statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: December 3, 2016

Keith Cadwallader

EXHIBIT A

Keith R. Cadwallader

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Education

University of Florida, Gainesville, FL	Fall, 1990	Ph.D. Food Science (Chem. minor)
University of Florida	Summer. 1987	M.S. Food Science
University of Georgia, Athens, GA	Spring, 1985	B.S. Food Science

Professional Experience

2007-present. Professor of Food Chemistry, Department of Food Science & Human Nutrition, University of Illinois. **Research Interests:** Chemistry and analysis of food flavor. Food flavor (aroma) characterization and flavor quality determination. Use of instrumental and sensory methods to identify and quantify character-impact flavor compounds in foods/agricultural/industrial materials. Development of process flavors. Recovery of flavor constituents from food processing by-products. Measurement and prediction of flavor diffusion, release and binding. Development of methods to stabilize labile flavors.
1999-2007. Associate Professor of Food Chemistry, University of Illinois.
1996-1999. Associate Professor of Food Science, Food Science & Technology, Mississippi State Univ.
1994-1996. Assistant Professor of Food Science, Mississippi State University
1991-1994. Assistant Professor of Food Science, Dept. Food Science, Louisiana State University.
1985-1990. Pre-doctoral Research Fellow, Dept. Food Science & Human Nutrition, Univ. Florida.

Current Appointment (UIUC)

Research – 60%; Teaching – 40%

Awards/Honors

- 2016 College of ACES Senior Faculty Award for Excellence in Research, University of Illinois,
- 2010 Elected Fellow, Agricultural and Food Chemistry Division of the American Chemical Society.
- Co-recipient, 2008 Donald A. Holt Achievement Award, Illinois Council on Food and Agricultural Research, presented to the Illinois Center for Soy Foods leadership team.
- 2007 Stephen S. Chang Award for Excellence in Lipid or Flavor Science, Institute of Food Technologists.
- Co-recipient, 2007 College of ACES Team Award for Excellence, University of Illinois, presented to the Illinois Center for Soy Foods leadership team
- Co-recipient, 2005 Bor S. Luh International Award, Institute of Food Technologists
- UIUC Incomplete list of teachers rated as excellent (Spr00, Fall00, Fall01, Spr03, Spr04, Spr05, Fall05; Spr06; Spr08; Spr10; Fall12; Fall14)
- 1996 Alpha Zeta Outstanding Teaching Award
- 1985-1990 FMC Corp. Research Fellowship Recipient
- 1984 U. Georgia Food Science Department Faculty Scholarship Recipient

Professional Memberships

Institute of Food Technologists & IFT, Chicago Section
American Chemical Society & ACS, Division of Agricultural and Food Chemistry

Teaching Interests and Experience

Teaching interests include food analysis, food chemistry, flavor chemistry and analysis, and sensory evaluation of foods. Courses taught are listed below.

University of Illinois

FSHN 418, *Food Analysis*: *2 h lecture: 6 h lab.* Principles and application of the chemical, physical, and instrumental methods used to determine the constituents of foods; special considerations applicable to the analysis of certain foods.

FSHN 519, Flavor Chemistry and Analysis: *2 h lecture: 6 h lab.* a graduate level course that involves the discussion/demonstration of the chemistry, manufacture/delivery and analysis (sensory, instrumental, and physical) of food flavor.

Mississippi State University

FST 4114/6114 *Analysis of Food Products*: *2 h. lecture; 6 h lab.* Principles, methods and techniques for quantitative, physical, chemical, and/or biological analysis of food products and ingredients.

FST 4241/6241 *Applied Food Chemistry*: *2 h lab.* Basic laboratory experiments to provide understanding of the function and interactions of chemical components of food.

FST 8163 *Flavor and Food Acceptance*: *2 h lecture; 1 h demonstration.* Theory and application of sensory evaluation and analytical chemistry techniques for the analysis of food flavor.

Louisiana State University

FDSC 4050 *Food Composition and Analysis*: *2 h. lecture; 6 h lab.* Principles, methods and techniques for quantitative, physical, chemical, and/or biological analysis of food products and ingredients.

FDSC 7040 *Flavor and Colors of Foods*: *2 h lecture; 3 lab.* Methods of chemical, physical, and instrumental analysis of food colors and flavors; natural and synthetic flavorings and colorants.

Research Publications

Books Edited (5)

Cadwallader, K.R. and Chang, S.K.C. (Eds.). 2010. *Chemistry, Texture and Flavor of Soy.* ACS Symposium Series 1059, American Chemical Society, Washington, DC.

Cadwallader, K.R., Drake, M.A. and McGorrin, R.J. (Eds.) 2007. *Flavor of Dairy Products.* ACS Symposium Series 971, American Chemical Society, Washington, DC.

Cadwallader, K.R. and Weenen, H. (Eds.). 2003. *Freshness and Shelf Life of Foods.* ACS Symposium Series 836, American Chemical Society, Washington, DC.

Rouseff, R.L. and Cadwallader, K.R. (Eds.). 2001. *Headspace Analysis of Foods and Flavor: Theory and Practice.* Kluwer Academic/Plennum Publishers, New York.

Shahidi, F. and Cadwallader, K.R. (Eds.). 1997. *Flavor and Lipid Chemistry of Seafoods.* ACS Symposium Series 674. American Chemical Society, Washington, DC.

Patent Applications (1)

Cadwallader, K.R. and Fang, M.-C. 2014. Stabilized compositions and methods of manufacture. US Patent Application US 2014/0275543 A1.

Book Chapters (57)

Cadwallader, K.R. 2016. Flavor challenges in functional beverages. Chapter 3. In *Handbook of Functional Beverages and Human Health*. Alasalvar, C. and Shahidi, F. (Eds.), CRC Press, pp. 27-34.

Yin, Y., Cadwallader, K.R. and Fang, M.-C. 2015. A novel method for the stabilization of the potent odorant 2-acety-1-pyrroline. In *Flavour Science: Proceedings of the XIV Weurman Flavour Research Symposium*, Taylor, A.J. and Mottram, D.S (Eds.), Context Products Ltd, Leicestershire, UK, pp. 571-574.

Burnside, E. and Cadwallader, K.R. 2014. Aroma components of commercial and self-prepared rum ethers. In *Current Topics in Flavor Chemistry and Biology: Proceedings of the 9th Wartburg Symposium on Flavor Chemistry & Biology*, T. Hofmann, W. Krautwurst, D., and Schieberle, P. (Eds.), Eigenverlag Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany, pp. 244-249.

Moran, L., Yin, Y. Cadwallader, K.R. and Padua, G.W. 2014. Testing tools and physical, chemical, and microbiological characterization of microencapsulated systems. In *Microencapsulation in the Food Industry: A Practical Implementation Guide*. Gaonkar, A., Vasisht, N, Khare, A. and Sobel, R. (Eds.). Academic Press/Elsevier Inc., Oxford, UK, pp. 323-352.

Lorjaroenphon, Y., Hausch, B.J. and Cadwallader, K.R. 2014. Formation and stability of 2,3-dehydro-1,8-cineol in a model carbonated beverage system. In *Flavor Science: Proceedings from the XIII Weurman Flavour Research Symposium*, Elsevier, Inc. Oxford, UK.

Fang, M. and Cadwallader, K.R. 2012. Improved synthesis of deuterium-labeled alkyl pyrazines for use in stable isotope dilution analysis. In *Recent Advances in the Analysis of Foods and Flavor*. Mussinan, C. et al. (Eds.). ACS Symposium Series 1098, American Chemical Society, Washington, DC, pp. 57-69.

Lahne, J. and Cadwallader, K.R. 2012. Streamlined analysis of potent odorants in distilled alcoholic beverages. In *Chemistry and Flavor of Alcoholic Beverages.* Qian, M. et al., (Eds.). ACS Symposium Series 1104, American Chemical Society, Washington, DC, pp. 37-53.

Cadwallader, K.R., Potts, D., Brisske-BeVier, L. and Mirarefi, S. 2011. Contribution of volatile sulfur compounds to the characteristic aroma of roasted garlic. In *Volatile Sulfur Compounds in Food.* Qian, M.C., Fan, X. and Mahattanatawee, K. (Eds.). ACS Symposium Series 1068, American Chemical Society, Washington, DC, pp. 137-151.

Genthner, L.R. and Cadwallader, K.R. 2011. Structure-odor relationships of selected esters with (Z,Z)-3,6-nonadiene backbone structures. In *Advances and Challenges in Flavor Chemistry & Biology: Proceedings of the 9th Wartburg Symposium on Flavor Chemistry & Biology*, T. Hofmann, W. Meyerhof, P. Schieberle, Eds., Eigenverlag Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany, pp. 123-128.

Kim, H.K. and Cadwallader, K.R. 2011. Instrumental analysis of seafood flavor. In *Handbook of Seafood Quality, Safety and Health Applications.* Alasalvar, C., Shahidi, F., Miyashita, K. and Wanasundara, U. (Eds.). Blackwell Publishing Ltd., UK, pp. 50-67.

Cadwallader, K.R., Moore, J.J., Zhang, Z. and Schmidt, S.J. 2010. Comparison of spray drying and Refractance Window™ drying technologies for the encapsulation of orange oil. In *Recent Advances in Food and Flavor Chemistry: Food Flavors and Encapsulation, Health Benefits, Analytical Methods, and Molecular Biology of Functional Foods.* Ho C.-T., Mussinan, C.J., Shahidi, F. and Tratras Contis, E. (Eds.). The Royal Society of Chemistry, Cambridge, UK, pp. 246-254.

Sun, C., Cadwallader, K.R. and Kim, H. 2010. Comparison of key aroma components between soymilks prepared by cold and hot grinding methods. In *Chemistry, Texture and Flavor of Soy.* Cadwallader, K.R. and Chang, S.K.C. (Eds.). ACS Symposium Series 1059, American Chemical Society, Washington, DC, pp. 361-373.

Suppavorasatit, I. and Cadwallader, K.R. 2010. Flavor-soy protein interactions. In *Chemistry, Texture and Flavor of Soy.* Cadwallader, K.R. and Chang, S.K.C. (Eds.). ACS Symposium Series 1059, American Chemical Society, Washington, DC, pp. 339-359.

Cadwallader, K.R., Tamamoto, L.C. and Sajuti, S.C. 2010. Aroma components of fresh and stored pomegranate (*Punica granatum* L.) juice. In *Flavors in Noncarbonated Beverages.* Da Costa, N.C. and Cannon, R.J. (Eds.). ACS Symposium Series 1036. American Chemical Society, Washington, DC, pp. 93-101.

Liu, G.-L. and Cadwallader, K.R. 2010. Aroma-impact components of "Carlos" muscadine grape juice. In *Flavor and Health Benefits of Small Fruits.* Qian, M.C. and Rimando, A.M. (Eds.). ACS Symposium Series 1035, American Chemical Society, Washington, DC, pp. 63-77.

Cadwallader, K.R. and Singh, T. 2009. Flavours and off-flavours in milk and dairy products. In *Advanced Dairy Chemistry-Volume 3. Lactose, Water, Salts and Minor Constituents.* McSweeny, P.L.H. and Fox, P.F. (Eds). Springer Science+Business Media, LLC, New York, pp. 631-690.

Cadwallader, K.R. and Puangpraphant, S. 2008. Volatile compounds in tree nuts. In Tree Nut s: Composition, Phytochemicals, and Health Effects. Alasalvar, C. and Shahidi, F. (Eds.) Taylor and Francis, LLC, Boca Raton, FL, pp. 109-125.

Singh, T. and Cadwallader, K.R. 2008. Cheese. In *Dairy Processing and Quality Assurance.* Chandan, R.C., Kilara, A. and Shah, N. (Eds.), Wiley-Blackwell, Danvers, MA, pp. 273-308.

Lorjaroenphon, Y., Cadwallader, K.R., Kim, H. and Lee, S-Y. 2008. Evaluation of key odorants in chipotle pepper by quantitative analysis, calculation of odor activity values and omission studies. In *Recent Highlights in Flavor Chemistry and Biology: Proceedings of the 8th Warburg Symposium on Flavor Chemistry & Biology,* Hofmann, T., Meyerhof, W. and Schieberle, P. (Eds.). Deutche Forschungsanstalt für Lebensmittelchemie, Garching, Germany, pp. 191-200.

Zhou, Q. and Cadwallader, K.R. 2008. Measurement of flavor-soy protein interactions in low-moisture solid food systems by inverse gas chromatography. Ch. 4. In *Food Flavor: Chemistry, Sensory Evaluation and Biological Activity.* Tamura, H., Ebeler, S.E., Kubota, K. and Takeoka, G.R. (Eds.). ACS Symposium Series 988. American Chemical Society, Washington, DC., pp. 45-54.

Cadwallader, K.R. 2007. Wood smoke flavor. In *Handbook of Meat, Poultry & Seafood Quality.* Nollet, L.M.L. (Ed.) and Boylsten, T., Chen, F. Coggins, P.C., Gloria, M.B., Hyldig, G., Kerth, C.R., McKee, L.H., Hui, Y.H. (Assoc. Eds.), Blackwell Publishing, Inc., Lancaster, PA, pp. 201-210.

Cadwallader, K.R. 2007. Measuring cheese flavour. In *Improving the Flavour of Cheese.* Weimer, B. (Ed.), Woodhead Publishing Ltd., Cambridge, UK, pp. 401-412.

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Singh, T.K., Cadwallader, K.R. and Drake, M.A. 2007. Flavor of dairy products. In *Handbook of Food Products Manufacturing: Health, Meat, Milk, Poultry, Seafood, and Vegetables.* Hui, Y.H. (Ed.), John Wiley & Sons, Inc., Hoboken, NJ, pp. 715-748.

Drake, M.A., Miracle, R.E., Caudle, A.D. and Cadwallader, K.R. 2007. Relating sensory and instrumental analyses. In *Sensory-Directed Flavor Analysis.* Marsili, R. (Ed.), CRC Press/Taylor & Francis Group, LLC, Boca Raton, FL, pp. 23-54.

Cadwallader, K.R., Gnadt, T.A. and Jasso, L. 2007. Aroma components of chipotle peppers. In *Chemistry and Flavor of Hispanic Foods.* Tunick, M. and Gonzales de Mejia, E. (Eds), ACS Symposium Series 946, American Chemical Society, Washington, DC, pp. 57-66.

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Cadwallader, K.R., Drake, M.A., Carunchia-Whetstine, M.E. and Singh, T.J. 2006. Characterisation of Cheddar cheese flavour by sensory directed instrumental analysis and model studies. In *Flavour Science: Recent Trends.* Bredie, W.P. and Peterson, M.A. (Eds.), Developments in Food Science 43, Elsevier, New York, pp. 157-160.

Jarunrattanasri, A., Cadwallader, K.R., Theerakulkait, C. 2005. Aroma and amino acid composition of hydrolyzed vegetable protein from rice bran. In *Process and Reaction Flavors*, Weerasinghe, D.K. (Ed.), ACS Symposium Series 905, American Chemical Society, Washington, DC, pp. 83-97.

Cadwallader, K.R., Benitez, D., Pojjanapimol, S., Syriyaphan, O. and Singh, T. 2005. Characteristic aroma components of the cilantro mimics. In. *Natural Flavors and Fragrances*, Frey, C. and Rouseff, R. (Eds.), ACS Symposium Series 908, American Chemical Society, Washington, DC, pp. 117-128.

Cadwallader, K.R. 2005. Flavor and volatile metabolism in produce. Chapter 6. In *Produce Degradation: Pathways and Prevention,* Lamikanra, O., Imam, S.H. and Ukuku, D.O. (Eds.), CRC Press LLC, Boca Raton, FL, pp 155-189.

Pojjanapimol, S., Chaiseri, S. and Cadwallader, K. 2004. Heat-induced changes in aroma components of holy basil (*Ocimum sanctum* L.). Ch. 15. In *Handbook of Flavor Characterization: Sensory analysis, chemistry, and physiology*, Diebler, K.D. and Delwiche, J. (Eds.), Marcel Dekker, Inc., New York, pp. 217-230.

Singh, T.K. and Cadwallader, K.R. 2004. Ways of measuring shelf-life and spoilage. Ch. 9. In *Understanding and Measuring the Shelf-life of Food*, Steele, R. (Ed.). CRC Press LLC, Boca Raton, FL, pp. 165-183.

Cadwallader, K.R., Lopez, J.R., Menke, S.D. and Surakarnkul, R. 2003. Aroma components of wines from Chardonel - A French-American hybrid grape. In *Nutraceutical Beverages. Chemistry, Nutrition, and Health Effects*, Shahidi, F. and Weerasinghe, D.K. (Eds.), ACS Symposium Series 871. American Chemical Society, Washington, DC, Chapter 27.

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Cadwallader, K.R. 2001. Flavor chemistry of saffron. In *Carotenoid-derived Aroma Compounds.* Winterhalter, P. and Rouseff, R.L. (Eds.). ACS Symposium Series 802, American Chemical Society, Washington, DC, pp. 220-239.

Cadwallader, K.R. and Heo, J. 2001. Aroma of roasted sesame oil: Characterization by direct thermal desorption-gas chromatography-olfactometry and sample dilution analysis. In *Gas Chromatography-Olfactometry: The State of the Art.* Leland, J.V.; Schieberle, P.; Buettner, A.; Acree, T.E. (Eds.). ACS Symposium Series 782; American Chemical Society, Washington, DC, pp. 187-202.

Cadwallader, K.R. and Shahidi, F. 2001. Identification of potent odorants in seal blubber oil by direct thermal desorption-gas chromatography-olfactometry. In *Omega-3 Fatty Acids: Chemistry, Nutrition, and Health Effects.* Shahidi, F. and Finley, J.W. (Eds.) ACS Symposium Series 788; American Chemical Society, Washington, DC, pp. 221-234.

Rouseff, R. and Cadwallader K. 2001. Headspace techniques in foods, fragrances and flavors. In *Headspace Analysis of Foods and Flavor: Theory and Practice.* Rouseff, R.L. and Cadwallader, K.R., (Eds.). Advances in Experimental Medicine and Biology, Volume 488, Kluwer Academic/Plennum Publishers, New York, pp. 1-8.

Webb, T.E. and Cadwallader, K.R. 2001. Aroma components of an oil-based grill flavoring by direct thermal desorption-gas chromatography-olfactometry and sample dilution analysis. In *Headspace Analysis of Foods and Flavor: Theory and Practice.* Rouseff, R.L. and Cadwallader, K.R., (Eds.). Kluwer Academic/Plennum Publishers, New York, pp. 143-150.

Cadwallader, K.R. 2000. Flavor recovery from crustacean processing by-products. In *Seafood in Health and Nutrition. Transformation in Fisheries and Aquaculture: Global Perspectives.* Shahidi, F. (Ed.). ScienceTech Publishing Co., St. John`s, Canada, Ch. 12, pp. 159-168.

Cadwallader, K.R. 2000. Enzymes and flavor biogenesis in fish. In *Seafood Enzymes.* Haard, N.F. and Simpson, B.K. (Eds.). Marcel Dekker, Inc., New York, pp. 365-383.

Cadwallader, K.R., Surakarnkul, R., Yang, S.-P., and Webb, T. 1999. Character-impact aroma components of coriander (*Coriandrum sativum*) herb. In *Flavor and Chemistry of Ethnic Foods.* Shahidi, F. and Ho, C.-T. (Eds.). Plenum Publishing Corporation, New York.

Cadwallader, K.R. and Baek, H.H. 1998. Aroma-impact components in cooked tailmeat of freshwater crayfish (*Procambarus clarkii*). In *Food Flavors: Formation, Analysis and Packaging Influences*. Contis, E.T., Ho, C.T.; Mussinan, C.J., Parliment, T.H., Shahidi, F., Spanier, A.M. (Eds.), Elsevier Science B.V., Amsterdam.

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Baek, H.H. and Cadwallader, K.R. 1997. Character-impact aroma components of crustaceans. In *Flavor and Lipid Chemistry of Seafoods,* Shahidi, F. and Cadwallader, K.R. (Eds.). ACS Symposium Series. 674. American Chemical Society, Washington, DC, pp. 85-94.

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Journal Articles (96)

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Kelly, L. and Cadwallader, K.R. 2016. Identification and quantitation of potent odorants in spearmint oil. J. Agric. Food Chem. (ubmitted).

Donovan J, Cadwallader K, Lee Y, 2016. Volatile retention and morphological properties of microencapsulated tributyrin varied by wall material and drying method. J. Food Sci. 81: E643-E650.

Suteebut, N., Chanthachum, S., Intarapichet, K., Cadwallader, K.R. and Miller, M.J. 2016. Factors affecting conjugated linoleic acid production by *Lactobacillus plantarum* GSI 303. Intern. Food Res. J. 23: 1739-1746.

Erten, E.S and Cadwallader, K.R. 2017. Identification of predominant aroma components of raw, dry roasted and oil roasted almonds. Food Chem. 217:244-253.

