josefa Escribano, Fernando Gandía-Herrero, Nuria Caballero, and Maria Angeles Pedreño*

6130 **Statistical Differentiation of Bananas According to Their Mineral Composition**
*Markus Paul Forster, Elena Rodríguez Rodríguez, Jacinto Darias Martín, and Carlos Díaz Romero**

6136 **Effects of Different Origins and Harvesting Time on Vitamin C, Tocopherols, and Tocotrienols in Sea Buckthorn (*Hippophaë rhamnoides*) Berries**
Heikki Kallio, Baoru Yang, and Pekka Peippo*

6143 **Nonenzymatic Antioxidant Activity of Four Organosulfur Compounds Derived from Garlic**
Mei-chin Yin, San-wen Hwang, and Kung-chi Chan*

6148 **Cell Cytotoxicity and Mycotoxin and Secondary Metabolite Production by Common Penicillia on Cheese Agar**
Thomas Ostenfeld Larsen, Manfred Gareis, and Jens Christian Frisvad*

6153 **Thermal Inactivation of Pectin Methylesterase, Polygalacturonase, and Peroxidase in Tomato Juice**
Gordon E. Anthon, Yukio Sekine, Nobuo Watanabe, and Diane M. Barrett*

6160 **Possible Mechanism for Involvement of Cysteine in Aroma Production in Wine**
Stéphanie Marchand, Gilles de Revel, Joseph Vercauteren, and Alain Bertrand*

6165 **Rheological Properties of Yoghurt Made with Milk Submitted to Manothermosonication**
*Antonio Vercet, Rosa Oria, Pedro Marquina, Simon Crelier, and Pascual Lopez-Buesa**

Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

6172 **Color and Antioxidant Properties of Cyanidin-Based Anthocyanin Pigments**
Florian C. Stintzing, Angela S. Stintzing, Reinhold Carle, Balz Frei, and Ronald E. Wrolstad*

6182 **Antioxidant Activity of Grains**
*Kafui Kwami Adom and Rui Hai Liu**

6188 **Identification of Conjugated Linoleic Acids in Hydrogenated Soybean Oil by Silver Ion-Impregnated HPLC and Gas Chromatography-Ion Impacted Mass Spectrometry of Their 4,4-Dimethyloxazoline Derivatives**
Mun Yhung Jung and Mi Ok Jung*

6194 **Bound Malondialdehyde in Foods: Bioavailability of the *N*-2-Propenals of Lysine**
Julio Girón-Calle, Manuel Alaiz, Francisco Millán, Valentina Ruiz-Gutiérrez, and Eduardo Vioque*

6199 **Application Potency of Engineered G159 Mutants on P1 Substrate Pocket of Subtilisin YaB as Improved Meat Tenderizers**
Chuan-Mei Yeh, Ming-Chuai Yang, and Ying-Chieh Tsai*

6205 **In Vitro Fermentability of Differently Substituted Xylo-oligosaccharides**
*Mirjam A. Kabel, Linda Kortenoeven, Henk A. Schols, and Alphons G. J. Voragen**

6211 **Coffee Drinking Influences Plasma Antioxidant Capacity in Humans**
Fausta Natella, Mirella Nardini, Irene Giannetti, Cristina Dattilo, and Cristina Scaccini*

NUTRITIONAL ACTIVITY

6217 **Procyanidins from *Vitis vinifera* Seeds: In Vivo Effects on Oxidative Stress**
Paolo Simonetti, Salvatore Ciappellano, Claudio Gardana, Lorenzo Bramati, and Piergiorgio Pietta*

6222 **Mammalian Lignan Formation in Rats Fed a Wheat Bran Diet**
Catherine Nicolle, Claudine Manach, Christine Morand, Witold Mazur, Herman Adlercreutz, Christian Rémésy, and Augustin Scalbert*

6227 **In Vitro Anti-inflammatory and Anti-proliferative Activity of Sulfolipids from the Red Alga *Porphyridium cruentum***
*J. P. Bergé, E. Debiton, J. Dumay, P. Durand, and C. Barthomeuf**

6233 **Organic Acids Influence Iron Uptake in the Human Epithelial Cell Line Caco-2**
*Susan Salovaara, Ann-Sofie Sandberg, and Thomas Andlid**