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Thompson-Witrick, K.A., Rouseff, R.L., Cadwallader, K.R., Duncan, S.E., Eigel, W.N., Tanko, J.M. and O'Keefe, S.F. 2015. Comparison of two extraction techniques, solid-phase microextraction versus continuous liquid-liquid extraction/solvent-assisted flavor evaporation, for the analysis of flavor compounds in gueuze lambic beer. J. Food Sci. 80: C571-C576.

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Hausch, B.H., Lorjaroenphon, Y. and Cadwallader, K.R. 2015. Flavor chemistry of lemon-lime carbonated beverages. J. Agric. Food Chem. 63: 112-119.

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Yang, X., Lorjaroenphon, Y., Cadwallader, K.R., Wang, X., Zhang, Y., Lee, J. 2014. Analysis of particle-borne odorants emitted from concentrated animal feeding operations. Sci. Total Environment 490, 322-333.

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Invited Talks

Cadwallader, K.R., Yin, Y., Hausch, B. and Chen, F. 2016. Assuring accuracy in the quantitation of the unstable odorant 2-acetyl-1-pyrroline in aromatic rice. ACS Symposium: *Flavor Stability: Chemical Changes in Flavor Molecules, Food-Flavor Matrix Interactions, Flavor Encapsulation.* 252nd Americal Chemical Society Meeting. Philadelphia, PA, August 21-25.

Cadwallader, K.R. 2016. Occurrence, formation and stabilization of the potent odorant 2-acetyl-1-pyrroline. Meeting of the Illinois Heartland Local Section of the American Chemical Society, Bloomington, IL, May 16, 2016.

Ickes, C. and Cadwallader, K.R. 2016. Characterizing aroma components of rum. ACS Symposium: *Flavor Chemistry of Alcoholic Beverages.* 251st American Chemical Society Meeting, San Diego, CA, March 13-17.

Cadwallader, K.R., Hausch, B. and Yin, Y. Quest for stability of 2-acetyl-1-pyrroline: The key odorant in aromatic rice and pandan. 17th Food Innovation Asia Conference 2015, Bangkok, Thailand, June 18-19.

Ickes, C. and Cadwallader, K.R. 2015. Understanding the effects of ethanol-flavor interactions on flavor perception in alcoholic beverages. ACS Symposium: *Challenges in Applied Flavor Sciences.* ACS National Meeting, Boston, MA, August 16-20, AGFD paper no. 297.

Pang, X., Wu, J. and Cadwallader, K.R. 2014. Identification of predominant contributors to off-odors formed in thermally processed melon juice. ACS Symposium: *Chemical Changes During Processing.* 248th American Chemical Society Meeting, San Francisco, CA, August 10-14. AGFD paper no. 177.

Cadwallader, K.R. and Lapsongphon, N. 2012. Identification of key odorants in fish sauce. US-Korea Conference (UKC) 2012, Los Angeles, August 8-11.

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Hausch, B. and Cadwallader, K.R. 2010. Flavor chemistry of lemon-lime flavored carbonated beverages. ACS Symposium: *Symposium Honoring Prof. Terry E. Acree on the Occasion of His 70th Year.* 240th ACS National Meeting, Boston, MA, August 22-26, AGFD paper no. 40.

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Cadwallader, K.R. 2010. Flavor chemistry of tree nuts. IFT Symposium: *Nuts: Phytochemicals, Health Effects and Flavor.* IFT Annual Meeting and Food Expo, Chicago, IL, July 17-20, paper no. 054-05.

Lozano, P.R. and Cadwallader, K.R. 2009. Effect of deamidation on binding of flavor compounds by dehydrated soy protein isolates. ACS Symposium: *Food Proteins and Food-based Protein Chemistry* 237th ACS National Meeting, Finley, J., organizer, Salt Lake City, UT, March 22-26.

Cadwallader, K.R. 2008. Sensory evaluation of aroma models for flavor characterization. *Symposium in Honor of Ronald Buttery G. Kenneth Recipient of the 2008 Spencer Award for Outstanding Achievement in Agricultural and Food Chemistry*, Kansas City Section, American Chemical Society, Kansas City, MO, Oct 27.

Cadwallader, K.R. 2008. AGFD and 100 years of chemical research on grains and legumes. ACS Symposium: *AGFD 100th Anniversary Symposium*, Brine, C. and Tunick, M.H., organizers, 236th ACS National Meeting, Philadelphia, PA, August 17-21.

Liu, Y and Cadwallader, K.R. 2008. Aroma components of "Carlos" muscadine (*Vitis rotundifolia* Michx) grape juice. ACS Symposium: Flavor and Health Benefits of Small Fruits, Rimando, A.M. and Qian, M.C., organizers, 236th ACS National Meeting, Philadelphia, PA, August 17-21.

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Cadwallader, K.R. Lozano, P.R. and Kim, H. 2007. Challenges in the isolation and identification of key aroma components of complex functional foods. ACS Symposium: *Food Bioactives and Nutraceuticals: Production, Chemistry, Analysis and Health Effects: Symposium Honoring Fereidoon Shahidi*, Rimando, A.M., organizer. 234th ACS National Meeting, Boston, MA, August 19-23, AGFD Paper 92.

Cook, S., Cadwallader, K.R. and Kim, H. 2007. Identification of key thermally-derived odorants in cooked chicken liver. ACS Symposium: *Thermal Generation of Flavors and Off-flavors,* Ho, C.-T., Raghavan, S., McGorrin, R.J., organizers. 234th ACS National Meeting, August 19-23. AGFD Paper 206.

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Cadwallader, K.R. 2006. Modern techniques to characterize complex food flavor systems. International Symposium on *Functional Foods*, ATAM (Nutrition Society of Mexico) and Institute of Food Technologists, Mexico City, Mexico, August 17.

Rotsachakul, P., Cadwallader, K.R. and Chaiseri, S. 2005. Comparison of DHS and solvent extraction-SAFE for GCO-dilution analysis of key aroma components of Thai fried chili paste. ACS Symposium: *Aroma-active Compounds in Foods and Reaction Flavors: Formation, Measurement and Stability.* (Ho, C.-T., Weerasinghe, D.K. and Cadwallader, K.R., organizers). 330th ACS National Meeting, Washington, DC, August 28 – September 1, AGFD paper 9.

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Gnadt, T.A., Jasso, L. and Cadwallader, K.R. 2005. Characteristic aroma components of chipotle peppers. ACS Symposium: *Chemistry and Flavor of Hispanic Foods* (de Mejia, E. and Tunick, M.H., organizers). 229th ACS National Meeting, San Diego, CA, March 13-17.

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Cadwallader, K.R. Importance of sample preparation technique on the composition of aroma extracts from fresh herbs. ACS Sympsosium: *Challenges in Herb Research – Chemical Analysis and Quality* (Ho, C.T. and Hwang, L.S., organizers). 227th ACS National Meeting, Anaheim, CA, March 28-April 1, 2004.

Cadwallader, K.R., Jarunrattanasri, A. and Theerakulkait, C. 2003. Aroma components of HVP made from rice bran protein. ACS Symposium: *Processed/Reaction Flavors, Ingredients and Intermediate Reactions.* (Weerasinghe, D.K. and Sucan, M.K., organizers). 226th ACS National Meeting, New York, NY, September 7-11.

Cadwallader, K.R. and Pojjanapimol, S. 2003. Characteristic aroma components of the cilantro mimics. ACS Symposium: *Natural Flavor and Fragrance Ingredients: Chemistry, Production and Application.* (Frey, C. and Rouseff, R.L., organizers). 226th ACS National Meeting, New York, NY, September 7-11.

Cadwallader, K.R. Relating Cheddar cheese flavor to chemical components. IFT Symposium: *Flavor Chemistry of Dairy Products.* (Drake, M.A., organizer). Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 12-16, #52-5.

Cadwallader, K.R., Pojjanapimol, S. and Chaiseri, S. 2002. Heat-induced changes in holy basil aroma components. ACS Symposium: *Challenges in the Characterization of Flavor Compound.* (Deibler, K.D. and Delwiche, J., organizers). 224th ACS National Meeting, Boston, MA, August 18-22.

Cadwallader, K.R., Zhou, Q. and Huda, M.S. 2002. Measuring flavor release from microencapsulants under controlled environmental conditions. ACS Symposium: *Controlled Release Systems for Foods.* (Shefer, S. and Yildiz, M.E., organizers). 223rd ACS National Meeting, Orlando, FL, April 7-11.

Cadwallader, K.R., Brehart, D.M., Schmidt, S.J. and Huda, M.S. 2002. Volatile release from a sucrose encapsulated flavoring. IFT Symposium: *Use of Carbohydrates for Encapsulation and Sustained Release.* (Obanni, M. and Asquith, T.N., organizers). Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 15-19, #67-1.

Cadwallader, K.R., Lopez, J.R. and Menke, S.D. 2001. Aroma components of wines from Chardonel, a French-American hybrid grape variety. ACS Symposium: *Chemistry and Flavor of Nutritional Beverages.* (Shahidi, F. and Weerasinghe, D., organizers). 222nd ACS National Meeting, Chicago, IL, August 26-31.

Guthrie, B. and Cadwallader, K.R. 2001. Measuring flavor retention in dry food systems. IFT Symposium: *Developments in the Analysis of Complex Flavor Systems*. (Cadwallader, K.R. and McGorrin, R.J., organizers). Annual Meeting of the Institute of Food Technologies, New Orleans, LA, June 23-27, #76-2.

Cadwallader, K.R. 2001. Use of SPME Technology to Evaluate Antioxidant Effectiveness. Symposium: *Antioxidant Analysis*, (Engeseth, N.J., organizer). 92nd AOCS Annual Meeting and Expo, Minneapolis, MN, May 13-16.

Karagul-Yuceer,Y., Drake, M.A. and Cadwallader, K.R. 2001. Aroma characterization of fresh and stored nonfat dry milk. ACS Symposium: *Freshness and Shelf-life of Foods.* (Weenen, H. and Cadwallader, K.R., organizers). 222nd ACS National Meeting, Chicago, IL, August 26-30.

Cadwallader, K.R. 2000. Flavor Chemistry of Saffron. Symposium: *Carotenoid-Derived Aroma Compounds.* (Winterhalter, P. and Rouseff, R., organizers). 219th ACS National Meeting, San Francisco, CA, March 26-30, paper #46 AGFD.

Cadwallader, K.R. 1999. Sample dilution analysis for identification of aroma-impact components of edible oils. ACS Symposium: *Advances in Gas Chromatography/Olfactometry.* (Leland, J., Acree, T.E., organizers). 218th ACS National Meeting, New Orleans, LA, August 22-26.

Cadwallader, K.R. and Shahidi, F. 1999. Identification of potent odorants in seal blubber oil by direct thermal desorption-gas chromatography-olfactometry. ACS Symposium: *Chemistry and Nutrition of Highly Unsaturated Fatty Acids.* (Shahidi, F. and Finley, J.W., organizers). 217th ACS National Meeting, Anaheim, CA, March 21-25.

Cadwallader, K.R. and Howard, C.L. 1997. Analysis of aroma-active compounds in light-activated milk. ACS Symposium: *Challenges in the Isolation and Characterization of Flavor Compounds.* (Mussinan, C.J. and Morello, F., organizers). 214th National Meeting of the American Chemical Society, Las Vegas, NV, September 7-11.

Baek, H.-H. and Cadwallader, K.R. 1996. Character-impact aroma compounds of crustacea. ACS Symposium: *Flavor and Lipid Chemistry of Seafoods.* (Shahidi, F. and Cadwallader, K.R., organizers). 212th National Meeting of the American Chemical Society, Orlando, FL, August 25-29.

Cha, Y.J., Lee, G.H. and Cadwallader, K.R. 1996. Aroma-active compounds in salt-fermented anchovy. ACS Symposium: *Flavor and Lipid Chemistry of Seafoods.* (Shahidi, F. and Cadwallader, K.R., organizers). 212th National Meeting of the American Chemical Society, Orlando, FL, August 25-29.

Wang, H.-J., Cadwallader, K.R., Baek. H.H., D'Abramo, L.R. and Sullivan, J.A. 1996. Manipulating the flavor of crustacea using postharvest seawater acclimation. ACS Symposium: *Flavor and Lipid Chemistry of Seafoods.* (Shahidi, F. and Cadwallader, K.R., organizers). 212th National Meeting of the American Chemical Society, Orlando, FL, August 25-29.

Cadwallader, K.R. and Howard, C.L. 1996. Characterization of saffron flavor by aroma extract dilution analysis. ACS Symposium: *Spices: Flavor Chemistry and Antioxidant Properties.* (Risch, S.J. and Ho, C.T., organizers). 211th National Meeting of the American Chemical Society, New Orleans, LA, March 24-28.

Cadwallader, K.R., Baek, H.H., Chung, H.Y. and Moody, M.W. 1993. Contribution of lipid-derived components to the flavor of alligator meat. ACS Symposium: *Lipids and Food Flavors.* (Ho, C.T. and Hoffman, T.G., organizers). 205th National Meeting of the American Chemical Society, Denver, CO.

Braddock, R.J. and Cadwallader, K.R. 1993. Bioconversion of citrus d-limonene. Symposium paper presented at the 206th National Meeting of the American Chemical Society, Chicago, IL.

Braddock, R.J. and Cadwallader, K.R. 1991. Citrus by-products manufacture for food use. Symposium paper presented at the 52nd Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 1-5.

Volunteered Abstracts

Kelley, L. and Cadwallader, K.R. 2016. Identification and quantitation of potent odorants in spearmint oils. 11th Wartburg Syposium on Flavor Chemistry & Biology, Eisenach, Germany, June 21-24.

McKenna, S. and Cadwallader, K.R. 2016. Identification and characterization of mint lactones: Trace-level odor-important compounds in peppermint oil. IFT Annual Meeting and Food Expo, Chicago, IL, July 17-19, paper no. PO2-020

Ickes, C., Lee, S. and Cadwallader, K.R. Novel creation of rum flavor lexicon through the use of web-based material. IFT Annual Meeting and Food Expo, Chicago, IL, July 17-19, paper no. P03-111

Yu, L., Thomas, L., Yin, L, Jerrell, J., Cadwallader, K. and Schmidt, S. 2015. Impact on sucrose crystal composition and chemistry on its thermal behavior. IFT Annual Meeting and Food Expo, Chicago, IL, July 11-14, paper no. 046-093.

Yu, L., Thomas, L., Yin, L, Jerrell, J., Cadwallader, K. and Schmidt, S. 2015. Impact on sucrose crystal composition and chemistry on its thermal behavior. IFT Annual Meeting and Food Expo, Chicago, IL, July 11-14, paper 046-093.

Cadwallader, K.R. and Fang, M.-C. 2014. A novel and attractive method for the stabilization of the potent odorant 2-acetyl-1-pyrroline. 14th Weurman Flavor Research Symposium, Cambridge, UK, September 15-19.

Donovan, J.D., Cadwallader, K.R. and Lee, Y. 2014. Improvement of protein microcapsule structure and core retention through the use of inulin. IFT Annual Meeting and Food Expo, New Orleans, LA, June 20-25, paper 181-01.

Erten, E. and Cadwallader, K.R. 2014. Characterization of aroma components of raw, dry roasted, and oil roasted almonds using solvent assisted flavor evaporation (SAFE) and aroma extract dilution analysis (AEDA). IFT Annual Meeting and Food Expo, New Orleans, LA, June 20-25, paper 206-26.

Lu, Y., Lee, J., Thomas, L., Jerrell, J., Cadwallader, K.R. and Schmidt, S.J. 2014. Investigating the thermal decomposition differences between beet and cane sugars using HPLC. IFT Annual Meeting and Food Expo, New Orleans, LA, June 20-25, paper 050-07.

Rosales, E., Cadwallader, K.R. and Engeseth, N. 2014. Effect of ultrasonic application on fatty acid composition and aroma compounds of dark chocolate. IFT Annual Meeting and Food Expo, New Orleans, LA, June 20-25, paper 206-94.

Burnsides, E. and Cadwallader, K. 2012 Comparison of aroma compounds in rums and rum ethers. IFT Annual Meeting and Food Expo, Las Vegas, NV, paper no. 193-28.

Su, C., Padua, G. and Cadwallader, K. 2012. Zein applications in flavor encapsulation. IFT Annual Meeting and Food Expo, Las Vegas, NV, paper no. 228-23.

Cadwallader, K.R., Hausch, B. and Lorjaroenphon, Y. 2011. Identification, characterization and relative aroma impact of 2,3-dehydro-1,8-cineol in lemon-lime flavored carbonated beverages. 13th Weurman Flavor Research Symposium, Zaragoza, SPAIN, September 26-30.

Suppavorasatit, I., deMejia, E. and Cadwallader, K.R. 2011. Effect of enzymatic deamidation of soy protein isolate by protein-glutaminase on the functional properties of the protein. IFT Annual Meeting and Food Expo, New Orleans, LA, June 11-14, paper no. 236-57.

Lapsongphon, N., Yongsawatdigul, J. and Cadwallader, K.R. 2011. Aroma characterization of premium fish sauce. IFT Annual Meeting and Food Expo, New Orleans, LA, June 11-14, paper no. 236-61.

Tinchan, P., Chaiseri, S. and Cadwallader, K.R. 2011. Quantification of δ-lactones in canned coconut milk by stable isotope dilution assay-GC-MS. IFT Annual Meeting and Food Expo, New Orleans, LA, June 11-14, paper no. 236-64.

Lorjaroenphon, Y. and Cadwallader, K.R. 2011. Identification of potent odorants in cola flavored carbonated beverages. IFT Annual Meeting and Food Expo, New Orleans, LA, June 11-14, paper no. 236-73.

Gao, Y., Cadwallader, K.R. and Schmidt, S.J. 2011. Investigating the chemical and physical stability of glass encapsulated spray-dried flavors stored below their glass transition temperatures. IFT Annual Meeting and Food Expo, New Orleans, LA, June 11-14, paper no. 290-18

Lee, H., Kim, H., Cadwallader, K.R. and Feng, H. 2011. Analysis of aroma compounds of apple cider treated by thermal-pasteurization, manosonication, thermosonication, and manothermosolication. IFT Annual Meeting and Food Expo, New Orleans, LA, June 11-14, paper no. 293-14.

Cadwallader, K.R. 2010. Potent odorants in soymilk as affected by processing and storage. *Nutraceuticals, Ingredients, Challenges, Formulations, Flavours and Health Effects.* International Conference, Exhibition and Short Course on Nutraceuticals and Functional Foods, Denpasar, Bali, Indonesia, October 11 – 12, paper no. 92.

Genthner, E.R. and Cadwallader, K.R. 2010. Characterizing aroma components of fresh-cut watermelon. 9th Wartburg Symp. on Flavor Chemistry & Biology, Eisenach, Germany, April 13-16.

He, Y., Garces, M., Toshkov, S., Kim, H., Cadwallader, K.R., Engeseth, N., Parker, T. and Jackson, M. 2010. Impact of irradiation processing on papaya nectar flavor. IFT Annual Meeting and Food Expo, Chicago, IL, July 17-20, paper no. 188-11

Kim, H. and Cadwallader, K.R. 2010. Comparison of key odorants between commercial aseptic Korean and American soy milks. IFT Annual Meeting and Food Expo, Chicago, IL, July 17-20, 188-05

Tinchan, P., Chaiseri, S. and Cadwallader, K.R. 2010. Changes in the volatile profile of canned coconut milk during storage. IFT Annual Meeting and Food Expo, Chicago, IL, July 17-20, 188-14.

Cadwallader, K.R. 2009. Flavor challenges in functional and nutraceutical beverages. International Conference and Exhibition on Nutraceuticals and Functional Foods 2009, International Society for Nutraceuticals & Functional Foods, San Francisco, CA, October 31-November 4, paper 87.

LaFond, S., Cadwallader, K. and Artz, W. 2009. The formation of 4-HNE in frying oils: A controlled study using a French fry model. 110th AOCS Annual Meeting & Expo, Orlando, FL, May 3-6.

Choi, I.-S., Kim, H. and Cadwallader, K.R. 2009. Comparison of the aroma components between dairy and soy yogurt. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 6-9, #024-02.

Lorjaroenphon, Y., Moy, M., Rosales, E. and Cadwallader, K.R. 2009. Identification of the key aroma components of fresh and baked Bartlett pear. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 6-9, #057-15.

Lozano, P.R. and Cadwallader, K.R. 2009. Effect of nonenzymatic deamidation of soy protein isolate on the binding of selected flavor compounds. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 6-9, #024-12

Lozano, P. and Cadwallader, K. 2008. Binding interactions of selected sulfur compounds with soy protein isolate under controlled relative humidity. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 28 – July 1, #006-12.

Carrasco Hernandez, G. and Cadwallader, K.R. 2008. Headspace reduction by different terpenoids of the main volatile sulfur compounds responsible for garlic breath. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 28 – July 1, #006-24.

Waimaleongora-Ek, P, Herrera Corredor, J., Prinyawiwatkul., W., Suvanich, V. and Cadwallader, K. Sensory attributes driving acceptance and purchase intent of watermelon juice containing no added sugar. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 28 – July 1, #138-12

Cadwallader, K.R. and Puangpraphant, S. 2008. Changes in the aroma components of pecans during roasting. 12th Weurman Flavour Research Symposium, Interlaken, Switzerland, July 1-4

Kim, H., Cadwallader, K.R. and Kido, H. 2008. Effect of commercial rosemary extract powders on formation of lipid-derived odorants in cooked pork sausages during cold storage. 235th ACS National Meeting, New Orleans, LA, April 6-10.

Carrasco, G. and Cadwallader, K.R. 2007. Mechanism of action of menthone in ameliorating oral cavity malodor caused by consumption of garlic. 234th ACS National Meeting, Boston, MA, August 19-23.

Lozano, P.R., Miracle, E.R., Krause, A.J. and Drake, M.A. 2007. Effect of cold storage and packaging material on the major aroma components of sweet cream butter. 234th ACS National Meeting, Boston, MA, August 19-23.

Gnadt, T.A., Lee, S.-Y. and Cadwallader, K.R. 2007. Effects of commercial masking agents and lactones on soymilk aroma and flavor. Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 28 - August 2, #4-224.

Hong, L., Sajuti, S.C. and Cadwallader, K.R. 2007. Identification of characteristic aroma components of Pomegranate (Punica granatum). Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 28 - August 2, #3-187

Mosca, C., Esgro, S. and Cadwallader, K.R. 2007. Flavor Characterization of unrefined shea butters. American Oil Chemists' Society's (AOCS) 98th Annual Meeting and Expo, Quebec City, QC, Canada, May 13-16.

Andrae, L.M., Cadwallader, K.R. and Engeseth, N.J. 2006. Flavor changes of stored dark chocolate by dynamic headspace sorption – gas chromatography/mass spectrometry. Annual Meeting of the Institute of Food Technologists, Orlando, FL, June 24-28, #39F-12.

Lozano, P. and Cadwallader, K.R and Drake, M.A. 2006. Instrumental and sensory characterization of heat-induced odorants in aseptic packaged soymilk. Annual Meeting of the Institute of Food Technologists, Orlando, FL, June 24-28, #39F-12.

Gnadt, T.A., Jasso, L. and Cadwallader, K.R. 2005. Comparison of direct solvent extraction and dynamic headspace sampling for the analysis of characteristic aroma components of chipotle pepper. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, July 16-20, #17D-34.

Klein, B.P., Khanna, P., Cadwallader, K.R. and Endres, J.M. 2005. Reducing fat in school lunches by using soy-enhanced entrees. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, July 16-20, #70E-27.

Nowak, M.C., Singh, T.K. and Cadwallader, K.R. 2005. Identification of potent odorants in commercial hickory and mesquite liquid smoke. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, July 16-20, #17D-36.

Song, H. and Cadwallader, K.R. 2005. Aroma components of American country-style ham. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, July 16-20, #17D-35

Yu, X., Song, Y.S. and Cadwallader, K.R. 2005. Identification of the characteristic aroma components of commercial dried California bay leaves (*Laurus nobilis* L.). Annual Meeting of the Institute of Food Technologists, New Orleans, LA, July 16-20, #17D-19.

Zhou, Q. and Cadwallader, K.R. 2005. Application of IGC for measurement of binding of volatile flavor compounds by soy proteins in low-moisture food systems. 229th ACS National Meeting, San Diego, CA, March 13-17.

Bohn, D.M. Cadwallader, K.R. and Schmidt, S.J. 2004. Kinetic analysis of benzaldehyde release in artificial cherry Durarome® upon humidification using dynamic vapor sorption-fast gas chromatography-flame ionization detection. Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, July 12-16, #114B-27.

Lopez, J.R., Cadwallader K.R. and Menke, S.D. 2004. Menke Aroma-impact components of Illinois Frontenac wine. Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, July 12-16, #83D-3.

Singh, T.K., Young, N.D., Drake, M.A., Cadwallader, K.R. 2004. Production of a bitter peptide from casein and its sensory characterization. Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, July 12-16, #17B-17.

Ugarte-Romero, E.E. Feng, H, Martin, S.E. and Cadwallader, K.R. 2004. Power ultrasound as an alternative for apple cider pasteurization. Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, July 12-16, #32-10.

Yoon, Y., Carunchia-Whetstine, M.E., Friedeck, K.G., Drake, M.A. and Cadwallader, K.R. 2004. Characterization of aroma-active compounds in fresh sweet cherry cultivars. Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, July 12-16, #114B-3.

Zhou, Q. and Cadwallader, K.R. 2004. Investigation of flavor-soy protein interactions under controlled relative humidity by inverse gas chromatography (IGC). Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, July 12-16, # 114B-2.

Brisske, L.K., Lee, S.Y., Klein, B.P. and Cadwallader, K.R. 2003 Consumer ratings of energy bars: Should the bar be raised? Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 12-16, #12-2.

Bohn, D.M., Cadwallader, K.R. and Schmidt, S.J. 2003. Measurement of volatile release from an amorphous carbohydrate matrix as a function of relative humidity using dynamic vapor sorption technology. Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 12-16, #12-2.

Singh, T.K., Drake, M.A. and Cadwallader, K.R. 2003. Characterization of aroma components of Muenster cheese by aroma extract dilution and headspace dilution analyses. Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 12-16, #71-3.

Suriyaphan, O., Zhou, Q. and Cadwallader, K.R. 2003. Identification of key aroma components of *Piper betel* L. leaf. Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 12-16, #45C-16.

Drake, M.A., Karagul-Yuceer, Y., Civille, G.V., Cadwallader, K.R. and Tong, P.S. 2002. Development of a descriptive sensory language for milk powders. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 15-19, #100D-2.

Karagul-Yuceer, Y., Drake, M.A. and Cadwallader, K.R. 2002. Evaluation of the character-impact odorants in nonfat dried milk by sensory studies on model mixtures. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 15-19, #12-10.

Karagul-Yuceer, Y., Vlahovich, K., Drake, M.A. and Cadwallader, K.R. 2002. Compounds responsible for musky off-odor of rennet casein. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 15-19, #15B-12.

Karagul-Yuceer, Y, Cadwallader, K.R. and Drake, M.A. 2002. Odor-active compounds of liquid whey. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 15-19, #61B-4.

Pojjanapimol, S., Chaiseri, S. and Cadwallader, K.R. 2002. Aroma components of distilled essential oil of Holy basil. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 15-19, #61B-12.

Zhou, Q., Cadwallader, K.R. and Guthrie, B.D. 2002. Simultaneous measurement of permeability of aroma compound mixtures through polymer packaging films. Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 15-19, #100B-8.

Benitez, D. and Cadwallader K.R. 2001. Aroma-active components of Vietnamese coriander (*Polygonum odoratum*). Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #88A-14.

Benitez, D., Kappes, S. and Cadwallader, K.R. 2001. Aroma of dried peaches. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #44C-34.

Chen, D., Andrae, L., Suriyaphan, O., Weingartner, K. and Cadwallader, K. 2001. Characteristic aroma components of vegetable soybean (edamame). Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #88A-17.

Drake, M.A., Gerard, P.D., Wright, S., Cadwallader, K.R. and Civille, G.V. 2001. Cross validation of a sensory language for Cheddar cheese. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #73G-19.

Grady, J., Klein, B.P. and Cadwallader, K.R. 2001. Bitterness and astringency thresholds for soy isoflavones. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #98-2.

Karagul-Yuceer, Y, Cadwallader, K.R. and Drake, M.A. 2001. Identification of volatile off-flavor compounds of nonfat dried milks. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #88A-3.

Suvanich, V., Maciel-Pedrote, I., Cadwallader, K.R., Moser, E.B., Thongthai, C. and Prinyawiwatkul, W. 2001. Aroma indices of Southeast Asian fish sauces. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #73F-16.

Suvanich, V., Maciel-Pedrote, I., Cadwallader, K.R., Moser, E.B., Thongthai, C. and Prinyawiwatkul, W. 2001. Developnebt if fish sauces quantitative descriptive sensory aroma profile. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #73G-34.

Zhou, X., Winterstein, C. and Cadwallader, K.R. 2001. Aroma of buckwheat honey. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #88A-12.

Zhu, L., Robbins, K. and Cadwallader, K.R. 2001. Effects of soy protein isolate on headspace aroma components of cooked ground beef patties. Annual Meeting of the Institute of Food Technologists, New Orleans, LA, June 23-27, #88A-18.

Drake, M.A., McIngvale, S.C., Cadwallader, K.R. and Civille, G.V. 2000. Development of a descriptive language for Cheddar cheese. Book of Abstracts, Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 10-14, #11-3.

Suriyaphan, O., Cadwallader, K.R. and Drake, M.A. 2000. Lipid oxidation of deoiled soy lecithin in fermented milk systems. Book of Abstracts, Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 10-14, #14B-32.

Cadwallader, K.R. and Thongthai, C. 2000. Comparison of aroma-active headspace components of three types of fish sauces. Book of Abstracts, Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 10-14, #36-3.

Chen, X.Q., Drake, M.A. and Cadwallader, K.R. 2000. Characteristic aroma components of English Farmhouse Cheddar Cheeses. Book of Abstracts, Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 10-14, #78C-4.

Karagul, Y., Drake, M.A. and Cadwallader, K.R. 2000. Aroma-active components of nonfat dry milk powders. Book of Abstracts, Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 10-14, #78C-6.

Cadwallader, K.R. and Jung, J. 1999. Evaluation of warmed-over flavor in chicken wings by sensory and instrumental methods. 60th Annual Meeting of the Institute of Food Technologists, Chicago, IL, #50A-13, p. 122.

Wu, Y.F., Baek, H.H. and Cadwallader, K.R. 1999. Development of a meat-like process flavoring from soybean-based hydrolyzed vegetable protein. 60th Annual Meeting of the Institute of Food Technologists, Chicago, IL, #50A-9, p.121.

Suriyaphan, O., Cadwallader, K.R. and Drake, M.A. 1999. Lecithin associated off-flavors in fermented dairy products—A model study. 60th Annual Meeting of the Institute of Food Technologists, Chicago, IL, #17-2, p. 31.

Cadwallader, K.R. and Suriyaphan, O. 1998. Aroma-impact components of loroco. 59th Annual Meeting of the Institute of Food Technologists, Atlanta, GA, #45-2, p. 97.

Surakarnkul, R. and Cadwallader, K.R. 1998. Release of glycosidically-bound aroma compounds during fermentation of muscadine juice. 59th Annual Meeting of the Institute of Food Technologists, Atlanta, GA, #72-18, p. 180.

Suriyaphan, O., Drake, M.A., and Cadwallader, K.R. 1998. Identification of volatile off-flavors in reduced fat cheeses containing lecithin. 59th Annual Meeting of the Institute of Food Technologists, Atlanta, GA, #72-31, p. 183.

Baek, H.H. and Cadwallader, K.R. 1997. Free and glycosidically bound volatile aroma compounds in muscadine grape juice. 58th Annual Meeting of the Institute of Food Technologists, Orlando, FL, #23D-6, p.63.

Cai, M. and Cadwallader, K.R. 1997. (Z)-3-Nonenal and (Z,Z)-3,6-nonadienal: Character-impact aroma compounds in watermelon. 58th Annual Meeting of the Institute of Food Technologists, Orlando, FL, #23D-9, p.64.

Cha, Y.J., Kim, H., and Cadwallader, K.R. 1997. Aroma-active compounds in Kimchi during aging. 58th Annual Meeting of the Institute of Food Technologists, Orlando, FL, #23D-13, p.65.

Baek, H.H., Kim, C.J., Ahn, H.S., Nam, H.S., and Cadwallader, K.R. 1997. Aroma extract dilution analysis of beef-like process flavor from extruded enzymatically hydrolyzed soybean protein. 58th Annual Meeting of the Institute of Food Technologists, Orlando, FL, #23D-24, p.67.

Fu, S.G., Baek, H.H., and Cadwallader, K.R. 1997. Comparison of aroma-active compounds extracted from blue crab meat with processing by-products hydrolysate using aroma extract dilution analysis. 58th Annual Meeting of the Institute of Food Technologists, Orlando, FL, #23D-25, p.67.

Aryana, K.J., Cadwallader, K.R., and Haque, Z.U. 1996. Isolation of flavor volatiles in lowfat Cheddar cheese by vacuum simultaneous distillation-solvent extraction. 73rd Annual Meeting of the Southern Branch, American Dairy Science Association, February 3-7.

Baek, H.H. and Cadwallader, K.R. 1996. Evaluation of the enzymatic hydrolysis of defatted soybean meal by response surface methodology. 57th Annual Meeting of the Institute of Food Technologists, New Orleans, LA, #53A-16, p.120.

Baek, H.H., Cadwallader, K.R., Marroquin, E., and Silva, J.L. 1996. Gas chromatography/olfactometric analysis of muscadine grape aroma. 57th Annual Meeting of the Institute of Food Technologists, New Orleans, LA, #34-7, p.74.

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Cha, Y.J., Kim, E.J., Baek, H.H., and Cadwallader, K.R. 1996. Development of a flavoring agent from sea mussel. 57th Annual Meeting of the Institute of Food Technologists, New Orleans, LA, #80D-22, p.197.

Kim, H.R., Ahn, C.B., Jung, K.J., Baek, H.H., Cadwallader, K.R. 1996. Characterization and utilization of waste water from oyster processing plants as flavoring agents. 57th Annual Meeting of the Institute of Food Technologists, New Orleans, LA, #80D-21, p.197.

Aryana, K.J., Cadwallader, K.R., and Haque, Z.U. 1996. Development of methodology for measuring+ flavor volatiles in Cheddar cheese made with and without fat replacers. International Conference on Food Science and Technology of The Fine Particle Society, Chicago, IL, August 24-26.

Cha, Y.J., Baek, H.H., and Cadwallader, K.R. 1995. Volatile flavor components in oyster hydrolysate produced with protease. 56th Annual Meeting of the Institute of Food Technologists, Anaheim, CA, #54E-16, p.166.

Conner, T.M., McGregor, J.U., and Cadwallader, K.R. 1995. Identifying flavor components in light-activated milk. J. Dairy Sci. 48(supplement 1): 317.

Baek, H.H. and Cadwallader, K.R. 1994. Comparison of volatile compounds in flavor concentrates produced from crawfish processing by-products with and without protease treatment. 55th Annual Meeting of the Institute of Food Technologists, June 25-29, Atlanta, GA, #79C-5, p.244.

Cadwallader, K.R., Tan, Q., Chen, F., and Meyers, S.P. 1994. Identification of character-impact aroma compounds in spiny lobster (*Panulirus argus*) tail meat. 55th Annual Meeting of the Institute of Food Technologists, June 25-29, Atlanta, GA, #7-7, p.13.

Chung, H.Y. and Cadwallader, K.R. 1994. Comparison of the aroma of three different extracts prepared from blue crab (*Callinetes sapidus*) processing by-product. 55th Annual Meeting of the Institute of Food Technologists, June 25-29, Atlanta, GA, #79C-7, p.244.

Chung, H.Y., Chen, F., and Cadwallader, K.R. 1994. Evaluation of aroma of blue crab (*Callinetes sapidus*) claw and lump meats. 55th Annual Meeting of the Institute of Food Technologists, June 25-29, Atlanta, GA, #79C-6, p.244.

Tan, Q. and Cadwallader, K.R. 1994. Biotransformation of ß-pinene by *Aspergillus cellulosae*. 55th Annual Meeting of the Institute of Food Technologists, June 25-29, Atlanta, GA, #24C-7, p.67.

Andrews, L.S. and Cadwallader, K.R. 1993. Microbial and sensory quality of ground ginger as affected by gamma irradiation. 54th Annual Meeting of the Institute of Food Technologists, July 10-14, Chicago, IL, Technical Program: Book of Abstracts, #817, p.206.

Baek, H.H. and Cadwallader, K.R. 1993. Identification of characteristic volatile flavor components of roasted chicory by aroma extract dilution analysis. 54th Annual Meeting of the Institute of Food Technologists, July 10-14, Chicago, IL, Technical Program: Book of Abstracts, #506, p.129.

Cha, Y.J. and Cadwallader, K.R. 1993. Volatile flavor components in Korean fermented fish paste. 54th Annual Meeting of the Institute of Food Technologists, July 10-14, Chicago, IL, Technical Program: Book of Abstracts, #510, p.130.

Cha, Y.J., Baek, H.H., and Cadwallader, K.R. 1993. Comparison of DHS and SDE for the analysis of volatile compounds in fermented shrimp paste. 54th Annual Meeting of the Institute of Food Technologists, July 10-14, Chicago, IL, Technical Program: Book of Abstracts, #511, p.131.

Chung, H.Y. and Cadwallader, K.R. 1993. Aroma extract dilution analysis of freshly picked cooked blue crab meat. 54th Annual Meeting of the Institute of Food Technologists, July 10-14, Chicago, IL, Technical Program: Book of Abstracts, #563, p.144.

Chung, H.Y., Chou, C.-C., and Cadwallader, K.R. 1993. Changes in the volatile flavor constituents of coconut during roasting. 54th Annual Meeting of the Institute of Food Technologists, July 10-14, Chicago, IL, Technical Program: Book of Abstracts, #504, p.129.

Yoo, S.K. and Cadwallader, K.R. 1993. Bioconversion of α- and ß-pinene. 54th Annual Meeting of the Institute of Food Technologists, July 10-14, Chicago, IL, Technical Program: Book of Abstracts, #516, p.132.

Baek, H.H. and Cadwallader, K.R. 1992. Enzymatic hydrolysis of crawfish waste for bioflavor production. Ninth International Biotechnology Symposium and Exposition, August 16-21, Crystal City, VA, Book of Abstracts, #472.

Baek, H.H., Pike, M.M., Bidner, T.D., Moody, M.W., and Cadwallader, K.R. 1992. Evaluation of the off-odor associated with wild alligator. 53rd Annual Meeting of the Institute of Food Technologists, June 20-24, New Orleans, LA. Book of Programs and Abstracts, #359, p.93.

Cha, Y.J., Baek, H.H., and Cadwallader, K.R. 1992. Volatile flavor components in concentrated snow crab extracts. 53rd Annual Meeting of the Institute of Food Technologists, June 20-24, New Orleans, LA, Book of Abstracts, #357, p.92.

Chung, H.Y. and Cadwallader, K.R. 1992. Comparison of volatile compounds in freshly picked crabmeat and crab processing waste. 53rd Annual Meeting of the Institute of Food Technologists, June 20-24, New Orleans, LA, Book of Abstracts, #358, p.93.

Kim, H.R., Baek, H.H., Cadwallader, K.R., Meyers, S.P., and Godber, J.S. 1992. Improved flavor extractibility from crab waste using the hepatopancreatic enzyme of crawfish, *Procambarus clarkii*. 53rd Annual Meeting of the Institute of Food Technologists, June 20-24, New Orleans, LA, Book of Abstracts, #360, p.93.

Cadwallader, K.R. and Braddock, R.J. 1990. Enzymatic hydration of limonene to α-terpineol. 51st Annual Meeting of the Institute of Food Technologists, June 16-20, Anaheim, CA, Book of Programs and Abstracts, #354, p.170.

Cadwallader, K.R. 1988. Bioconversion of (+)-limonene. 49th Annual Meeting of the Institute of Food Technologists, June 19-22, New Orleans, LA, Book of Programs and Abstracts, #293.