6239 **Influence of Temperature and Ontogeny on the Levels of Glucosinolates in Broccoli (*Brassica oleracea* Var. *italica*) Sprouts and Their Effect on the Induction of Mammalian Phase 2 Enzymes**
Fernanda Maria Valente Pereira, Eduardo Rosa, Jed W. Fahey, Katherine K. Stephenson, Rosa Carvalho, and Alfredo Aires*

6245 **Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Wakame (*Undaria pinnatifida*) and Their Antihypertensive Effect in Spontaneously Hypertensive Rats**
Minoru Sato, Takao Hosokawa, Toshiyasu Yamaguchi, Toshiki Nakano, Koji Muramoto, Takashi Kahara, Katsura Funayama, Akio Kobayashi, and Takahisa Nakano*

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U.S. Patent No. 11,224,241

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Possible Mechanism for Involvement of Cysteine in Aroma Production in Wine

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Under conditions close to those of wine, that is, low pH, aqueous medium, and low temperatures, this work describes *N*-(2-sulfanylethyl)-2-oxopropanamide (**1**), a new intermediate in the formation of 2-acetylthiazole from methylglyoxal and cysteine. **1** was characterized by MS, derivatization MS, and ^{1}H and ^{13}C NMR and was synthesized from 2-sulfanylethanamine and ethyl pyruvate. A formation pathway for 2-acetylthiazole from methylglyoxal and cysteine is proposed, in which **1** is a new intermediate in Maillard-type reactions in systems under mild conditions.

KEYWORDS: ***N*-(2-Sulfanylethyl)-2-oxopropanamide; Maillard reaction; cysteine; 2-acetylthiazole; wine aroma**

INTRODUCTION

Maillard (*1*) was the first to observe a reaction between sugars and amino acids both at high temperatures and at 37 °C or below. At present, the term "Maillard reaction" describes a group of chemical reactions between amino and carbonyl functions present in food upon high-temperature treatment. According to Potman and Van Wijk (*2*), the Maillard reaction is responsible for the characteristic flavor and colors of many processed food products (baked, fried, or roasted).

Most of the odor products from the Maillard reaction are sulfur-, oxygen-, and nitrogen-containing heterocycles (*3*). Recently, Pripis-Nicolau et al. (*4*) identified some of these products in cysteine/α-dicarbonyl model solutions in mild conditions close to those of wines. Thereafter, we quantified these heterocycles in different wines (*5*). Among these molecules, we were especially interested in the formation of 2-acetylthiazole and 2-acetyl-2-thiazoline in cysteine and methylglyoxal wine-like solutions. 2-Acetylthiazole has been found in ground and canned beef (*6*, *7*), in boiled and baked potatoes (*8*), and in rice bran (*9*). 2-Acetyl-2-thiazoline was reported for the first time as a volatile constituent of beef broth (*10*). The compound was also identified in roast beef (*11*) and in overpasteurized beer (*12*). Later, Hofmann and Schieberle (*13*) described the formation and stability of 2-acetyl-2-thiazoline. These heterocycles are characterized by persistent roasted hazelnut notes, and their odor thresholds are very low (<5 μg/L in water) (*5*). The frequency of the occurrence of 2-acetylthiazole in wine is variable. Pomerol and Saint Emilion are the red wines with the highest mean levels (7 μg/L), whereas among the white wines, Burgundy, Alsace, and Champagne contain on average the highest concentrations (1.4−1.8 μg/L). Among the nitrogen-containing heterocyclic compounds with olfactory impact, 2-acetylthiazole is often encountered in foods. Moreover, it could play a role in wine flavor. Small quantities of 2-acetyl-2-thiazoline have also been detected in wines (*5*).

Maillard compounds with strong odor notes have long been thought to arise from simple amino acid degradation and subsequent interactions. Among these, the Strecker mechanism induces the degradation of cysteine, leading to the formation of the specific aldehyde. Griffith and Hammond (*14*) proposed a reaction pathway for the formation of 2-acetylthiazole from cysteine and methylglyoxal at conditions close to those of wine (25 °C, aqueous medium) with the exception of the pH (8 compared to 3.5 in wine). They suggested the condensation of the amino acid and the dicarbonyl to form 2-acetylthiazolidine, which is then oxidized twice into 2-acetylthiazole.