Students/Postdoctoral/Visiting Scholar Researchers Supervised (1991-1999)

Visiting Scientists
Yong-Jun Cha (LSU, MSU) – Chang Won National University
Hetty Voermans (LSU) – University of Wegeningen, The Netherlands

Postdoctoral Researchers
Hyung-Hee Baek (MSU)

Graduate Students Completed (LSU/MSU)

MS Students
Sun-Kyun Yoo (LSU)
Min Cai (MSU)
Hsin-Jen (Connie) Weng (MSU)
Shi-Guei (Scott) Fu (MSU)
Shin-Pei (Peggy) Yang (MSU)
Yi-Fang (Grace) Wu (MSU)
Leyla Yuksel (MSU)
JiHyun Jung (MSU)
Min-Sheng Su (MSU)
Gwo-Yuh Li (MSU)
Jeong Heo (MSU)

Ph.D. Students
Hyung-Hee Baek (LSU)
Hau Yin Chung (LSU)
Gyu-Hee (Chang) Lee (MSU)
Ranee Surakarnkul (MSU)
Yonca Karagul-Yuceer (MSU)

Students/Postdoctoral/Visiting Scholar Researchers Supervised (UIUC, 2000-present)

Visiting Scholars
Byung Hak Ahn (2006-2007) - Korea Food Research Institute, South Korea
Supeeraya Arsa (2013-2014) – Kasetsart University, Bangkok, Thailand
Mantana Buttara (2011-present) – Suranaree Univeristy of Technology, Thailand
Dong Chen (2015-present) – China Agricultural University, Beijing, China

Rongrong Chen (2016-present) - Ever Maple Food Science and Technology Co., Ltd., China
Arporn Jarunrattanasri (2003-2004) – Naresuan University, Thailand
Young-Suk Kim (2015-present) – Ewha Womans University
Patthamawadi Kiatbenjaku. (2009-2011, 2012) – Suranaree University of Technology, Thailand
Fareeya Kulapichitr (2016-present) – Chulalongkorn University, Thailand
Sasinee Kunyaboon (2016-present) - Suranaree University of Technology, Thailand
Nawaporn Lapsongphon (2010-2011) - Suranaree University of Technology, Thailand
Yuping Liu (2014-2015) – Beijing Technology and Business University, China
Yaowapa Lorjaroenphone (2006-2007) – Kasetsart University, Thailand
Xueli Pang (2013-2014) – China Agricultural University, Beijing, China
Sompoche Pojjanapimol (2003-2004) - Kasetsart University, Thailand
Primseri Rotsachakul (2004-2005) – Kasetsart University, Thailand
Huanlu Song (2005-2006) – Beijing Technology and Business University, Beijing, China
Patcharaporn Tinchan (2010-2012) – Kasetsart University, Thailand
Jihong Wu (2011-2012) – China Agricultural University, Beijing, China

Postdoctoral Researchers (UIUC)
Masud Huda (2002-2003)
Orasa Suriyaphan (2001, 2003)
Tanoj Singh (2001-2005)
Hun Kim (2005-2012)

Graduate Students Completed (UIUC)
Daniel Benitez (MS 2003)
Laura Brisske (MS 2003)
Erin Burnside (MS 2012)
Gabriela Carrasco (MS 2007)
Shondra Cook (MS 2007)
Edibe Erten (Ph.D. 2016)
Ming Chih Fang (Ph.D.2013)
Samantha Gardiner (MS 2015)
Elizabeth Genthner (MS 2010; Ph.D. 2014)
Theresa Gnadt (MS 2007)
Julia Gregg (Nonthesis MS, CHEFS Program, 2009)
Bethany Hausch (MS 2010)
Lauren Kelley (MS)
Sean LaFond (MS 2009)
Jacob Lahne (MS 2010)
Yaowapa Lorjaroenphone (Ph.D. 2012)
Patricio Lozano (MS 2005 & Ph.D. 2009)
Samantha McKenna (MS, 2016)
Joshua Moore (MS 2006)
Linda Moran (MS 2013)
Ana Carolina Mosca (MS 2007)
Jason Niermann (Nonthesis MS, CHEFS Program 2001)
Sirima Puangpraphant (MS 2007)
Bethany Richardson (MS 2013)
Inthawoot Suppavorasatit (Ph.D. 2012)
Chenxing Sun (3+2 program, Nonthesis MS)
Qiaoxuan Zhou (MS 2001 & Ph.D. 2005)

Current Graduate Students (2016)
Courtney Beckett (MS)
Chelsea Ickes (Ph.D.)
Fengjia Chen (MS, 3+1)
Bethany Hausch (Ph.D.)
Nagiat Hwasa (Ph.D.)
Jing (Jenny) Jin (MS, 3+1)
Tiandan Wu (MS)
Wenqi Zhu (Ph.D.)
Yun Yin (Ph.D.)

Undergraduate Student Researchers
Kelsey Choquette (2012-2013)
Diniene Chreaton, Rap II (2001)
Ashley Davis, Rap II (ACES) (2001) & UIUC Ronald E. McNair Summer Research Program (2004)
Yaozheng (Tess) Liu (2013-present)
Rosemary Nash (2011-2012)
Mary Nowak (2004-2005)
Jieun Oh (2013)
Abigail Pishevar (2014-2015)
David Potts (2008-2010)
Stephanie Sajuti (2005-2007)
Dylan Troth (2015-present)
Katrina Vlahovich (2001-2002)

Involvement in Professional Organizations (2000-present)

Leadership

Chair and Program Chair for the Division of Agricultural and Food Chemistry (AGFD) of the American Chemical Society, 2003-2005. Responsibilities include organizing AGFD technical programs for two ACS National Meetings (fall 2004 and spring 2005 meetings).

Editorial Board

Flavour and Frangrance Journal

Associate Editor

Journal of Food Science – *Food Chemistry*

Symposia Organized

Cadwallader, K.R. and Shahidi, F. 2017. ACS Symposium: *Chemistry of Clean Ingredients.* Washington, DC, August 20-24.

Cadwallader, K.R. and Chang, S.K.C. 2008. ACS Symposium: *Chemistry, Texture and Favor of Soy,* Philadelphia, PA, August 17-21.

Cadwallader, K.R. 2008. ACS Symposium: *Recent Developments in Flavor Chemistry*. 235th ACS National Meeting, New Orleans, LA, April 6-10.

Ho, C.-T., Weerasinghe, D.K. and Cadwallader, K.R. 2005. ACS Symposium: *Aroma-active Compounds in Foods and Reaction Favors: formation, measurement and stability*. 330th ACS National Meeting, Washington, DC, August 28 – September 1.

Cadwallader, K.R., P. Schieberle and M.J. Morello. 2005. ACS Symposium: *Grain-based Products: Health and Flavor, and Safety Aspects*. 330th ACS National Meeting, Washington, DC, August 28 – September 1.

Cadwallader, K.R. *Obesity Management: Role of Soy Foods*. University of Illinois, Champaign, IL, May 25-26, 2005.

Cadwallader, K.R., Drake, M.A. and McGorrin, R.J. 2004. ACS Symposium: *Chemistry and Flavor of Dairy Products*. 228th ACS National Meeting, Philadelphia, PA, August 22-26.

Weenen, H. and Cadwallader, K.R. (organizers). 2001. ACS Symposium: *Freshness and Shelf-life of Foods*. 222nd ACS National Meeting, Chicago, IL, August 26-30.

Cadwallader, K.R. and McGorrin, R.J. (organizers). 2001. IFT Symposium: *Developments in the Analysis of Complex Flavor Systems*. Annual Meeting of the Institute of Food Technologies, New Orleans, LA, June 23-27.

Rouseff, R.L. and Cadwallader, K.R. 1998. ACS Symposium: *Headspace Analysis of Foods and Flavors*. 214th ACS National Meeting, Boston, MA, August 23-27.

Shahidi, F. and Cadwallader, K.R. 1996. ACS Symposium: *Flavor and Lipid Chemistry of Seafoods*. 212th National Meeting of the American Chemical Society, Orlando, FL, August 25-29.

Workshops/Short courses (organized)

A Place for Soy in a Better School Lunch Program. University of Illinois, Urbana, IL, March 12, 2003.

Traditional and Emerging Methods for the Manufacture and Analysis of Food Flavorings. Faculty of Agriculture Industry, King Mongkut's Institute of Technology Ladkraband (KMITL), Bangkok, Thailand, July 29, 2002

Soy Flavor Workshop. ICSF Workshop (Klein, B.P. et al. coorganizers). Illinois Center for Soy Foods, University of Illinois, Urbana, IL, April, 2002/2003.

Workshops/Short courses (lecturer)

Nutraceuticals, Ingredients, Challenges, Formulations, Flavours and Health Effects. International Society for Nutraceutical and Functional Foods. Denpasar, Bali, Indonesia, October 11 – 12.

Flavor Chemistry. (course co-taught with Drs. Chi-Tang Ho and Soo-Yeun Lee), Taiwan National University, May 10-19, 2008.

Flavor Research Workshop. ACS Short Course (Acree, T.E., course director), American Chemical Society, Boston, MA, August 17-18, 2007.

Flavor Research Workshop. ACS Short Course (Acree, T.E. and Deibler, K., organizers), American Chemical Society, August 25-26, 2005.

Cadwallader, K.R. Flavoring soy products: masking and creating. Preconference AOAC Short Course. *Soyfoods: Ingredients, Preparation, and Utilization,* Koseoglu, S. and Shahidi, F. (organizers), Cincinnati, OH, May 9, 2005.

Cadwallader, K.R. 2004. Aroma and flavor aspects of soy beverages. Preconference Shortcourse: *Functional Beverages,* Koseoglu, S and Shahidi, F. (organizers), Worldnutra: International Conference and Exhibition on Nutraceutacals and Functional Foods, San Francisco, CA, Nov 8, 2004

Flavor Research Workshop. ACS Short Course (Acree, T.E., organizer), American Chemical Society, Boston, MA, August 16-17, 2002; New York, NY, September 7-11, 2003.

Cheese Flavor and Texture: Sensory and Instrumental Analysis. SDFRC Short Course (Drake, M.A., course director), Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, NC, August 7-9, 2002.

Proposal Reviewer/Panel Participation (2010-present)

Panelist/Reviewer (2015) - Office of Scientific Quality Review (OSQR)-USDA-ARS, NP 306 Panel 14: Functional Foods (2015)

Panelist/Reviewer (2015) - Office of Scientific Quality Review (OSQR)-USDA-ARS, NP 107 Panel 14: Grain Engineering (2014)

Panelist/Reviewer (2014) - Office of Scientific Quality Review (OSQR)-USDA-ARS, NP 107 Panel 5: Food Chemistry Analysis (2014)

Panelist/Reviewer (2013) – 2012/2013 King Abdulaziz City for Science and Technology (KACST) Research Proposal Competition

EXHIBIT B

MATERIALS CONSIDERED

- U.S. Patent Application No. 14/797,006 ("the '006 application")
- Office Action mailed June 6, 2016
- Proulx et al. (2006, J. Agric. Food Chem., 54:1518-22) ("Proulx")
- Hsieh et al. (US 2012/0093994) ("the '994 application")
- Declaration under 37 CFR § 1.132 of Dr. Celeste Holz-Schietinger ("the Holz-Schietinger Declaration")
- Lane and Nursten, "Variety of odors produced in Maillard model systems and how they are influenced by reaction conditions" in Maillard Reaction in Foods and Nutrition, Waller and Feather, eds., ACS Symposium Series No. 215, pp 141-158
- Kokkinidou and Peterson, 2013, Food Funct., 4:1093-104
- Elmore et al., 2001, "A comparison of headspace entrainment on Tenax with solid phase micro-extraction for the analysis of the aroma volatiles of cooked beef," in Headspace Analysis of Foods and Flavors; Rouseff and Cadwallader, eds, Springer
- Elmore et al. (2002, J. Agric. Food Chem., 50:1126-32)
- Chen et al. (2003, "Influence of DNA on Volatile Generation from Maillard Reaction of Cysteine and Ribose," *in* Nutraceutical Beverages, ACS Symposium Series Vol 871)
- Mottram and Nobrega (2002, J. Agric. Food Chem., 50(14):4080–86)
- Schieberle and Hofmann ("Characterization of Key Odorants in Dry-Heated Cysteine—Carbohydrate Mixtures: Comparison with Aqueous Reaction Systems," *in* Flavor Analysis, ACS Symposium Series, Vol 705)

- Mottram and Mottram (2002, "An Overview of the Contribution of Sulfur-Containing Compounds to the Aroma in Heated Foods," *in* Heteroatomic Aroma Compounds, ACS Symposium Series, Vol 826)
- Shu-C and Ho (1989, "Parameter Effects of the thermal reaction of cysteine and 2,5-dimethyl-4-hydroxy-3(2H)-furanone," *in* Thermal Generation of Aromas, ACS Symposium Series, Vol 409)
- Chemistry and Technology of Flavours and Fragrances, 2005, Ed David Rowe, Blackwell Publishing Ltd
- Flavor of Meat and Meat Products, 1994, Ed Shahidi, Springer-Science+Business Media, BV
- Handbook of Meat and Meat Processing, 2012, 2nd Ed, Ed Hui, CRC Press
- Chen and Ho (2002, J. Agric. Food Chem., 50(8):2372–76)
- Kerscher and Grosch (1998, J. Agric. Food Chem., 46:1954-58)
- Tang et al. (2013, J. Sulfur Chem., 34:38-47)
- Withcombe and Mussinan (1988, J. Food Sci., 53:658-60)

Attorney's Docket No.: 38767-0015001 / M3CON-Track 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Rachel Fraser	Art Unit	: 1791
Serial No.	:	14/797,006	Examiner	: Elizabeth A. Gwartney
Filed	:	July 10, 2015	Conf. No.	: 8496
Title	:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR § 1.132

I, Devin Grant Peterson, PhD, hereby declare as follows:

1. I am currently a Professor in the Department of Food Science and Technology at the Ohio State University in Columbus, Ohio. I have held this position since early 2016. I have worked and taught in the field of Food Science since 2001. Prior to my current position, I held Professor/Associate Professor positions at the University of Minnesota in Saint Paul, Minnesota from 2009 to 2016; and was a faculty member in the Department of Food Science at the Pennsylvania State University in University Park, Pennsylvania from 2001 to 2009. In addition, I have been the Director of the Flavor Research and Education Center for the past seven years, at both the University of Minnesota and the Ohio State University.

2. I am a nationally recognized expert and scholar in the field of flavor science. In 2015, I received the Excellence in Flavor Science Award from the Flavor and Extract Manufacturers Association. In 2008, I received a Pan-American Nutrition, Food Science and Technology Award. In 2004, during my tenure at Pennsylvania State University, I was awarded the Presidential Early Career Award for Scientists and Engineers (PECASE), which was presented by the White House Office of Science and Technology Policy.

3. I received a Bachelor's of Science (B.S.) degree in Food Science in 1994 from the University of Manitoba in Winnipeg, Manitoba. I earned a Master's of Science (M.S.) degree in 1997 and a Doctor of Philosophy (Ph.D.) degree in 2001 in Flavor Chemistry from the Department of Food Science from the University of Minnesota in Saint Paul, Minnesota. I was named to the Dean's List during my academic career at both the University of Manitoba and at the University of Minnesota. While I was obtaining my Ph.D., I completed a six-month Internship as a Research Flavor Scientist at Givaudan Flavors in Cincinnati, Ohio.

4. A copy of my curriculum vitae is attached in **Exhibit A**.

5. My research focuses on food flavor and related chemistry, with an emphasis on identification of flavor stimuli (e.g., taste, aroma, chemesthetic, mouthfeel), pathways of formation and degradation of flavor stimuli, taste and aroma modulation, and mechanisms of delivery.

6. In preparing my Declaration, I have reviewed U.S. Patent Application No. 14/797,006 ("the '006 application"), as well as the Office Action mailed June 6, 2016, in which the claims of the '006 application were rejected as being anticipated by Proulx et al. (2006, J. Agric. Food Chem., 54:1518-22) ("Proulx") and as being obvious considering Hsieh et al. (US 2012/0093994) ("the '994 application") in view of Proulx. I also reviewed the Declaration under 37 CFR § 1.132 of Dr. Celeste Holz-Schietinger ("the Holz-Schietinger Declaration"). A list of the materials I reviewed in preparing this Declaration is provided in **Exhibit B**.

7. In forming my opinion, I reviewed and considered Applicants' experiments related to their discovery and demonstration that the addition of heme protein (in some cases as

leghemoglobin or LegH) surprisingly contributes to the development of meaty aroma in a "ground beef-like food product" comprising no animal products. These experiments are described in the '006 application and in the Holz-Schietinger Declaration.

8. Flavor is a complex sensation, consisting of different modalities such as olfaction (aroma), gustation (sweet, sour, salty, bitter, umami), and chemesthesis (heat, cooling, pain), as well as others. The aroma attributes are known to be a key component of flavor perception and have been the most widely studied by flavor chemists.

9. In my opinion, the scientific protocols in the '006 application and in the Holz-Schietinger Declaration for a) sample preparation and treatment; and b) chemical analysis of aqueous thermal reaction samples are reasonable and consistent with methods that would be used by skilled individuals in the art. Each of these aspects are reviewed in more detail below.

10. The Hotz-Schietinger Declaration describes chemical analysis of aqueous thermal reaction samples using art recognized instrumental aroma analysis methods, specifically HS-SPME combined with GC-MS, to monitor the effect of LegH addition on the formation of volatile compounds, specifically those with meaty aroma characteristics, during heating (i.e., cooking). The objective was to demonstrate that LegH was capable of promoting or catalyzing the formation of meaty aroma compounds via aqueous thermal reactions between ribose and cysteine. Three flavor reaction systems were compared, consisting of: 1) 1% (10 mg/mL) LegH alone (**LegH control**), 2) 50 mM ribose + 50 mM cysteine alone (**Rib + Cyst**) and 3) 1% (10 mg/mL) LegH + 50 mM ribose + 50 mM cysteine (**LegH + Rib + Cyst**). The above reaction mixtures were diluted in a buffer (50 mM potassium phosphate and 100 mM sodium chloride)

and adjusted to pH 6.5, which is near the physiological pH of ground beef. A 2 mL aliquot of each reaction mixture was transferred to sample vials (sealed) which were subsequently heated (150°C for 3 min) and then analyzed by HS-SPME-GC-MS.

11. The concentrations (i.e. 0.1 to 50 mM) of flavor precursors (i.e., ribose and cysteine) of the aqueous thermal reaction samples described in the '006 application and the Hotz-Schietinger Declaration are consistent with peer-reviewed publications that have used similar aqueous reaction samples to investigate the thermal generation of volatile aroma compounds contributing to the flavor sensation of a food product. While aqueous thermal reaction samples are simplistic compared to food matrices, they provide relevant guidance and generalized outcomes for understanding the influence of flavor precursors on the formation of volatile compounds contributing to aroma, and to the flavor sensation generally in a number of different food products.

12. For example, a widely utilized and referenced publication for understanding the impact of flavor precursor composition on the formation of volatile aroma compounds generated therefrom is Lane and Nursten, "Variety of odors produced in Maillard model systems and how they are influenced by reaction conditions" in Maillard Reaction in Foods and Nutrition, Waller and Feather, eds., ACS Symposium, Series No. 215, pp 141-158. This publication describes aqueous combinations of sugar and amino acid mixtures heated under different time and temperature conditions. Lane and Nursten provides directional guidance for flavor development, and how flavor is impacted by flavor precursors, in various types of food matrices.

13. It is my opinion that the experimental approach and subsequent data analysis techniques described in the Holz-Schietinger declaration are appropriate and acceptable for the purpose of evaluating and comparing aroma compound formation in the three aqueous thermal reaction systems under heating conditions. The aqueous thermal reaction samples described in the Holz-Schietinger Declaration are comparable with those described by Lane and Nursten. It is also my opinion that these conditions would simulate the cooking of a "ground beef-like food product" containing no animal products as described in the '006 application, and that the data obtained from the experimental approach would be reasonably predictive of the expected aroma compound formation from a "ground beef-like food product" containing no animal products and including the flavor reaction system components LegH, ribose, and cysteine.

14. It is well known that thermal generation of volatile aroma compounds in an aqueous reaction system is influenced by reaction temperature and pH. When utilizing aqueous reaction samples, the reaction systems often are sealed so they can be pressurized, which increases the temperature of the sample to simulate temperatures in excess of 100°C, which would be experienced with conventional food heating methods such as baking, grilling or pan-frying. The aqueous thermal reaction samples described in the '006 application and in the Holz-Schietinger Declaration were heated in sealed vials at 150°C, which would be consistent with temperatures experienced by common meat preparation (heating) techniques.

15. My research program has similarly utilized the benchtop autosampler apparatus utilized in the '006 application and the Holz-Schietinger Declaration to rapidly heat sealed vials of aqueous samples to between 100-150°C for the purpose of evaluating and comparing aroma

compound formation. See, for example, Kokkinidou and Peterson, 2013, Food Funct., 4:1093-104.

16. The slightly acidic pH value of 6 - 6.5 for the aqueous thermal reaction samples used in the '006 application and the Holz-Schietinger Declaration also are consistent with the typical intrinsic pH values of meat products, as well as with peer-reviewed publications evaluating flavor development, and how it is impacted by flavor precursors, in various types of food matrices, including in meat food products.

17. It is my opinion that the experimental approach and analytical methods used in the '006 application and the Holz-Schietinger Declaration for evaluating and comparing aroma compound formation in aqueous thermal reaction samples were consistent with those used by flavor scientists of ordinary skill in the art. For example, the headspace solid phase micro-extraction (SPME) sampling technique coupled with GCMS utilized in the '006 application and the Holz-Schietinger Declaration has been widely utilized for characterizing the aroma of foodstuffs, such as meat food products. See, for example, Elmore et al., 2001, "A comparison of headspace entrainment on Tenax with solid phase micro-extraction for the analysis of the aroma volatiles of cooked beef," in Headspace Analysis of Foods and Flavors; Rouseff and Cadwallader, eds, Springer. Indeed, more than 100 articles are reported in SciFinder when the key words "meat, aroma, SPME" are set as filters to screen the existing scientific literature.

18. It is well known that the aroma profiles of meat food products are impacted by sulfur-containing compounds (typically, reaction products of sulfur-containing amino acids) and lipid degradation/oxidation products that are generated during heating. The different chemical

classes of volatile compounds formed in the aqueous thermal reaction samples of the '006 application and the Holz-Schietinger Declaration are consistent with volatile aroma compounds reported in the literature evaluating volatile compound formation contributing to meat aroma and flavor sensation. See, for example, Elmore et al. (2002, J. Agric. Food Chem., 50:1126-32); Chen et al. (2003, "Influence of DNA on Volatile Generation from Maillard Reaction of Cysteine and Ribose," *in* Nutraceutical Beverages, ACS Symposium Series Vol 871); Mottram and Nobrega (2002, J. Agric. Food Chem., 50(14):4080-86); Schieberle and Hofmann ("Characterization of Key Odorants in Dry-Heated Cysteine—Carbohydrate Mixtures: Comparison with Aqueous Reaction Systems," *in* Flavor Analysis, ACS Symposium Series, Vol 705); Mottram and Mottram (2002, "An Overview of the Contribution of Sulfur-Containing Compounds to the Aroma in Heated Foods," *in* Heteroatomic Aroma Compounds, ACS Symposium Series, Vol 826); and Shu-C and Ho (1989, "Parameter Effects of the thermal reaction of cysteine and 2,5-dimethyl-4-hydroxy-3(2H)-furanone," *in* Thermal Generation of Aromas, ACS Symposium Series, Vol 409).

19. In my opinion, Applicant's experiments in the '006 application and the Holz-Schietinger Declaration demonstrate that the addition of LegH to an aqueous thermal reaction mixture comprising ribose and cysteine has the surprising result of enhancing the formation of volatile compounds associate with a meaty aroma. Significantly, the 2,851-fold increase in concentration of 2-acetylthiazole when LegH was combined with Ribose and Cysteine, which is shown in the Holz-Schietinger Declaration, in my opinion signifies that novel and unexpected chemical reactions occur in the presence of LegH. 2-acetylthiazole is particularly interesting as

it has been associated in the literature with a meaty/roasty aroma. See, for example, Chemistry and Technology of Flavours and Fragrances, 2005, Ed Rowe, Blackwell Publishing Ltd; Flavor of Meat and Meat Products, 1994, Ed Shahidi, Springer-Science+Business Media, BV; Handbook of Meat and Meat Processing, 2012, 2nd Ed, Ed Hui, CRC Press; Mottram and Mottram (2002, "An Overview of the Contribution of Sulfur-Containing Compounds to the Aroma in Heated Foods," *in* Heteroatomic Aroma Compounds, ACS Symposium Series, Vol 826); and Chen and Ho (2002, J. Agric. Food Chem., 50(8):2372–76).

20. Yield changes of 0.5- to 10-fold would be consistent with optimizing a reaction pathway, however, changes of greater than 1000-fold, as is seen with 2-acetylthiazole, indicate that new pathways of formation for meaty aromas result from the inclusion of LegH in the aqueous thermal reaction mixture.

21. Additionally, the Holz-Schietinger Declaration demonstrates that 2-methyl-3-furanthiol (MFT) and the major degradation product of MFT, the dimer, bis(2-methyl-3-furyl) disulfide, were detected only in the aqueous thermal reaction samples that contained Leghemoglobin. MFT is a very potent meaty odorant, with an extremely low odor threshold at 0.007 μg/kg in water, and is known to have an important contribution to meat aroma. See, for example, Kerscher and Grosch (1998, J. Agric. Food Chem., 46:1954-58); Tang et al. (2013, J. Sulfur Chem., 34:38-47); and Withcombe and Mussinan (1988, J. Food Sci., 53:658-60). It is surprising to me that an intense meaty aroma compound such as MFT was uniquely identified in the reaction mixture upon the addition of LegH.

22. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: Dec 2, 2016

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EXHIBIT A

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Devin G Peterson

Experience:

Aug/16 **Professor** **Food Science & Technology** **Director of the Flavor Research & Education Center**	The Ohio State University	Columbus, OH
June/12 – Aug/16 **Professor** **Food Science & Nutrition** **Director of the Flavor Research & Education Center**	The University of Minnesota	St. Paul, MN
Aug/09 – June/12 **Associate Professor** **Food Science & Nutrition** **Director of the Flavor Research & Education Center**	The University of Minnesota	St. Paul, MN
July/07 – Aug/09 **Associate Professor/Food Science**	The Pennsylvania State University	University Park, PA
March/01 – June/07 **Assistant Professor/Food Science**	The Pennsylvania State University	University Park, PA
Dec/99 – Feb./01 **Research Assistant**	University of Minnesota	St. Paul, MN
July/99 - Dec./99 **Research Flavor Scientist (Internship)**	Givaudan Flavors	Cincinnati, OH
Sept./94 - July/99 **Research Assistant**	University of Minnesota	St. Paul, MN
June/94 –Aug./94 **Information Technician**	The Canadian Wheat Board	Winnipeg, MB
May – Aug./92&93 **QC Technician**	Coca-Cola Bottling Company	Winnipeg, MB

Education:	1997 - 2001 **PhD/Food Science** (Dean's List)	University of Minnesota	St. Paul, MN
	1994 - 1997 **MS/Food Science** (Dean's List)	University of Minnesota	St. Paul, MN
	1990 – 1994 **BS/Food Science** (Dean's List)	University of Manitoba	Winnipeg, MB

Awards:	Flavor and Extract Manufacturers Association - Excellence in Flavor Science Award	2015
	Waters Centers of Innovation Award	2012
	Pan-American Nutrition, Food Science and Technology Award	2008
	Presidential Early Career Award for Scientist and Engineers (PECASE)	2004
	John Brandt Memorial Foundation Scholarship; National	2000
	M. E. Franks Scholarship; National	1998, 1999 & 2000
	IFT Grad. Fellowship–Soc. of Flavor Chemists Memorial; National	1998/99 & 2000/01
	AACC Graduate Fellowship; National	1997/98
	IFT Scholarship; Minnesota	1997 & 2000
	Triticus Award; Departmental	1996
	The Canadian Wheat Board Scholarship; National	1994

Peer Reviewed Publications (*italics* is corresponding author):

1. Raithore, S. and ***Peterson, D.G.*** 2016. Delivery of taste and aroma components in sugar-free chewing gum: mass balance analysis. Chemosensory Perception. *Accepted.*
2. Paravisini, L. and ***Peterson, D.G.*** 2016. Characterization of Browning Formation in Orange Juice during Storage. ACS Symposium Proceedings, Browned Flavors: Analysis, Formation, & Physiology. *Accepted.*
3. Kokkinidou, S. ***Peterson, D.G.*** 2016. Identification of compounds that contribute to trigeminal burn in aqueous ethanol solutions. Food Chem. 211: 757-762 http://www.sciencedirect.com/science/article/pii/S0308814616308068
4. Bin, Q. and ***Peterson, D.G.*** 2016. Identification of Bitter Compounds in Whole Wheat Bread Crumb. Food Chem. 203: 8–15, http://www.sciencedirect.com/science/article/pii/S0308814616301182
5. Yeo, L., Thompson, D. and ***Peterson, D.G.*** 2016. Inclusion Complexation of Flavor Compounds by Dispersed High-Amylose Maize Starch (HAMS) in an Aqueous Model System. Food Chem. *199*: 393–400. http://www.sciencedirect.com/science/article/pii/S0308814615303320
6. Yucel, U. and ***Peterson, D.G.*** 2015. Effect of Protein–Lipid–Salt Interactions on Sodium Availability in the Mouth and Consequent Perception of Saltiness: In Solutions. J. Agric. Food Chem., 63: 7487–7493. Impact Factor: 3.1 http://pubs.acs.org/doi/abs/10.1021/acs.jafc.5b02311
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8. Forero, D.P, Orrego, C.E. **Peterson, D.G.** and *Osoria, C.* 2015. Chemical and sensory comparison of fresh and dried lulo (*Solanum quitoense* Lam.) fruit aroma. Food Chemistry, 169: 85–91. http://www.sciencedirect.com/science/article/pii/S0308814614011595
9. Kokkinidou, S. and ***Peterson, D.G.*** 2015. Characterization of compounds that influence alcohol trigeminal burn in spirits. Weurman Symposium Proceedings Flavour Science Proceedings of the XIV Weurman Flavour Research Symposium, Andrew Taylor and Donald Mottram (Eds), pg 31-37.
10. Ronningen, I. and ***Peterson, D.G.*** 2015. Application of untargeted LC/MS techniques (Flavoromics) to identify changes related to freshness of food, *in* The Chemical Sensory Informatics of Food: Measurement, Analysis, Integration. Eds. Guthrie, et al.; ACS Symposium Series; American Chemical Society: Washington, DC, Vol. 1191, pg 269-277. http://pubs.acs.org/doi/pdf/10.1021/bk-2015-1191.ch019
11. Zhang, L., Xia, Y. and ***Peterson, D.G.*** 2014. Identification of bitter modulating Maillard-catechin reaction products. J. Agric. Food Chem. 62, 10092−10100. http://pubs.acs.org/doi/abs/10.1021/jf502040e
12. Karametsi, K., Kokkinidou, S., Ronningen, I. and ***Peterson, D.G.*** 2014. Identification of Bitter Peptides in Aged Cheddar Cheese. J. Agric. Food Chem. 62, 8034−8041. http://pubs.acs.org/doi/abs/10.1021/jf5020654
13. Troise, A. D. Fiore, A. Colantuono, A. Kokkinidou, S., **Peterson, D.G.** and *Fogliano, V.* 2014. Effect of olive mill wastewater phenol compounds on reactive carbonyl species and Maillard reaction end-products in ultrahigh-temperature-treated milk. J. Agric. Food Chem. 62, 10092−10100. http://pubs.acs.org/doi/abs/10.1021/jf501919y
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15. Lui, X., Jiang, D. and ***Peterson, D.G.*** 2014. Identification of Bitter Peptides in Whey Protein Hydrolysate. J. Agric. Food Chem. 62, 5719−5725. http://pubs.acs.org/doi/abs/10.1021/jf4019728
16. Kokkinidou, S. and ***Peterson, D.G.*** 2013. Response Surface Methodology as Optimization Strategy for Reduction of Reactive Carbonyl Species in Heat-processed Foods by Means of Phenolic Chemistry. Food and Function. 4(7): 1093-1104. http://pubs.rsc.org/en/content/articlelanding/2013/fo/c3fo60032g
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Patents (inventor):
International Application (PCT). April 21st, 2015. Peterson, D. and Kokkinidou, S. Methods of Making Modified Alcohol Containing Products.

USA application. An umami-enhanced food product and method of enhancing the umami taste of food products.

Courses Taught:
Flavor Chemistry (FScN 8337), Flavor Technology (FScN 5521); Current Issues in Food Science (FScN 8318); Food Chemistry and Functional Foods (FScN 4112)

Professional Memberships:
American Chemical Society
Institute of Food Technologists

EXHIBIT B

MATERIALS CONSIDERED

- U.S. Application Serial No. 14/797,006, Fraser, R. Brown, P.O., Karr, J. and Holz-Schietinger, C., 2014, Publication No. US 2015/0351435
- Hsieh, F-h and Huff, H.E. 2012. Meat analog compositions and process. U.S. Patent Application No. US 2012/0093994 ("the '994 application")
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Attorney's Docket No. TBD / TBD

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Patrick O'Reilly Brown M.D., Ph.D.	Art Unit	:	1792
			Examiner	:	Assaf Zilbering
Serial No.	:	14/152,499	Conf. No.	:	1137
Filed	:	January 10, 2014			
Title	:	METHODS AND COMPOSITIONS FOR CONSUMABLES			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR §1.132

I, S. Christopher Davis, D.Phil, hereby declare as follows:

1. I am currently a Senior Director at Impossible Foods in Redwood City, California. I have held this position since 2012, and I am responsible for long term research, intellectual property strategies, and research partnerships with global businesses. I have lead the product development, pilot and engineering team in their work to discover, develop and scale-up food products. I have also lead the research team responsible for examining the flavor profile of animal products and for developing similar flavor profiles in products derived from plant materials.

2. Prior to my position at Impossible Foods, I was a Director at Codexis, Inc. in Redwood City, California from 2002 to 2012. In that position, I was responsible for the analytical biochemistry group, which was focused on the quantitative analysis of chemical and biological compositions to support the creation of proteins and microbial biocatalysts for the production of pharmaceutical drugs, drug intermediates, chemicals, and biofuels. I was also responsible for creating the process chemistry, analytical chemistry and fermentation groups and for leading a series of product development programs. My work was recognized by two Presidential Green Chemistry Challenge Awards (2006 and 2012). Prior to my position at Codexis, I was a Scientist III at Maxygen, Inc. in Redwood City, California from 1998 to 2002. In that position, I developed new tools and methods for directed evolution.

3. I received Bachelors of Science (B.S.) degrees in Chemistry and Biochemistry in 1989 from Southampton University, and I received a Doctor of Philosophy (D.Phil) degree in Organic Chemistry in 1992 from Oxford University. I was a postdoctoral research associate at the University of California at San Francisco from 1993 to 1998, where I worked on the control

of catalysis by cytochrome P450 enzymes and on the development of novel gene therapy methods.

4. A copy of my curriculum vitae is attached as **Exhibit A**.

5. In the preparation of my declaration, I have reviewed U.S. Application Serial No. 14/152,499 ("the '499 application"), as well as the Office Action mailed March 10, 2017, in which the claims of the '499 application were rejected as being obvious over Van den Briel et al. (EP 1759593)("Van den Briel") in view of Proulx et al. (2006, *J. Agric. Food Chem.*, 54:1518-22)("Proulx"), Asgar et al. (2010, Comp. Rev. Food Sci. Food Safety, 9:513-29)("Asgar") and Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814).

6. Proulx examined the bioavailability of heme iron derived from soy root nodule and purified soy leghemoglobin sources in a corn flour matrix (a tortilla made with corn flour and water) using a Caco-2 cell model system.

7. We performed a number of compositional analyses on corn flour. To perform these experiments, I purchased a 4.84 pound bag of Maseca masa instant corn flour ("the corn flour") from the Casa Lucas Market on 7/16/2016. Casa Lucas Market is located at 2934 24th St. in San Francisco, California. I brought the bag of the corn flour back to Impossible Foods.

8. On July 27, 2016, I weighed and then placed 500 grams of the corn flour into a clean Ziplock bag and shipped the bag to Silliker's Northern California Laboratory (referred to hereafter as "Silliker"). Silliker is located at 5262 Pirrone Court in Salida, California.

9. I instructed Silliker to analyze the elemental composition of the corn flour. Specifically, I instructed Silliker to perform compositional analysis of the corn flour for ash, carbohydrates, fats, total protein, sulfur, minerals (using ICP-MS to quantitate heavy metals), free amino acids, moisture, and monosaccharides and disaccharides including fructose, glucose, sucrose, maltose and lactose.

10. A copy of the Certificate of Analysis prepared by Silliker is provided in **Exhibit B**. Included in the Certificate of Analysis is a literature citation for each method that was used in the analysis determining the elemental composition of the corn flour. Silliker follows a validated sample preparation method appropriate for measurement of each compound identified in the

Certificate of Analysis and a validated method to determine the amount of each compound identified in the Certificate of Analysis. Silliker performs all of their analyses under good laboratory practice (GLP) conditions.

11. As shown in **Exhibit B**, the amount of each free amino acid, including methionine, was at or below the limit of quantitation for this assay. The limit of quantitation of 0.01% w/w corresponds to a concentration of 0.7 millimolar (mM) for methionine, which has a molecular weight of 149. Therefore, methionine is present at a concentration of less than 0.7 mM in the corn flour.

12. The methods utilized by Silliker cause cysteine and cystine (also known as disulfide-bonded cysteine) to degrade and so are inappropriate for quantitation of cysteine and cystine. To further evaluate the content of amino acids, including cysteine and cystine, in the corn flour, we asked Alphalyse Inc. (referred to hereafter as "Alphalyse") to determine the amounts of individual amino acids (i.e., free amino acids) in the corn flour.

13. On October 27, 2016, I placed 10 g grams of the corn flour into a clean Ziplock bag and shipped the bag to Alphalyse. Alphalyse is located at 200 Page Mill Road, Suite 100, in Palo Alto, California. I instructed Alphalyse to determine the amounts of the free amino acids in the corn flour.

14. A copy of the Amino Acid Analysis Report prepared by Alphalyse is provided in **Exhibit C**. Included in **Exhibit C** is a description of the methods that were used to analyze the free amino acids in the corn flour. Briefly, Alphalyse extracted the free amino acids by treating the corn flour with dilute acid, followed by chromatographic separation of the compounds contained within the resulting extract. The specific chromatographic conditions and the resulting chromatograms provided by Alphalyse also are shown in **Exhibit C**.

15. As shown in **Exhibit C**, none of methionine, cysteine, or cystine were detected in the corn flour at a limit of quantitation of 50 ppm. 50 ppm of methionine (molecular weight of 149) corresponds to a concentration of 0.3 mM, 50 ppm of cysteine (molecular weight of 121) corresponds to a concentration of 0.4 mM, and 50 ppm of cystine (molecular weight of 240) corresponds to a concentration of 0.2 mM. Therefore, methionine, cysteine and cystine, as free

amino acids, are present in the corn flour at a concentration of less than 0.3 mM, 0.4 mM and 0.2 mM, respectively, or less than 0.9 mM in total.

16. In a letter dated November 23, 2016 (**Exhibit D**), Alphalyse confirmed their results in respect to cysteine/cystine, stating that their analysis "did not find any trace of free Amino Acids, including cysteine/cystine" in the corn flour.

17. The methods utilized by Silliker and Alphalyse did not specifically detect for the presence of thiamine. Thiamine, however, is known to be present in white masa flour (e.g., white corn flour) at a level of 0.22 mg/100 g flour (see Suri et al., 2016, Comprehensive Reviews in Food Science and Food Safety, 15:912-926, see pg. 915; **Exhibit E**), which corresponds to a concentration of 8 micromolar (μM) thiamine in corn flour.

18. To further examine the composition of the masa flour, and, particularly, the sulfur-containing free amino acid composition of the masa flour, I weighed and then placed about 100 grams of the corn flour into three different clean Ziplock bags and shipped the bags to the Proteomics & Mass Spectrometry Facility at the Donald Danforth Plant Science Center (referred to hereafter as "Danforth") on December 13, 2016. Danforth is located at 975 North Warson Road in St. Louis, Missouri.

19. I instructed Danforth to analyze the elemental composition of the corn flour. Specifically, I instructed Danforth to perform free amino acid analysis of the corn flour.

20. A copy of the Report prepared by Danforth is provided in **Exhibit F**. Included in the Report is a description of the method that was used to analyze the free amino acids in the corn flour. Danforth follows a validated sample preparation method appropriate for measurement of free fatty acids and a validated method to determine the amount of each free fatty acid. Danforth performs all of their analyses under good laboratory practice (GLP) conditions.

21. Briefly, Danforth extracted the free amino acids by treating the flour samples with several different extraction solvents as indicated, followed by treatment with a mild acid and chromatographic separation of the compounds contained within the resulting extract. The specific chromatographic conditions and the resulting chromatograms provided by Danforth also are shown in **Exhibit F**.

22. As shown in **Exhibit F**, the amount of cysteine in the three flour samples (i.e., 0.00000%, 0.00000% and 0.00000%) were all below the limit of quantitation for this assay (i.e., 0.00050%). For cysteine, which has a molecular weight of 121, the limit of quantitation corresponds to a concentration of 0.04 mM. Therefore, cysteine is present at a concentration of less than 0.04 mM in the corn flour.

23. As shown in **Exhibit F**, the amount of cysteic acid (Cya) in the three flour samples (i.e., 0.00000%, 0.00000% and 0.00000%) were all below the limit of quantitation for this assay (i.e., 0.00050%). For Cya, which has a molecular weight of 169, the limit of quantitation corresponds to a concentration of 0.03 mM. Therefore, Cya is present at a concentration of less than 0.03 mM in the corn flour.

24. As shown in **Exhibit F**, the amount of disulfide bonded cysteine (Cys-Cys), also known as cystine, in the three flour samples (i.e., 0.00000%, 0.00000% and 0.00000%) were all below the limit of quantitation for this assay (i.e., 0.00050%). For Cys-Cys, which has a molecular weight of 240, the limit of quantitation corresponds to a concentration of 0.02 mM. Therefore, Cys-Cys is present at a concentration of less than 0.02 mM in the corn flour.

25. As shown in **Exhibit F**, the amount of methionine in the three flour samples (i.e., 0.00041%, 0.00037% and 0.00040%) were all below the limit of quantitation for this assay (i.e., 0.00050%). For methionine, which has a molecular weight of 149, the limit of quantitation corresponds to a concentration of 0.03 millimolar (mM). Therefore, methionine is present at a concentration of less than 0.03 mM in the corn flour.

26. As shown in **Exhibit F**, the amount of methionine sulfoxide (MetS) in the three flour samples (i.e., 0.00000%, 0.00000% and 0.00000%) were all below the limit of quantitation for this assay (i.e., 0.00050%). For MetS, which has a molecular weight of 165, the limit of quantitation corresponds to a concentration of 0.03 mM. Therefore, MetS is present at a concentration of less than 0.03 mM in the corn flour.

27. In summary, based on the analyses described above and the published literature, it is my opinion that the corn flour tortilla of Proulx contains no more than 0.15 mM of free amino acids that contain sulfur (i.e., methionine, cysteine, disulfide bonded cysteine, cysteic acid, and methionine sulfoxide).

28. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: 20th July 2017

S. Christopher Davis, Ph.D.

EXHIBIT A

S. Christopher Davis

Summary

The application of science to improve the quality of life with minimal environmental impact has been the focus of my career. I am an experienced industrial biotechnologist, with a passion for building and mentoring scientific teams to achieve strategic goals in a safe, cost effective, focused manner. Broadly experienced, but with a focus on the optimization of strains and proteins to meet commercial targets. Proven ability to conceive, implement and lead challenging research projects to completion. An independent thinker and team player, with excellent interpersonal and leadership skills who maintains high standards and adapts rapidly to changing business needs.

Professional Experience

Impossible Foods, Redwood City, CA Senior Director

Accomplishments:

- Responsible for research partnerships with global businesses to rapidly expand production of our foods.
- Responsible for long term research and intellectual property strategy
- Built and lead the product development, pilot and engineering team from 2012 to 2015, leading the discovery, development and scale up of Impossible Foods products.
- Built and lead the research team from 2012 to 2016 to understand why animal products are so delicious and how to recreate the composite structures and flavors of animal products from plant derived materials.
- Creation and maintenance of a safe and collaborative environment for innovation

Codexis, Redwood City, CA Director

Accomplishments:

BioIndustrials

- Responsible for the development of Codexol™, leading the strain and process development to transform cellulosic sugars into detergent alcohols.
- Built and led the Analytical Biochemistry-Bioindustrials group of 25 scientists. The group is responsible for analysis, ranking and characterisation of protein and strain variants. Core skills include high throughput screening, microfermentation, microbiology, protein chemistry, rheology, analytical chemistry, protein secretion, and enzymology.
- Concurrently built and led the Fermentation Research and Development group of 11 scientists., responsible for the development and tech transfer of safe, cost effective fermentation processes for the production of biofuels. Core skills involve fermentation process and media development, adaptive evolution and microbial physiology.

Pharmaceuticals

- Presidential Green Chemistry Challenge award for Greener Synthetic Pathways 2012 for "An Efficient Biocatalytic Process to Manufacture Simvastatin"
- Led the research team that developed the first commercial biocatalytic process enabled by shuffling, currently running at the multi-ton scale. This work has been recognized by:
    - Presidential Green Chemistry Challenge award for Greener Reaction Conditions 2006 for "Directed Evolution of Three Biocatalysts to Produce the Key Chiral Building Block for Atorvastatin, the Active Ingredient in Lipitor®"
    - The cover of Chemistry and Engineering News and Nature Biotechnology
    - The award of 5 patents, publication in Nature Biotech.
- Built and led the Analytical Biochemistry-Pharmaceuticals group of 30 scientists. The group is responsible for analysis, ranking and characterization of variants for every directed evolution campaign. Core skills include HTS, chemistry, analytical chemistry, heterologous protein expression, enzymology, process miniaturization, statistics and high throughput experimentation.
- Started the Biocatalysis, Chemical development and Analytical Chemistry groups in Codexis, responsible for chemical process development, biocatalysis and method development and analytical support for all projects.
- Founding member of Codexis in 2002

Maxygen, Redwood City, CA

Accomplishments:

- Led a team of 4 scientists focused on the development of new assay tools for use in directed evolution, including HTP chiral analysis and protein purification/quantitation and patch arrays for protein activity analysis.
- Developed and implemented methods for directed evolution in *E. coli*, *P. pastoris* and the baculovirus expression system enabling product opportunities in protein therapeutics, agriculture and chemical processing.
- Coinvented the ProSAR algorithm incorporating statistical sequence-function analysis into directed evolution.

Education

University of California at San Francisco Prof. P. Ortiz de Montellano and Prof F.C. Szoka Jr.

D.Phil., Organic Chemistry, Oxford University, UK Professor Sir J. E. Baldwin, F.R.S.

US Patents

9,228,223 Structure-activity relationships
8,849,575 Methods, systems, and software for identifying functional biomolecules
8,535,910 Halohydrin dehalogenases and related polynucleotides
8,252,554 Halohydrin dehalogenases and related polynucleotides
8,101,395 Halohydrin dehalogenases and related polynucleotides

8,014,961 Integrated systems and methods for diversity generation and screening
7,939,309 Glucose dehydrogenase polypeptides and related polynucleotides
7,833,767 Ketoreductase polypeptides and related polynucleotides
7,824,898 Halohydrin dehalogenases and related polynucleotides
7,816,111 Glucose dehydrogenase polypeptides and related polynucleotides
7,807,423 Enzymatic processes for the production of 4-substituted 3-hydroxybutyric acid derivatives
7,783,428 Methods, systems, and software for identifying functional biomolecules
7,751,986 Methods, systems, and software for identifying functional biomolecules
7,747,391 Methods, systems, and software for identifying functional biomolecules
7,629,157 Ketoreductase polypeptides and related polynucleotides
7,588,928 Halohydrin dehalogenases and related polynucleotides
7,541,171 Halohydrin dehalogenases and related polynucleotides
7,462,469 Integrated system for diversity generation and screening
7,132,267 Enzymatic processes for the production of 4-substituted 3-hydroxybutyric acid derivatives and vicinal cyano, hydroxy substituted carboxylic acid esters
7,125,693 Enzymatic processes for the production of 4-substituted 3-hydroxybutyric acid derivatives
6,605,430 DNA shuffling of monooxygenase genes for production of industrial chemicals

Published Patent Applications

WO/2016/054375 Methods for extracting and purifying non-denatured proteins
WO/2015/038796 Secretion of heme-containing polypeptides
US/20150017696 Recombinant host cells and processes for producing 1,3-butadiene through a crotonol intermediate
US/20130165341 Structure-activity relationships
US/20130157900 Structure-activity relationships
US/20120208259 Halohydrin Dehalogenases and Related Polynucleotides
US/20110257023 Methods, systems, and software for identifying functional biomolecules
US/20110161265 Methods, systems, and software for identifying functional bio-molecules
US/20100304459 Glucose dehydrogenase polypeptides and related polynucleotides
US/20100167345 Halohydrin dehalogenases and related polynucleotides
US/20100028972 Ketoreductase polypeptides and related polynucleotides
US/20090298125 Halohydrin dehalogenases and related polynucleotides
US/20090118130 Structure-activity relationships
US/20070161094 Enzymatic processes for the production of 4-substituted 3-hydroxybutyric acid derivatives
US/20060195947 Ketoreductase polypeptides and related polynucleotides
US/20050272064 Halohydrin dehalogenases and related polynucleotides
US/20050153417 Halohydrin dehalogenases and related polynucleotides
US/20050095619 Glucose dehydrogenase polypeptides and related polynucleotides

Peer reviewed articles

A green-by-design biocatalytic process for atorvastatin intermediate. Steven K. Ma, John Gruber, Chris Davis, Lisa Newman, David Gray, Alica Wang, John Grate, Gjalt W. Huisman and Roger A. Sheldon Green Chem., 2010, 12, 81-86

Bayesian credible intervals for response surface optima Richard J. Fox, David Elgart, S. Christopher Davis. Journal of Statistical Planning and Inference (2009), 139(3), 2498–2501

Improving catalytic function by ProSAR-driven enzyme evolution. Fox, Richard J.; Davis, S. Christopher; Mundorff, Emily C.; Newman, Lisa M.; Gavrilovic, Vesna; Ma, Steven K.; Chung, Loleta M.; Ching, Charlene; Tam, Sarena; Muley, Sheela; Grate, John; Gruber, John; Whitman, John C.; Sheldon, Roger A.; Huisman, Gjalt W. Nature Biotechnology (2007), 25(3), 338-344.

Novel enzyme activities and functional plasticity revealed by recombining highly homologous enzymes. SunAi Raillard, Anke Krebber, Yonghong Chen, Jon E Ness, Ericka Bermudez, Rossana Trinidad, Rachel Fullem, Christopher Davis, Mark Welch, Jennifer Seffernick, Lawrence P Wackett, Willem P.C Stemmer, Jeremy Minshull. Chemistry & Biology 8 (2001) 891-898

Conditionally stable liposomes for gene delivery. Davis, S. C.; Szoka, F. C., Jr. ACS Symposium Series (1999), 728(Intelligent Materials for Controlled Release), 179-189.

Cholesterol Phosphate Derivatives: Synthesis and Incorporation into a Phosphatase and Calcium-Sensitive Triggered Release Liposome. Davis, S. C.; Szoka, F. C. Jr. Bioconjugate Chemistry (1998), 9(6), 783-792.

Glu-325 and Asp-328 Are Determinants of the Cyp4A1 Hydroxylation Regiospecificity and Resistance to Inactivation by 1-Aminobenzotriazole. Dierks, Elizabeth A.; Davis, S. Christopher; Ortiz de Montellano, Paul R. Biochemistry (1998), 37(7), 1839-1847.

Effects of cytochrome P450 inhibitors on EDHF-mediated relaxation in the rat hepatic artery. Zygmunt, Peter M.; Edwards, Gillian; Weston, Arthur H.; Davis, S. Christopher; Hoegestaett, Edward D. British Journal of Pharmacology (1996), 118(5), 1147-1152.

Oxidation of ω-oxo fatty acids by cytochrome P450BM-3 (CYP102). Davis, S. Christopher; Sui, Zhihua; Peterson, Julian A.; Ortiz de Montellano, Paul R. Archives of Biochemistry and Biophysics (1996), 328(1), 35-42.

Cleavage of the 5-amino-5-carboxy-2-oxapentanoyl side chain from enzymically synthesized penicillins and cephalosporins. Baldwin, Jack E.; Davis, S. Christopher; Forrest, Andrew K.; Schofield, Christopher J. Bioorganic & Medicinal Chemistry Letters (1995), 5(21), 2507-12.

EXHIBIT B

MERIEUX NutriSciences

CERTIFICATE OF ANALYSIS

SILLIKER, Inc.
Northern California Laboratory
5262 Pirrone Court, Salida, CA 95368
Tel. 209/ 549 7508 Fax. 209/ 545 0245

COA No:	CCA-39255182-0
Supersedes:	None
COA Date	8/9/16
Page 1 of 3	

TO:
Mr. Tuan Truong
Sr. Quality Control Specialist
Impossible Foods Inc.
525 Chesapeake Drive
Redwood City, CA 94063

Received From:	Redwood City, CA
Received Date:	7/28/16
P.O.# / ID:	CD072716/1234
Location of Test: (except where noted)	Salida, CA

Analytical Results

Desc. 1: Maseca Harina
Desc. 2: 500g Powder

Laboratory ID: 362549446
Condition Rec'd: NORMAL
Temp Rec'd (°C): 27

Analyte	Result	Units	Method Reference	Test Date	Loc.
Ash	1.21	% (w/w)	AOAC 945.46	8/2/16	
Carbohydrates - Calculation	74.26	% (w/w)	Calculation	8/4/16	
Fat - Mojo, Acid Hydrolysis	4.49	% (w/w)	AOAC 933.05	8/4/16	
Free Amino Acids			USDA MSS2 (1993)	8/8/16	CHG
Aspartic Acid	0.02	% (w/w)			
Threonine	0.03	% (w/w)			
Serine	<0.01	% (w/w)			
Glutamic Acid	0.02	% (w/w)			
Glycine	<0.01	% (w/w)			
Alanine	0.01	% (w/w)			
Valine	<0.01	% (w/w)			
Methionine	<0.01	% (w/w)			
Isoleucine	<0.01	% (w/w)			
Leucine	<0.01	% (w/w)			
Tyrosine	<0.01	% (w/w)			
Phenylalanine	<0.01	% (w/w)			
Lysine	<0.01	% (w/w)			
Histidine	<0.01	% (w/w)			
Arginine	<0.01	% (w/w)			
ICP MS Full Mineral Screen			EPA 3050/6020 USP730	8/3/16	CHG
ICPMS Prep	Acid Digest	-			
Aluminum	2.1	ppm (w/w)			
Antimony	<0.01	ppm (w/w)			
Arsenic	0.02	ppm (w/w)			
Barium	0.12	ppm (w/w)			
Beryllium	<0.01	ppm (w/w)			
Bismuth	<0.02	ppm (w/w)			
Boron	1.4	ppm (w/w)			
Cadmium	0.006	ppm (w/w)			
Calcium	552	ppm (w/w)			
Chromium	0.03	ppm (w/w)			
Cobalt	0.03	ppm (w/w)			

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Page 2 of 3	

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Analytical Results

Desc. 1: Maseca Harina
Desc. 2: 500g Powder

Laboratory ID: 362549446
Condition Rec'd: NORMAL
Temp Rec'd (°C): 27

Analyte	Result	Units	Method Reference	Test Date	Loc.
Copper	1.41	ppm (w/w)			
Iron	14.6	ppm (w/w)			
Lead	<0.01	ppm (w/w)			
Lithium	<0.1	ppm (w/w)			
Magnesium	886	ppm (w/w)			
Manganese	5.14	ppm (w/w)			
Mercury	<0.005	ppm (w/w)			
Molybdenum	0.38	ppm (w/w)			
Nickel	0.20	ppm (w/w)			
Phosphorus	2090	ppm (w/w)			
Potassium	2810	ppm (w/w)			
Selenium	<0.1	ppm (w/w)			
Silver	<0.02	ppm (w/w)			
Sodium	231	ppm (w/w)			
Strontium	1.01	ppm (w/w)			
Thallium	<0.01	ppm (w/w)			
Thorium	<0.1	ppm (w/w)			
Tin	<0.01	ppm (w/w)			
Titanium	4.12	ppm (w/w)			
Uranium	<0.01	ppm (w/w)			
Vanadium	0.01	ppm (w/w)			
Zinc	17.9	ppm (w/w)			
Zirconium	<0.1	ppm (w/w)			
ICP Sample Prep - Microwave	Microwave	-	AOAC 984.27	8/2/16	CHG
Moisture - Vacuum Oven	11.38	% (w/w)	AOAC 927.05	7/29/16	
Protein - Kjeldahl			AOAC 991.20.I	8/2/16	
Protein Factor	6.25	-			
As Received	8.66	% (w/w)			
Reducing Sugar	0.30	% (w/w)	AOAC 968.28	8/8/16	CHG
Sugars			AOAC 980.13	8/8/16	CHG
Fructose	<0.25	% (w/w)			
Glucose	0.30	% (w/w)			

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Desc. 1: Maseca Harina
Desc. 2: 500g Powder

Laboratory ID: 362549446
Condition Rec'd: NORMAL
Temp Rec'd (°C): 27

Analyte	Result	Units	Method Reference	Test Date	Loc.
Sucrose	1.29	% (w/w)			
Maltose	<0.25	% (w/w)			
Lactose	<0.25	% (w/w)			
Sulfur			EPA 6010	8/4/16	CHG
As Received	87.1	mg/100g			
Sulfur	87.1	mg/100g			

Mike Erickson — Laboratory Director

Noted Test Locations: CHG-Silliker, Inc. Illinois Laboratory, 3600 Eagle Nest Drive, North Building, Crete, IL 60417

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Except as otherwise stated, Mérieux NutriSciences Terms and Conditions for Testing Services apply.

EXHIBIT C

Amino Acid Analysis Report

Order 16967

PICK 'n POST

Experimental

Samples received

The following sample was received at Alphalyse for protein analysis.

flour

Amino Acid Analysis

Amino acid analysis is a method to determine the absolute amounts of individual amino acids in a sample. The method can be applied to samples containing free amino acids, as well as to peptides and protein samples after hydrolysis into amino acids. Amino acid analysis can be used for determination of the relative composition of amino acids in a protein, for determination of the absolute amount of a protein or peptide if the amino acid sequence is known, and for purity estimation of a purified protein.

The acid hydrolysis is performed for 20 hours at 110 degrees Celcius, in 6 M HCl, 0.1% phenol, 0.1% thioglycolic acid. The hydrolysis takes place under reduced pressure in an atmosphere of argon. Identification and quantification of the amino acids takes place on a BioChrom 30 amino acid analyzer using ion exchange chromatography, post-column derivatization with ninhydrin and detection at two wavelengths, 570 nm and 440 nm. A known amount of the natural amino acid sarcosine (Sar) is added as an internal control standard. The common 20 amino acids, except tryptophan and cysteine, are determined. Under the used conditions tryptophan is degraded, and the yield of cysteine is so variable that it is not calculated. Asparagine is determined as aspartic acid and glutamine as glutamic acid.

Data interpretation

In the amino acid analysis it should be noted that:

1. Serine and threonine are degraded slightly during acid hydrolysis, and recoveries can be 10% lower than expected.
2. Methionine can be oxidized during hydrolysis, usually less than 10% is oxidized.
3. Valine and isoleucine bonds (Val-Val, Ile-Val, Val-Ile, Ile-Ile) are difficult to hydrolyse and recoveries can be 5-15% lower than expected.
4. Glycine content is often higher than expected because it is a frequent contaminant due to its use in many buffers.
5. The results table contains the columns Raw pmol and Corrected pmol. Analysis of known amounts of BSA standards is used to determine a Compensation factor to correct for differences in ninhydrin reactivity and hydrolysis efficiency (Data from the standard can be provided on request).
6. The raw chromatography data are provided in page 3.

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Amino Acid Analysis Report

Order 16967

PICK 'n POST

Results

2,16mg of the sample was subjected protein removal by acetone precipitation and supernatant was used for free amino acid analysis.

Order # 16967
AAA order # 848
Signal strength None
Appendix: 20161108_011_Method Report

	ANALYSIS INFO	ANALYSIS DATA		CALCULATIONS	
Amino acids	**MW of free AA**	**Raw pmole**	**Corrected pmole**	**Calc. % of AA**	**µg AA in loaded sample**
Asp/Asn	133	0	0		0,00
Thr	119	0	0		0,00
Ser	105	0	0		0,00
Sar					
Glu/Gln	147	0	0		0,00
Gly	75	0	0		0,00
Ala	89	0	0		0,00
Cys	121				
Val	117	0	0		0,00
Met	149	0	0		0,00
Ile	131	0	0		0,00
Leu	131	0	0		0,00
Tyr	181	0	0		0,00
Phe	165	0	0		0,00
His	155	0	0		0,00
Lys	146	0	0		0,00
Arg	174	0	0		0,00
Pro	115	0	0		0,00
Trp	204				
Total		**0**	**0**		**0,00**

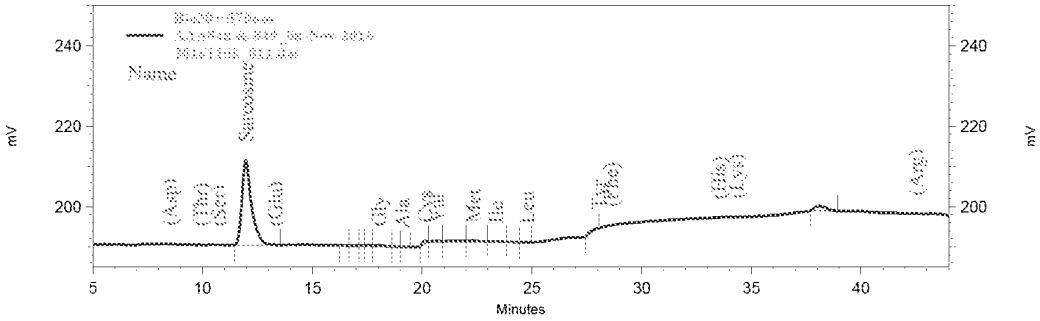
Compensation factor 1,04
Loaded on instrument 2,16 mg

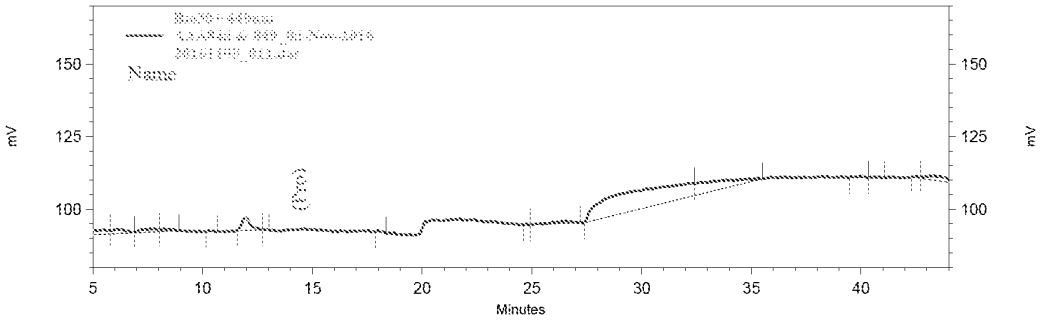
Amount in sample (µg AA/mg sample)	**0,00**

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Alphalyse - Biochrom 30+

Method: C:\Biochrom\OpenLAB Projects\Default\Method\NaHP alphalyse 0215.met
Standard: C:\Biochrom\OpenLAB Projects\Default\Result\20161108_011.dat
Name: **AAA848 & 849_16967_flour2** Date : 08/11/2016 23:26:21 (GMT -00:00)
Instrument Serial No : 121820 Column No : b-5080 Resin No : 20966





Bio30+ 570nm Results

Amino acid	Retention time [min.]	Area	ESTD amount [pmol]
Asp			**0.0 BDL**
Thr			**0.0 BDL**
Ser			**0.0 BDL**
Sarcosine	12.0	64055154	**0.0**
Glu			**0.0 BDL**
Gly	18.0	700058	**52.8**
Ala	19.1	63856	**22.9**
Cys	20.1	997802	**0.0**
Val	20.6	459100	**0.0**
Met	22.3	1451145	**59.0**
Ile	23.4	913855	**70.6**
Leu	24.8	117841	**99.3**
Tyr	28.1	1213658	**225.7**
Phe			**0.0 BDL**
His			**0.0 BDL**
Lys			**0.0 BDL**
Arg			**0.0 BDL**
Totals		69972469	**530.3**

Bio30+ 440nm Results

Amino acid	Retention time [min.]	Area	ESTD amount [pmol]
Pro			**0.0 BDL**
Totals			

EXHIBIT D

Impossible Foods Inc.