In wine-like solutions, we have found an as yet unknown compound when methylglyoxal and cysteine are presents. On the other hand, the same compound was detected at a higher level than that of 2-acetylthiazole during the degradation of 2-acetyl-2-thiazoline solution. We have used GC, GC-MS, and NMR to unambiguously determine the structure of this new molecule.

MATERIALS AND METHODS

Materials. Cysteine, methylglyoxal, thiazole, 2-acetylthiazole, and 2-acetyl-2-thiazoline were purchased from Sigma Aldrich Chemical Co. Inorganic reagents and solvents were all commercial products of analytical grade. All mixtures were prepared with aqueous ethanolic solution 12% volume with 4 g/L of tartaric acid and adjusted to pH 3.5 with 1 N H_2SO_4 and 1 N NaOH. 2-Acetyl-2-thiazoline solutions were 1 mM, and cysteine/methylglyoxal mixtures were 20 mM for each.

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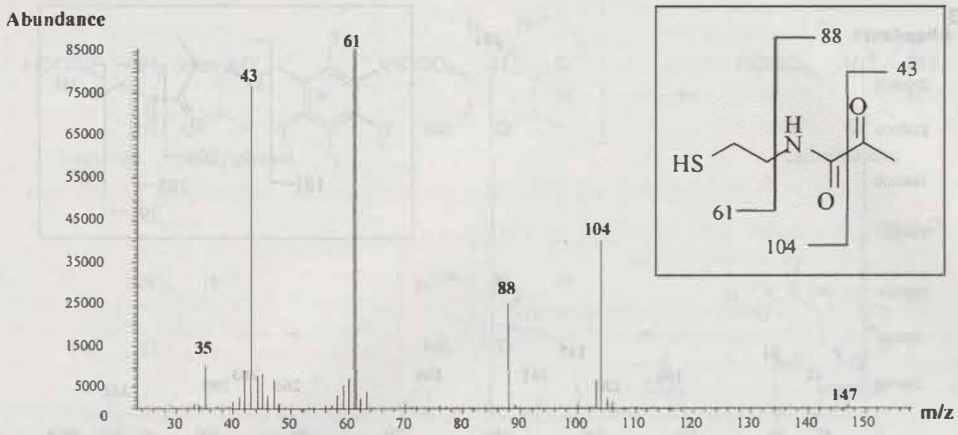

Figure 1. MS (EI) of *N*-(2-sulfanylethyl)-2-oxopropanamide.

Analytical Procedures. Reaction mixtures were analyzed by gas chromatography coupled with various detectors: FID, FPD, NPD, and mass spectrometry or by nose through an olfactometric system. A first gas chromatograph (Hewlett-Packard 5970) was coupled with an NPD, and separation was carried out with an HP5 column (50 m × 0.32 mm, 0.52 μm). A second gas chromatograph (Hewlett-Packard 5970) was coupled with an FID or a sniffing SDS system, and the same column was used. A third gas chromatograph (Hewlett-Packard 6890) was coupled with an FPD, and separations were carried out on an HP5 column (30 m × 0.58 mm, 5 μm) with hydrogen as a carrier gas.

All of the oven temperatures were programmed from 50 to 200 °C at a rate of 2 °C/min, the initial step lasting 1 min. The final isothermal time was 20 min.

GC-MS Analysis. A gas chromatograph (Hewlett-Packard 6890) was coupled with a mass spectrometer (HP 5972; electronic impact, 70 eV; eMV, 2.7 kV). The column was a BP1 (50 m × 0.32 mm, 0.2 μm). The oven temperature was programmed from 50 to 220 °C, the initial step lasting 1 min, at a rate of 2 °C/min to 200 °C and then at a rate of 5 °C/min to 220 °C, and the final step lasting 20 min. The carrier gas was helium (1.5 mL/min). The injector was a split/splitless system (splitless time was 30 s) and the split vent 30 mL/min. Detection was performed in the SCAN mode.

Derivatizations. Derivatization of sulfanyl groups was carried out by *p*-hydroxymercuribenzoic acid (*p*-HMB) (*15*) at 20 °C for 10 min at pH 10, to form a sulfanyl aryl mercuric derivative. Carbonyl functions were derivatized by reacting with perfluorobenzylhydroxylamine (PFBOA) at 20 °C for 1 h at pH 3 to form an oxime (*16*). Derivatization of α-dicarbonyl functions was done by *o*-diaminobenzene (DAB) at 60 °C for 3 h at pH 3 to form a quinoxaline (*17*).

Nuclear Magnetic Resonance Spectroscopy (NMR). 1D and 2D NMR experiments were performed on a spectrometer equipped with an inverse 5 mm broad-band probe at 400.13 and 100.6 MHz for ^{1}H and ^{13}C, respectively. All spectra were recorded using 2 mg of product dissolved in 0.5 mL of CF_3COOD-acidified (1%) H_2O-d_2 in a 5 mm tube. ^{1}H and ^{13}C chemical shifts were expressed in ppm relative to TMS. ^{1}H and ^{13}C chemical shifts were performed as previously described (*18*). NMR spectra were recorded on a Bruber DPX 400. H_2O-d_2 was used as solvent and TSP as the internal standard.

***N*-(2-Sulfanylethyl)-2-oxopropanamide Synthesis.** A chloroform solution (10 mL of chloroform and 1.2 g of 2-sulfanylethanamine) was stirred with an aqueous solution (10 mL of water and 0.5 g of NaOH). Then the mixture was stirred for 1.5 h at ~58 °C. Next, 1.16 g of ethyl pyruvate in tetrahydrofuran (THF; 10 mL) was dripped into the mixture. The resulting solution was further refluxed for 1.5 h. After the reaction was finished, an aliquot of the oil was dissolved in chloroform for GC-MS analysis.

RESULTS

In cysteine/methylglyoxal wine-like solutions, we have never detected the presence of 2-acetylthiazolidine or any specific aldehyde, but we have found an as yet unknown compound. On the other hand, the same compound was detected at a higher level than that of 2-acetylthiazole during the degradation of 2-acetyl-2-thiazoline solution. Now, we have unambiguously determined the structure of this new molecule.

Table 1. Derivatizations of 1 Chemical Functions

reactive	function	product	result
p-hydroxymercuribenzoic acid	sulfanyl	organometallic complex	+
pentafluorobenzylhydroxylamine	carbonyl	oxime	+
o-diaminobenzene	α-dicarbonyl	quinoxaline	+

Mass Spectrometry. When 2-acetyl-2-thiazoline was dissolved in aqueous alcoholic solution at 1 mM, an unknown compound appeared. By comparison of retention indexes and mass spectra, the unknown compound was sought and observed in cysteine and methylglyoxal model solutions at 20 mM. The GC retention indexes calculated on the HP5 and BP21 columns were 1151 and 1168, respectively. Four experienced researchers in olfactometry attributed the descriptors "roasted" and "rubber" to the odorous zones. Mass spectrometry in the SCAN mode showed fragmentation ions arising from the molecule (**Figure 1**). The ion at *m/z* 43 was characteristic of an acetyl group. The *m/z* ratio of the molecular ion was 147. On the other hand, the specific detectors FPD and NPD showed that the molecule contained nitrogen and sulfur atoms. Because the ion at *m/z* 147 was the molecular ion, the molecule contained an odd number of nitrogen atoms, according to the nitrogen rule. Therefore, the $C_5H_9NSO_2$ formula was proposed.

To determine the chemical functions carried by the molecule, we performed a series of specific derivatizations. The reagents used, the functions observed, and the products formed are presented in **Table 1**. Finally, mass spectrometry of the adducts led us to propose the structure of *N*-(2-sulfanylethyl)-2-oxopropanamide (**1**). This was the case, for example, for the oxime resulting from the derivatization of **1** by PFBOA and for the quinoxaline from derivatization of **1** by DAB. The spectra and fragments are presented in **Figure 2**. In addition to the pentafluorotropylium ion at *m/z* 181, we found ions at *m/z* 295, 283, and 266 in the oxime spectrum that are characteristic of the loss of sulfanylmethyl, 2-sulfanylethenyl, and *N*-(2-sulfanylethyl) radicals. The quinoxaline fragment ions are also characteristic of the loss of sulfanyl, sulfanylmethyl, and 2-sulfanylethenyl radicals, thus confirming the structure of **1**.