Att: Chris Davis

November 23, 2016

Regarding order 16967

This is to confirm that we have conducted amino acid analysis on sample: flour

2,16mg of the sample was subjected to protein removal by acetone precipitation and supernatant was used for free amino acid analysis. We did not find any trace of free Amino acids, including cysteine/cystine, in the supernatant at level >50 ppm.

Best regards,

Thomas Kofoed

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EXHIBIT E

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Comprehensive REVIEWS in Food Science and Food Safety

Effects of Different Processing Methods on the Micronutrient and Phytochemical Contents of Maize: From A to Z

Devika J. Suri and Sherry A. Tanumihardjo

Abstract: Maize is a staple human food eaten by more than a billion people around the world in a variety of whole and processed products. Different processing methods result in changes to the nutritional profile of maize products, which can greatly affect the micronutrient intake of populations dependent on this crop for a large proportion of their caloric needs. This review summarizes the effects of different processing methods on the resulting micronutrient and phytochemical contents of maize. The majority of B vitamins are lost during storage and milling; further loss occurs with soaking and cooking, but fermentation and nixtamalization (soaking in alkaline solution) can increase bioavailability of riboflavin and niacin. Carotenoids, found mainly in the kernel endosperm, increase in concentration after degermination, while other vitamins and minerals, found mainly in the germ, are reduced. Mineral bioavailability can be improved by processing methods that reduce phytic acid, such as soaking, fermenting, cooking, and nixtamalization. Losses of micronutrients during processing can be mitigated by changes in methods of processing, in addition to encouraging consumption of whole-grain maize products over degermed, refined products. In some cases, such as niacin, processing is actually necessary for nutrient bioavailability. Due to the high variability in the baseline nutrient contents among maize varieties, combined with additional variability in processing effects, the most accurate data on nutrient content will be obtained through analysis of specific maize products and consideration of *in vivo* bioavailability.

Introduction

Maize is one of the most produced cereal crops in the world, with over 120 million metric tons for human consumption in 2011 (FAO 2011), and it is a staple food for over 1.2 billion people (Lozano-Alejo and others 2007). Maize contributes almost one-third of the calories in the food supply of Central America and Southern Africa (FAO 2011), and even higher in some individual countries, such as Zambia, in which maize contributes over half of the calories in the food supply (FAOSTAT 2013). Such populations may be dependent on maize for micronutrients as well as macronutrients; among a group of Guatemalans, intake of maize tortillas was found to contribute a mean energy intake of 1164 kcal/d, and provided at least half of the daily requirement of calcium, iron, and zinc (Krause and others 1992).

Maize is a crop that develops ears that each contains 300 to 1000 kernels. Types of maize include sweet, popcorn, dent (starchy or floury), and flint, which is also used as animal feed (FAO 1992). Although the most commonly consumed varieties are white or yellow, maize can be found in other colors including red, blue, purple, and orange (FAO 1992; Gannon and others 2014). The kernel is mainly for storage of starch, but it also contains protein, fat, vitamins, and minerals. The major parts of the kernel are shown in Figure 1. Kernel weight is distributed among the endosperm (70% to 90%), germ (10% to 12%), and seed coat [pericarp plus tipcap (5% to 6%)] (FAO 1992; Bressani and others 2002). The minerals in maize are found mainly in the germ, which contains about 78% of that in the whole kernel (FAO 1992), yet many processing methods remove the germ, in part to improve shelf-life. The most abundant minerals in maize are phosphorus, magnesium, and potassium. Vitamins are found in all major parts of the kernel, including the endosperm (provitamin A carotenoids), germ (vitamin E), and aleurone (water-soluble vitamins) (FAO 1992). Maize contains no vitamin D or B_{12}, which must be obtained from animal foods. The vitamin and mineral contents of maize and some common maize products are listed in Table 1 and 2.

Maize is consumed across the world in a variety of whole and processed products. Whole-grain maize is consumed boiled or roasted on the cob, canned or frozen sweetcorn, and as popcorn. Different processing methods result in changes to the nutritional profile of maize products, which vary by nutrient and strain of maize. Types of maize processing methods and examples of maize products from around the world can be found in Table 3. The most common processing method is milling, which grinds the maize into coarse whole-grain pieces or fine flour and removes much of the bran and germ, as in the case of refined maize flour. Alternative types of milling produce different products; products of

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Motif Exhibit 1150, Page 347 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

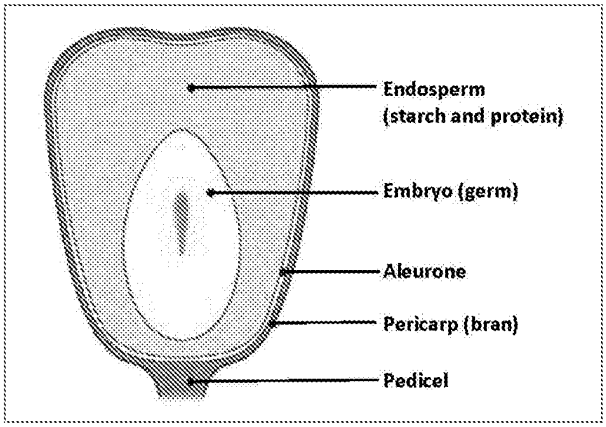


Figure 1–Major components of a maize kernel. Adapted from: Micklos, http://www.carolina.com/teacher-resources/Interactive/using-carolina-corn-ears-to-teach-genetic-imprinting/tr28902.tr.

dry milling include grits, meal, and flour; products of wet milling include starch, syrups, and dextrose, mainly for industrial uses; traditional grinding methods retain some of the maize germ (Slavin and others 2000). Milled maize can be processed by heat into foods such as porridge, polenta, grits, baked goods, and other locally named dishes. Traditional preparation methods often include further processing, such as soaking, fermentation, and nixtamalization (a process used in the production of tortillas in which maize is mixed with alkaline lime solution, heated, and soaked overnight, resulting in a chemically altered dough), which affects the content and bioavailability of several nutrients (Bressani and others 1958). Other maize products include snack foods, such as deep-fried maize chips, and industrially extruded, puffed, or flaked products. Extrusion is a quick, high-temperature technology used for processing maize that results in chemical and nutritional changes in the resulting maize products (Athar and others 2006). Each of these processing methods can affect the nutritional value of the maize.

Nutritional changes during processing are important to consider when assessing nutrient intake in populations who consume a large proportion of their daily intake as maize or maize products. Furthermore, nutrient losses have important implications in the nutritional value of processed maize products targeted toward populations at risk of nutrient deficiencies. The objective of this paper is to review the effects of different processing methods on the nutrient contents of maize products by individual micronutrients (including vitamins and minerals) and some other important phytochemicals. Effects on macronutrients are reviewed separately (Ai and Jane 2016).

Effect of Maize Processing on Vitamin and Mineral Contents

Provitamin A carotenoids: β-carotene, α-carotene, and β-cryptoxanthin

Vitamin A requirements are estimated using the concept of retinol activity equivalent (RAE), which, in addition to preformed retinol and retinyl esters found in animal products, supplements, and fortified foods, describes the amount of provitamin A carotenoids required to be converted to 1 μg retinol from a mixed diet in healthy people. The Inst. of Medicine (IOM) set RAEs at 12 μg for β-carotene, and 24 μg for α-carotene and β-cryptoxanthin (Otten and others 2006), which is based on the structural differences among them. Some studies have shown more efficient conversion factors (Muzhingi and others 2011), likely related to vitamin A status among other factors (Tanumihardjo 2008; Tanumihardjo and others 2010). The Recommended Daily Allowance (RDA) for vitamin A is 900 μg RAE and 700 μg RAE for men and women, respectively (Otten and others 2006).

Carotenoid content of maize is highly variable among different strains; some varieties can contain as much as 80 μg total carotenoids/g dry weight (Pixley and others 2013), while white maize contains little to no retinol activity (Kean and others 2008; USDA 2011; Žilić and others 2012). In the yellow maize kernel, over 90% of carotenoids are found in the endosperm (Lozano-Alejo and others 2007), specifically the horny endosperm, which contains 74% to 81%, and the floury endosperm, which contains 9% to 23%. In contrast, the bran and germ contain only 1% and 2% to 4% of the carotenoids, respectively (Blessin and others 1963). The carotenoid contents of maize and some maize products are shown in Table 4.

Biofortification. Biofortification is the process of improving nutritional profiles of crops through traditional breeding or biotechnology (Nestel and others 2006). Due to the natural variability in carotenoid content of maize, breeding efforts have aimed to increase the provitamin A carotenoids in maize, from less than 2 μg provitamin A carotenoids/g in yellow varieties to a target of 15 μg/g in orange varieties (Pixley and others 2013). One strain of high-β-carotene maize meeting this target contains 10 to 15 times the β-carotene of other strains, as well as increased amounts of α-carotene and β-cryptoxanthin (Li and others 2007; USDA 2011). Another example of provitamin A biofortified maize contains 12.7 μg β-carotene/g, 0.95 μg α-carotene/g and 1.62 μg/g β-cryptoxanthin (Li and others 2007). A randomized controlled study with high-β-carotene maize (about 20 μg β-carotene equivalents at harvest) found that children consuming the orange biofortified maize had similar total body vitamin A increases at the end of the trial compared with children given vitamin A supplements at the level of the RDA, indicating good bioefficacy of the β-carotene from biofortified maize (Gannon and others 2014).

Harvest, drying, and storage. Mugode and others (2014) examined the carotenoid content of biofortified provitamin A maize, harvested both at its "green" stage (60% moisture) and immediately boiled and roasted, and 2 mo later when dried on the stalk (12% moisture). Their analysis data showed that the green maize retained more total carotenoids than the dry maize, due mainly to higher levels of lutein and zeaxanthin, while the dry maize had higher concentrations of provitamin A carotenoids (for the majority of genotypes tested).

A recent review found carotenoid retention in maize during storage to be highly variable (39% to 78%), and in addition to storage time, retention can depend on environmental conditions, such as temperature, light exposure, and humidity, maize genotype, and initial carotenoid content (de Moura and others 2015). Mugode and others examined the effect of storage on dry maize stored shelled or on the ears over a period of 6 mo in a "traditional storage bin at ambient temperature" in Zambia. Provitamin A carotenoid levels dropped to about 50% within the first 30 d, after which the content stabilized, although levels continued to decrease slightly over time. There was little difference between maize kernels stored shelled or on the ear (Mugode and others 2014). Burt and others (2010) found that the carotenoid content of high-carotenoid maize lines stored in the dark at 25 °C remained stable for the first 3 mo but declined significantly by 6 mo

Motif Exhibit 1150, Page 348 of 1599

Table 1–Mineral contents of maize and common maize products.

Maize product	USDA NDB no.	Nutrient content/100 g[a] Calcium (mg)	Copper (mg)	Iron (mg)	Magnesium (mg)	Manganese (mg)	Phosphorus (mg)	Potassium (mg)	Selenium (mg)	Sodium (mg)	Zinc (mg)
Whole kernels											
Yellow maize (dent), dry	20014	7	0.314	2.71	127	0.485	210	287	15.5	35	2.21
Yellow maize (sweet), raw	11167	2	0.054	0.52	37	0.163	89	270	0.6	15	0.46
White maize, dry	20314	7	0.314	2.71	127	0.485	210	287	15.5	35	2.21
Milled products											
Flour, whole grain, yellow	20016	7	0.23	2.38	93	0.46	272	315	15.4	5	1.73
Flour, degermed, yellow	20018	2	0.142	0.91	18	0.056	60	90	8	1	0.37
Flour, whole grain, white	20316	7	0.23	2.38	93	0.46	272	315	15.4	5	1.73
Flour, masa, white	20019	138	0.209	1.47	93	0.376	231	262	10.5	5	1.8
Flour, masa, white, enriched	20017	138	0.209	8.5	93	0.376	231	262	10.5	5	1.8
Flour, whole-grain, blue	20315	5	0.154	1.74	110	0.54	263	381	2.2	5	2.24
Cornmeal, degermed, yellow	20422	3	0.076	1.1	32	0.174	99	142	10.5	7	0.66
Corn bran	20015	42	0.248	2.79	64	0.14	72	44	16.5	7	1.56
Processed products											
Grits, yellow	08160	2	0.075	1	27	0.106	73	137	17	1	0.41
Hominy, canned, yellow	20330	10	0.03	0.62	16	0.07	35	9	3	345	1.05
Tortillas	18363	81	0.154	1.23	72	0.326	314	186	6.1	45	1.31
Tortilla chips, yellow	25028	104	0.105	1.32	84	0.357	234	206	8.4	310	1.46
Chips, corn-based, extruded	19003	138	0.101	1.2	72	0.333	194	144	7.6	514	1.29
Popped corn, yellow	19034	7	0.262	3.19	144	1.113	358	329	0	8	3.08
Kellogg's corn flakes	08020	5	0.198	28.9	39	0.168	102	168	8.3	729	1
Recommended daily allowances[b]											
Men		1000	0.90	8	265	2.3	700	4700	55	1500	11
Women[c]		1000	0.90	18	420	1.8	700	4700	55	1500	8

[a]Source: USDA Nutrient Database (USDA 2011).
[b]Recommended daily allowances are for adults 19 to 30 y of age (Otten and others 2006).
[c]Nonpregnant, nonlactating.

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Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Table 2–Vitamin contents of maize and common maize products.

Maize product	USDA NDB no.	Nutrient content/100 g[a] Vitamin (μg)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Pantothenic (mg)	Vitamin B_6 (mg)	Folate (μg)	Vitamin B_{12} (μg)	Vitamin C (mg)	Vitamin D (μg)	Vitamin E (mg)	Vitamin K (μg)
Whole kernels													
Yellow maize (dent), dry	20014	11	0.39	0.20	3.63	0.42	0.62	19	0	0	0	0.49	0.3
Yellow maize (sweet), raw	11167	9	0.16	0.06	1.77	0.72	0.09	42	0	6.8	0	0.07	0.3
White maize, dry	20314	0	0.39	0.20	3.63	0.42	0.62	N/A	0	0	0	N/A	N/A
Milled products													
Flour, whole grain, yellow	20016	11	0.25	0.08	1.90	0.66	0.37	25	0	0	0	0.42	0.3
Flour, degermed, yellow	20018	11	0.07	0.06	2.66	0.05	0.10	48	0	0	0	0.15	0.3
Flour, whole grain, white	20316	0	0.25	0.08	1.90	0.66	0.37	25	0	0	0	0.42	0.3
Flour, masa, white	20019	0	0.22	0.10	1.63	0.19	0.48	29	0	0	0	0.12	0
Flour, masa, white, enriched	20017	0	1.48	0.81	9.93	0.19	0.48	209	0	0	0	0.12	0
Flour, whole-grain, blue	20315	N/A	0.16	0.23	2.60	0.55	0.47	N/A	N/A	N/A	N/A	N/A	N/A
Cornmeal, degermed, yellow	20422	11	0.14	0.05	1.00	0.24	0.18	30	0	0	0	0.12	0
Corn bran	20015	4	0.01	0.10	2.74	0.64	0.15	4	0	0	0	0.42	0.3
Processed products													
Grits, yellow	08160	11	0.13	0.04	1.20	0.49	0.15	5	0	0	N/A	N/A	0.3
Hominy, canned, yellow	20330	N/A	0.00	0.01	0.03	0.15	0.01	1	0	0	0	N/A	N/A
Tortillas	18363	0	0.09	0.07	1.50	0.11	0.22	5	0	0	0	0.28	0
Tortilla chips, yellow	25028	8	0.13	0.04	1.50	0.41	0.20	22	0	0	0	2.97	0.6
Chips, corn-based, extruded	19003	3	0.05	0.08	0.84	0.19	0.17	8	0	0	0	7.23	6.3
Popped corn, yellow	19034	10	0.10	0.08	2.31	0.51	0.16	31	0	0	0	0.29	1.2
Kellogg's corn flakes	08020	490	1.34	1.52	17.90	0.23	1.79	357	5	21	3.6	0.12	0
Recommended daily allowances[b]													
Men		900	1.2	1.3	16	5	1.3	400	2.4	90	5	15	120
Women[c]		700	1.1	1.1	14	5	1.3	400	2.4	75	5	15	90

[a] *Source:* USDA Nutrient Database (USDA 2011); biotin content not available; N/A, data not available.
[b] The recommended daily allowances listed are for adults 19 to 30 y of age (Otten and others 2006).
[c] Nonpregnant, nonlactating.

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Motif Exhibit 1150, Page 350 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Table 3–Types of maize processing methods and examples of food products.

Processing method	Brief description	Example products
Milling		
Dry milling and degermination	Particle size reduction, mechanical separation and removal of bran and germ from endosperm for a highly shelf-stable product	Quick grits, refined flour
Wet milling	Industrial process which separates maize into starch, protein, oil, and fiber constituents; not typically used at the household level	Starch, syrups, dextrose
Traditional stone-grinding	Retains some of the maize germ	Whole maize meal, whole kernel ("speckled") grits
Heat	Boiling, baking, roasting, deep-frying	Porridge, bread, chips, popped corn, whole ears of maize ("corn-on-the-cob"), canned and frozen kernels
Extrusion	Quick, high-temperature/pressure industrial process	Extruded chips and flakes
Nixtamalization	Maize is mixed with alkaline lime solution, heated, soaked, and rinsed, resulting in a chemically altered dough known as *masa*	Tortillas
Other traditional methods: soaking, germination, fermentation	Traditional maize preparation may involve one or more of these methods	*Ogi*, a fermented porridge eaten in Nigeria

Sources: Athar and others (2006), Bressani and others (1958), Gwintz and Garcia-Casal (2014), Nuss and Tanumihardjo (2010), and Slavin and others (2000).

Table 4–Carotenoid contents of maize and common maize products.

		Nutrient content/100 g [a]							
Maize product	USDA NDB no.	Vitamin A RAE (μg)	Retinol (μg)	β-Carotene (μg)	α-Carotene (μg)	β-Cryptoxanthin (μg)	Vitamin A (IU)	Lycopene (μg)	Lutein + Zeaxanthin (μg)
Whole kernels									
Yellow maize (dent), dry	20014	11	0	97	63	0	214	0	1355
Yellow maize (sweet), raw	11167	9	0	47	16	115	187	0	644
White maize, dry	20314	0	0	N/A	N/A	N/A	0	N/A	N/A
Milled products									
Flour, whole grain, yellow	20016	11	0	97	63	0	214	0	1355
Flour, degermed, yellow	20018	11	0	97	63	0	214	0	1355
Flour, whole grain, white	20316	0	0	1	0	1	3	0	5
Flour, masa, white	20019	0	0	2	0	2	5	0	6
Flour, masa, white, enriched	20017	0	0	2	0	2	5	0	6
Flour, whole-grain, blue	20315	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cornmeal, degermed, yellow	20422	11	0	97	63	0	214	0	1628
Corn bran	20015	4	0	32	21	0	71	0	1355
Processed products									
Grits, yellow	08160	11	0	97	63	0	214	0	1355
Hominy, canned, yellow	20330	N/A	0	N/A	N/A	N/A	110	N/A	N/A
Tortillas	18363	0	0	1	0	1	2	0	3
Tortilla chips, yellow	25028	8	0	39	56	57	158	0	1198
Chips, corn-based, extruded	19003	3	0	20	2	41	69	0	527
Popped corn, yellow	19034	10	0	89	58	0	196	0	1450
Kellogg's corn flakes	08020	490	481	83	54	0	1786	0	339

[a] *Source:* USDA Nutrient Database (USDA 2011). N/A, data not available.
RAE, retinol activity equivalents, which is 12 μg β-carotene or 24 μg α-carotene and β-cryptoxanthin estimated to yield 1 μg retinol for healthy, vitamin A-adequate individuals.

of storage, with a total carotenoid loss of 35% to 40%, but then remained stable up to 18 mo. Burt and others (2010) also compared drying 6 high-carotenoid maize lines at −80 (freeze-drying), 25, or 90 °C; among all but one of the lines, freeze-drying retained the most carotenoids initially, while maize dried at 25 or 90 °C exhibited similar initial losses. During 4 mo of dark storage, there was no further loss in the freeze-dried samples stored at −80 °C. The maize lines originally dried at 25 or 90 °C were stored at 25 °C in the dark, and after 4 mo showed losses ranging from 24% to 61%, with an inverse relationship between losses due to drying and storage for each genotype; those strains that lost more carotenoids during the drying process tended to lose less during storage, and vice versa. The carotenoid profiles (that is, relative amounts of each) of these lines were maintained throughout, while total carotenoid losses occurred during drying and storage (Burt and others 2010). In summary, unless maize was freeze-dried, it lost substantial quantities of carotenoids during long-term storage.