NMR Spectra. Figure 3 displays the ^{1}H NMR spectrum of **1**. The signal at 1.70 ppm, which integrates for three protons, is characteristic of an isolated methyl group. Each of the four ddd signals, centered, respectively, at 2.9, 3.1, 3.6, and 3.8 ppm,

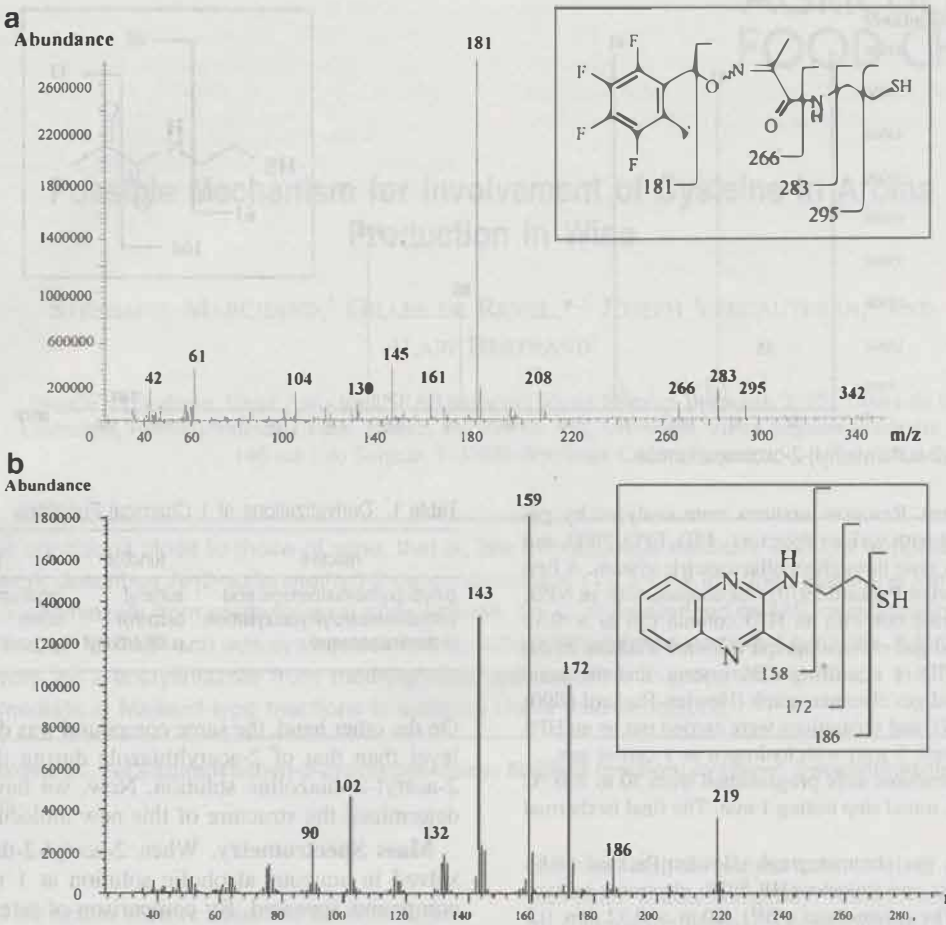

Figure 2. (a) Mass spectrum of PFBOA–[*N*-(2-sulfanylethyl)-2-oxopropanamide] derivative. (b) Mass spectrum of DAB–[*N*-(2-sulfanylethyl)-2-oxopropanamide] derivative.

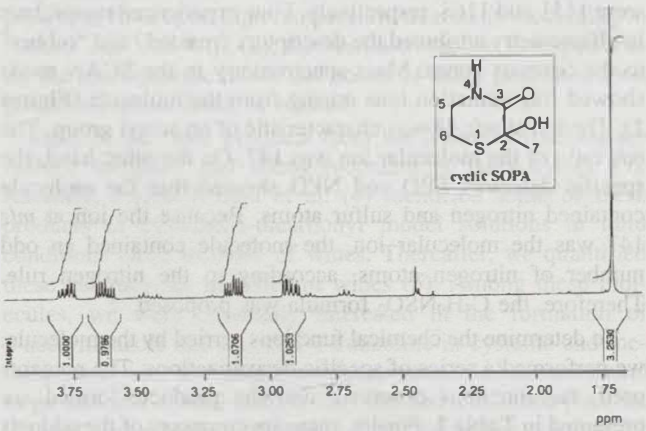

Figure 3. ^{1}H NMR spectrum of *N*-(2-sulfanylethyl)-2-oxopropanamide.

is integrated for one proton and corresponds to the four protons of an ethane chain. However, such diastereotopic protons can be explained only by a cyclic structure. Indeed, the ^{13}C NMR spectrum shows four peaks between 25 and 76 ppm and only one signal at low field attributed to the lactam carbonyl. One-bond and long-range ^{1}H−^{13}C reverse heteronuclear chemical shift correlations (HMQC and HMBC) made it possible to unambiguously assign all the resonances (**Table 2**): the carbon of the methyl group is responsible for the peak at 26.9 ppm; the carbons of the two methylenic groups are responsible for the peaks at 24.9 and 43.5 ppm; the peak at 76.8 ppm is due to the hemi-thioketal carbon atom; finally, the quaternary carbonyl group is responsible for the peak at 172.3 ppm. Therefore, this downfield signal is in favor of a cyclic structure for **1** (only one carbonyl and a quaternary carbon alcohol).

Table 2. Assignment of ^{1}H and ^{13}C NMR Signals

C no.	δ ^{13}C	δ ^{1}H (multiplicity)	HMBC
2	76.8 (C)		H-1, H-5
3	172.3 (C)		
5	43.5 (CH_2)	3.6 (ddd) 3.8 (ddd)	H-1
6	24.9 (CH_2)	2.9 (ddd) 3.1 (ddd)	H-2
7	26.8 (CH_2)	1.7 (s)	H-5

Chemical synthesis of **1** was performed, and the reaction products were analyzed by GC-MS. Retention times and fragmentation spectra were compared with those of **1**, thus confirming the structure.

DISCUSSION

Heterocyclic compounds, especially sulfur ones, may play a major role in the aroma of many processed products (*19*). The pathways for such heterocycle formations have been proposed as part of the Maillard reaction (*14*, *20*). The first step is Strecker degradation. It often involves the formation of a Schiff base, leading to the degradation of the amino acids to their corresponding aldehydes (*21*−*23*). Simultaneously, hydrogen sulfide reacts with the breakdown products of sugars (*24*, *25*). Because these reactions are often run at basic or neutral pH and at quite high temperature (>100 °C), they are efficient and rapid. Although our wine conditions are quite different, the reaction products are close to Maillard reaction adducts.

Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

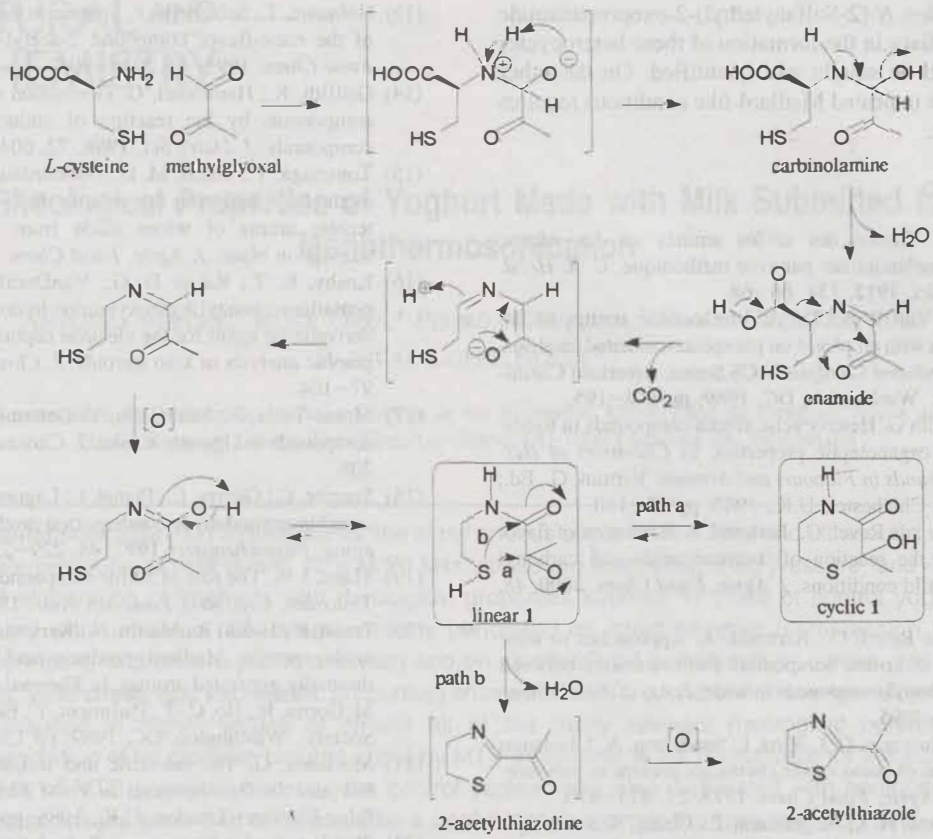

Figure 4. Proposed pathway leading from cysteine and methylglyoxal to 2-acetylthiazole through the new intermediate *N*-(2-sulfanylethyl)-2-oxopropanamide.

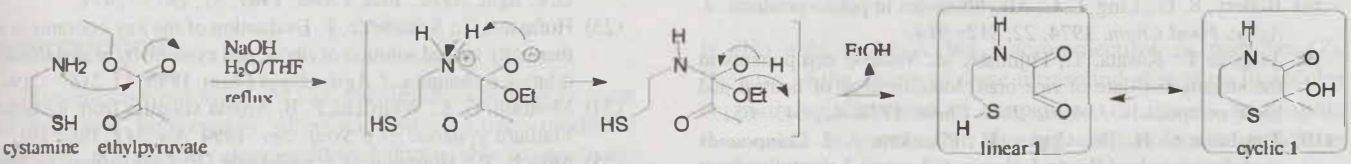

Figure 5. Chemical correlation of *N*-(2-sulfanylethyl)-2-oxopropanamide by direct synthesis.

We were unable to isolate a Schiff base, but we did isolate **1**, a possible intermediary between, on the one hand, cysteine and methylglyoxal, and, on the other hand, the thiazole-type heterocycles. **Figure 4** suggests the formation pathway of 2-acetylthiazole from cysteine and methylglyoxal in aqueous and acidic conditions (wine-type conditions). The pathway starts by the formation of a carbinol amine, which is dehydrated under acidic conditions to form an imine. The conjugation between the imine and the carbonyl functions (enamide) is the driving force that best explains the loss of the carboxyl group. The resulting decarboxylated enamide undergoes an oxidation reaction that ends up as a stable keto-amide. The oxidation step can occur in many ways, because it is not a major phenomenon but only the transformation of a tiny amount of the starting material. For instance, it could be the result of a dismutation with the starting aldehyde (present in a large excess) or with dioxygen dissolved in the solvents. Another pathway could be the cyclization of the imino-ketone to give 2-acetylthiazolidine, which is rapidly oxidized into 2-acetyl-2-thiazoline, and then the hydrolysis of 2-acetyl-2-thiazoline leading to **1**. This would involve the loss of conjugation between unsaturated bonds (imine and carbonyl). Moreover, 2-acetylthiazolidine has never been detected in solutions, so the pathway involving oxidation of the imino-ketone seems to be the most plausible. Further investigations on this point are required. The linear form of **1** gains in stability by the formation of a six-membered cycle after the nucleophilic attack of the sulfur on the ketone group (path a). To prove that **1** really is a compound resulting from two sequential events (first, a decarboxylation, and, second, an oxidation step), we tried to synthesize it directly from chemicals not bearing the carboxyl group (cystamine, rather than cysteine) and at the right number of oxidation (ethyl pyruvate instead of methylglyoxal). In such a case, the isolated compound was mainly the cyclic form of **1** (**Figure 5**) even though yields were relatively low, due to the concurrent reactivity of the amine with the ketone of pyruvate.

Alternatively, the linear form of **1** may close either directly or after reopening of the cyclic one, due to the attack of the sulfur on the carboxamide (path b, **Figure 4**), thereby leading to the even more stable five-membered ring (2-acetylthiazoline). Once formed, this thiazoline undergoes an easy oxidation step (in the same conditions as above) and yields the acetylthiazole, a compound that is formed naturally, even if resulting from many events, due to the low energy of this aromatic ring.

Whatever its conformation, **1** may be considered to be involved in the formation of sulfur- and nitrogen-containing heterocycles under "wine conditions". This compound could play the role of the Schiff base in a mechanism comparable to Strecker degradation but at low temperature, with acid pH and in an aqueous medium.

These data throw light on the involvement of **1** in the reaction mechanisms leading to the formation of very odorous hetero-

Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

cycles such as thiazoles. *N*-(2-Sulfanylethyl)-2-oxopropanamide could be an intermediary in the formation of these heterocycles in wines, so it could be sought and quantified. On the other hand, its possible role in heated Maillard-like conditions requires further investigation.

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