Milling and soaking. As the majority of carotenoids in maize are found in the endosperm, degermination has very little impact on the quantity and, thus, maize meal and flour contain more carotenoids than the bran and germ. Pillay and others (2014) found a higher retention of provitamin A carotenoids in biofortified maize meal (106% to 137%) compared with *samp* (broken maize grain, 95% to 118%), but both had values similar to un-milled maize or slightly higher, perhaps due to the breakdown of the maize kernel matrix. Li and others (2007) found soaking and wet

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Motif Exhibit 1150, Page 351 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

milling of high-β-carotene maize kernels resulted in 7% to 9.5% losses in α-carotene, β-carotene, and β-cryptoxanthin.

Boiling, baking, and extrusion. Pillay and others (2014) examined carotenoid retention among cooked porridges made from provitamin A-biofortified maize. *Phutu* (a very thick porridge made from boiling maize meal) and cooked *samp* retained 78% to 118% provitamin A carotenoids (89% to 129% of the unprocessed maize), while a thin porridge retained only 63% to 71% β-cryptoxanthin during cooking (76% to 92% that of unprocessed maize) and 72% to 78% of the β-carotene (78% to 97% that of unprocessed maize). Muzhingi and others (2008) found that both thick and thin yellow maize porridges retained, or even increased, their carotenoid contents, with the exception of β-cryptoxanthin, which decreased about 20% in the thin porridge. Li and others (2007) examined the effects of fermentation on carotenoid retention in high β-carotene maize porridge, and they found that while fermentation prior to cooking reduced losses during cooking, losses during fermentation made up for this resulting in similar total carotenoid losses of about 25% from cooking alone or fermenting and then cooking. Kean and others (2008), on the other hand, found that maize meal porridge retained only 52% to 60% of total carotenoids. Overall, maize porridge preparations retain 50% to over 100% of initial carotenoids. Values greater than 100% may be due to processing and cooking which increase bioaccessibility during extraction procedures (Tanumihardjo and others 2010).

There are different estimates of carotenoid retention for baked maize products, with Muzhingi and others (2008) reporting 25% to 50% retention of provitamin A carotenoids in baked corn muffins. Kean and others (2008), on the other hand, found that baked corn bread retained 65% to 75% of initial carotenoids. Kean and others (2008) analyzed extruded puffs made from maize meal and found carotenoid retention between 56% and 65%. Lozano-Alejo and others (2007) found that a little more than a third of provitamin A carotenoids was lost from deep-fried maize snacks. De La Parra and others (2007) found that deep-fried maize chips made from white or yellow maize had very low retention of provitamin A carotenoids (0% to 6%), while chips made from high-carotenoid maize retained about 20%. Thus, while maize porridge retains half to all its carotenoids, other heat treatments, such as baking, frying, and extrusion, have wide variation and, depending on the method used, may result in almost 0% to 75% retention.

Scott and Eldridge (2005) examined losses of carotenoids from canned and frozen maize. Canned yellow maize heated to 126.7 °C for 12 min suffered 62% losses of α-carotene, but changes to the contents of other provitamin A carotenoids were not significant. Maize processed to be sold frozen is also subjected to heat treatment. In the same study, yellow maize kernels were steam-blanched at 87.8 to 93.3 °C for about 3 min and then quickly frozen at temperatures of –17.8 to –23.3 °C. The resulting provitamin A content was similar to fresh maize, with the exception of α-carotene content which decreased by about 42% (Scott and Eldridge 2005).

Nixtamalization. Nixtamalization is often employed in Latin America for the preparation of tortillas. During this process, maize (including germ and endosperm) is cooked and then soaked in an alkaline lime solution (usually calcium hydroxide), thereby breaking down the pericarp (Serna-Saldivar and others 1993). The resulting mixture, "nixtamal," is then washed and ground into a dough called "masa" (Bressani and others 1958). The effect of nixtamalization on carotenoid content varies and may depend on what methods are used during processing. De La Parra and others (2007) found that the nixtamalization process significantly reduced levels of β-cryptoxanthin (84%) and β-carotene (38%), and further decreases occurred during additional processing into tortillas. Fried tortilla chips had effectively no provitamin A carotenoids remaining. Gutiérrez-Uribe and others (2014), on the other hand, found that the nixtamalization process released carotenoids from the kernels, yielding 100-fold higher β-carotene levels in the resulting masa; however, the subsequent processing of masa into tortillas brought carotenoid levels back down to the same level as the whole kernel content.

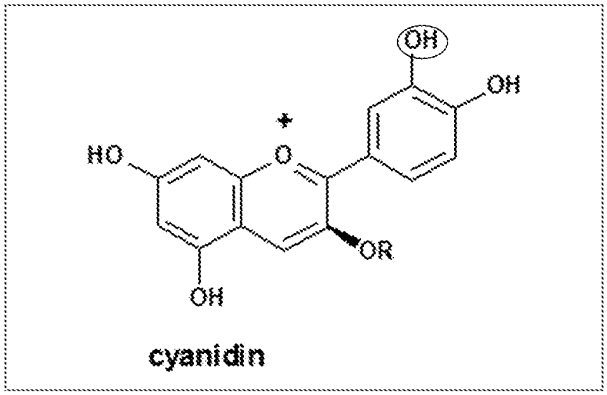


Figure 2–Polyphenols that are commonly quantified in purple, blue, or red maize include 7 anthocyanins (Collison and others 2015). The basic anthocyanin backbone is called cyanidin that contains the circled hydroxyl group. The circled functional group is not present in pelargonidin or it is methylated in peonidin. The R group can be various glycosides and include cyanidin-3-glucoside, pelargonidin-3-glucoside, cyanidin-3-(3-malonylglucoside), peonidin-3-glucoside, cyanidin-3-(6-malonylglucoside), pelargonidin-3-malonylglucoside, and peonidin-3-malonylglucoside.

Anthocyanins and other polyphenols

Different strains of maize contain varying amounts of antioxidant polyphenols. For example, colored maize strains contain high quantities of anthocyanins which confer deep pigmentation, such as blue, purple, and red, compared with white or yellow maize that has minimal quantities (De La Parra and others 2007; Žilić and others 2012). The most common anthocyanins in colored maize are cyanidins, perlargonidins, and peonidins (Figure 2) (Abdel-Aal and others 2006), which are found in the pericarp and aleurone layer of the maize endosperm (Escalante-Aburto and others 2013). Antioxidant capacity is not always directly related to total anthocyanin and polyphenol contents. For example, varieties of Mexican blue maize and American blue maize were found to contain similar amounts of anthocyanins, but the American strain contained higher levels of other polyphenols; however, despite this, the Mexican blue maize exhibited a higher antioxidant capacity (Del Pozo-Insfran and others 2006).

Total polyphenol and anthocyanin contents of whole and processed maize are shown in Table 5. Generally, thermal processing decreases phenolic content of foods; however, that decline may be due to leaching of water-soluble polyphenols into brine or syrup. Canned sweetcorn was found to have only 5% loss of phenolic content (Dewanto and others 2002). During processing of maize into tortillas and chips, often nixtamalization is employed that causes changes to the structure, chemical composition, and nutritional value of the resulting product (Bressani and others 1958).

Motif Exhibit 1150, Page 352 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Table 5–Polyphenol and anthocyanin contents of whole and processed blue maize.

Blue maize product	Polyphenols mg gallic acid/100 g Mean ± SD	Anthocyanins mg cyanidin 3-glucoside/100 g Mean ± SD
Whole kernels	266.2 ± 0.7[a]	36.87 ± 0.71[a]
Dry flour	343 ± 8.6[b]	99.5 ± 1.8[b]
		27.2[c]
	343.2 ± 6.8[d]	63.1 ± 1.4[d]
Masa	158.5 ± 1.2[a]	2.63 ± 0.12[a]
	195.4 ± 4.5[d]	27.9 ± 1.7[d]
Tortillas	161.8 ± 2.1[a]	3.81 ± 0.11[a]
	201.4 ± 5.8[d]	18.9 ± 2.9[d]
Chips	136.9 ± 1.2[a]	3.29 ± 0.10[a]

Sources:
[a]De La Parra and others (2007).
[b]Lopez-Martinez and others (2009).
[c]Cortés and others (2006).
[d]Lopez-Martinez and others (2011)

Preparation of tortillas and chips from blue maize results in overall polyphenol losses, with the nixtamalization process having the most detrimental effect. Masa made through traditional nixtamalization can lose 54% to 66% of polyphenols (Del Pozo-Insfran and others 2006).

Blue maize contains bound and free polyphenols. Bound phenolics are the primary contributors to antioxidant activity in blue maize (>80%); however, these decrease by about 75% after nixtamalization, while free phenolic antioxidant activity increases after lime-cooking and processing into tortillas and chips (De La Parra and others 2007). Further processing can decrease polyphenol content, but nixtamalization has the largest impact on losses. One study found that processing blue maize into tortillas and chips resulted in 75% and 81% losses of polyphenols, respectively (Del Pozo-Insfran and others 2006). However, the effects of traditional nixtamalization can be mitigated through an acidification treatment. Adding 0.2 g fumaric acid/100 g dry maize (reducing pH to 5.2) resulted in a 10% reduction in polyphenol losses in masa made by traditional nixtamalization (Del Pozo-Insfran and others 2006).

Up to 100% of anthocyanins in blue maize can be lost during nixtamalization, mainly by leaching into the lime solution, but different methods of nixtamalization can lead to higher retention of anthocyanins (Del Pozo-Insfran and others 2006; De La Parra and others 2007; Escalante-Aburto and others 2013). The pH is an important factor because it influences structural changes of anthocyanins during nixtamalization. One study found that nixtamalization of blue maize reduced anthocyanin content by 82%; however, when cooked without calcium hydroxide, anthocyanin loss was half of traditional nixtamalization, with 42% loss compared with dry maize flour (Cortés and others 2006). While nixtamalization causes most of the anthocyanin losses, additional processing steps can further reduce their content. Del Pozo-Insfran and others (2006) found anthocyanin losses were 37%, 54%, and 75% from nixtamal, tortillas, and chips, respectively; however, with acidification treatment, these losses were reduced to 9%, 11%, and 17%, respectively. In addition to nixtamalization, extrusion and heat treatment can reduce anthocyanin content of maize. Extrusion lowers anthocyanin content of blue maize by about 57% (Mora-Rochin and others 2010). Extracts of purple maize heated to 98 °C showed high degradation of anthocyanin content (Cevallos-Casals and Cisneros-Zevallos 2004).

B vitamins

Maize contains the B vitamins thiamin, riboflavin, niacin, pantothenic acid, B_6, folate, and biotin, but not B_{12} which is typically obtained from animal foods. These water-soluble vitamins are found in the maize endosperm and germ, with the highest concentration in the aleurone layer (Nuss and Tanumihardjo 2010). Differing amounts are present depending on the type of maize. The amounts of these nutrients found in maize and common maize products and the RDAs for adults are shown in Table 2. However, many of the B vitamins are present in a bound form in maize, therefore resulting in limited bioavailability (Hegedüs and others 1985). Processing may increase or decrease quantity and bioavailability of the B vitamins in maize.

Vitamin B_1 (thiamin). Whole grain yellow and white maize varieties contain 0.39 mg thiamin/100 g. The RDA for thiamin set by the IOM is 1.2 mg/d for adult men and 1.1 mg/d for women. Processed maize products contain less than whole grains; however, bioavailability of thiamin increases with processing. Utilization of thiamin from maize bran seems to increase with dry milling, but not wet milling, and fine particle size compared with coarse particle size, with size being more of a factor for wet-milled than dry-milled maize bran (Yu and Kies 1993). Extrusion reduces thiamin content of degermed maize grits by over 50% (Athar and others 2006). A study in Guatemala found that the nixtamalization process used in preparation of tortillas resulted in a loss of 60% and 65% of thiamin in white and yellow maize masa, respectively, compared with dry maize (Bressani and others 1958). Most of these losses were from the maize germ.

Vitamin B_2 (riboflavin). Whole yellow and white maize varieties contain 0.2 mg riboflavin/100 g, which is 15% of the RDA for adult men (1.3 mg) and 18% for women (1.1 mg). Processed maize tends to have less riboflavin, with non-enriched maize flours containing between 0.08 and 0.1 mg. Blue maize flour, however, has more riboflavin (that is, 0.23 mg/100 g) than whole yellow and white maize. Riboflavin is fairly evenly distributed in the maize kernel; thus milling results in lower loss of riboflavin compared with other B vitamins. Degermed maize loses a little over 50% of riboflavin content during processing (Hegedüs and others 1985). The production of maize masa was found to reduce levels of riboflavin 52% and 32% in white and yellow maize, respectively (Bressani and others 1958). Fermentation and germination (sprouting) of maize can increase its nutritive value. One study showed that maize fermented after germination had significantly higher levels of riboflavin (Lay and Fields 1981). Extrusion reduces riboflavin content of degermed maize grits by about 16% (Athar and others 2006).

Vitamin B_3 (niacin). Dry maize contains about 3.6 mg niacin/100 g. The RDA is 16 mg for men and 14 mg for women. Most of the niacin in maize is found in the aleurone, germ, and endosperm (Heathcote and others 1952). However, niacin is bound in maize, making it less available for absorption during digestion, that is, only about 0.4 μg niacin/g maize is found as free nicotinic acid or nicotinamide (Wall and Carpenter 1988). Processing of maize may reduce total niacin but can also release bound niacin, making it more bioavailable. One study showed that maize fermented after germination had significantly higher levels of niacin (Lay and Fields 1981). Roasting maize can release bound niacin (Kodicek and others 1974). Nixtamalization reduces total levels of niacin by about 30% in white and yellow varieties (Bressani and others 1958), but the remaining niacin becomes more bioavailable (Kodicek and others 1956). Extrusion reduces niacin content of degermed maize grits by 25% (Athar and others 2006).

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Motif Exhibit 1150, Page 353 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Dry-milling of maize increases bioavailability of niacin, while wet-milling decreases it (Yu and Kies 1993).

Pellagra is a deficiency disease resulting from inadequate dietary niacin and/or tryptophan (Prinzo 2000). Maize is low in both of these nutrients, putting populations dependent on maize for the majority of their calories at high risk of pellagra. Pellagra as related to maize was first documented over 250 y ago in Europe after maize was introduced from the Americas; however, there was very little pellagra documented in the populations of Central America despite high consumption of maize (Prinzo 2000). It was hypothesized, and subsequently confirmed, that this was due to the increased niacin bioavailability from traditional nixtamalization (Laguna and Carpenter 1951; Kodicek and others 1956; Harper and others 1958). Furthermore, typical maize is also deficient in tryptophan, which can spare niacin deficiency due to conversion of tryptophan to niacin, which is why other cereals low in niacin do not have the same pellagragenic effect as maize (Goldsmith and others 1961; Prinzo 2000).

Vitamin B_5 (pantothenic acid). Dry maize contains 0.42 mg pantothenic acid/100 g, about 8% of the adult RDA (5 mg/d) (Otten and others 2006; USDA 2011). The amount of pantothenic acid increases in whole-grain maize flour to 0.66 mg/100 g, and grits and popcorn contain slightly more than dry maize at approximately 0.5 mg/100 g, while other forms of processed maize contain less pantothenic acid than dry maize. Pantothenic acid utilization is higher in dry-milled or finely ground maize compared with wet-milled or coarse-ground maize (Yu and Kies 1993).

Vitamin B_6. Vitamin B_6 is present in food in free or bound forms of pyridoxine, pyridoxal, and pyridoxamine. In maize, more than half of its B_6 content occurs as pyridoxal (Roth-Maier and others 2002). Dry maize contains 0.62 mg vitamin B_6/100 g, almost half of the RDA for adults (1.3 mg). This amount decreases in processed maize. Prececal digestibility of B_6 from maize was found to be 67% in pigs (Roth-Maier and others 2002); however, B_6 in maize bran may be unavailable to humans (Kies and others 1984). Extrusion has little effect on pyridoxine content of degermed maize grits (Athar and others 2006).

Vitamin B_7 (biotin). Biotin content is not available from the USDA nutrient database. Animal studies have found that the biotin content of finely ground maize is 0.04 to 0.11 mg/kg (Anderson and others 1978), with high bioavailability estimated at 100% (Frigg 1976). Furthermore, microbiological assays determined the contents to be 7.3 μg/100 g in whole-grain maize flour and 1.4 μg/100 g in degermed maize flour. Accordingly, 100 g whole-grain flour provides about 4.1 to 10.8 μg of biotin, or 14% to 36% of the RDA (30 μg), with degermed flour providing only 1.4 μg/100 g, about 5% of the RDA.

Vitamin B_9 (folate). Dry maize contains little folate, about 19 μg/100 g. The RDA for folate is 400 μg for nonpregnant, nonlactating females, and for male adults. Whole grain maize flour contains 28 μg/100 g, which is reduced by 64% to 10 μg/100 g after degerming (Hegedüs and others 1985). Populations consuming maize as a staple may have higher rates of neural tube defect. This may explain, in part, why Hispanic populations in the United States have a higher rate of neural tube defects because they are not consuming as much folic acid from fortified wheat products (CDC 2010). Fortification of maize flour masa is a potential solution. One study modeled the effects of folic acid fortification on maize masa flour consumption, and this would increase Mexican Americans' folic acid intake by 22% from the fortificant (Hamner and Tinker 2014). There is little research on the effect of processing on folate content of maize, but in a study of spinach and broccoli, boiling resulted in folate retention of 49% and 44%, respectively, but steaming resulted in no significant decreases (McKillop and others 2002).

Calcium

The RDA for calcium is 1000 mg/d for adults aged 19 to 50 y (Otten and others 2006). Whole, dry maize is very low in calcium, containing only 7 mg/100 g (USDA 2011), with a higher concentration in the germ compared with the endosperm (Bressani and others 2004). Therefore, maize itself is not a good source of calcium. However, the nixtamalization process actually increases calcium content of maize (Mendoza and others 1998); one study found an average increased calcium content of several maize varieties to 170 mg/100 g, about an 18-fold increase from dry maize (Bressani and others 2002). Another study found a highly significant increase in calcium content associated with cooking, soaking, and soaking time of maize in lime water (calcium hydroxide solution). Furthermore, lime-soaking increased calcium concomitant with alkalinity increases (Bressani and others 2004). Thus, nixtamalized maize products can be a major source of calcium in populations consuming high amounts of these products, such as in Mexican and Central American diets (Bressani and others 2004; Hambidge and others 2005), meeting up to 78% of daily calcium requirements in Guatemalan women (Krause and others 1992).

Vitamin C

The RDA for vitamin C is 75 and 90 mg/d for women and men, respectively. Most sources indicate that processed maize does not contain detectable amounts of vitamin C (Saldana and Brown 1984; USDA 2011). However, raw sweetcorn has a content of 6.8 mg/100 g according to USDA (2011), and Dewanto and others (2002) found that raw sweet corn had a vitamin C content of 0.24 $\pm$ 0.02 μmol vitamin C/g corn; duration of heating and increased temperature had negative associations with vitamin C content. Canned sweetcorn is normally processed at 115 °C for 25 min, conditions which Dewanto and others (2002) found decreased vitamin C content by 25%. Interestingly, while vitamin C content decreased, total antioxidant activity increased with longer heating and higher temperatures, likely due to freeing of bound phenolics and ferulic acid.

Copper

The RDA for copper is 900 μg/d for adults 19 to 30 y of age (Otten and others 2006). Whole, dry maize contains 314 μg/100 g (USDA 2011). Maize is an important source of dietary copper in some populations; for example, one study found that Guatemalan women consumed an average of 880 μg copper from maize tortillas (Krause and others 1992). The effects of processing on copper content of maize have not been well-studied. Whole-grain maize products, such as popcorn and whole-grain flour, seem to retain between 60% and 80% of copper, while hominy, grits, and degermed cornmeal may retain only 10% to 24% (USDA 2011). Interestingly, phytic acid may not affect copper bioavailability to the same extent as it does iron, zinc, and other metals found in maize (Lonnerdal 2002).

Vitamin E

Vitamin E describes 8 fat-soluble compounds with α-tocopherol activity, grouped as tocopherols and tocotrienols, which serve as antioxidants (Rocheford and others 2002). The RDA for vitamin E (d-α-tocopherol) is 15 mg/d for adult men and women (nonpregnant, nonlactating) (Otten and others 2006).

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Motif Exhibit 1150, Page 354 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Dry maize contains about 0.5 to 1.5 mg d-α-tocopherol/100 g (Herting and Drury 1969; USDA 2011). Presence of tocopherols can vary by genotype, but the majority of the tocopherols in the maize kernel are found in the germ along with most of the stored oil which contains 70% to 86%, while the endosperm contains 11% to 27% (Rocheford and others 2002).

Tocopherols include α, β, δ, and γ, with α and γ as the predominant forms in maize (Kurilich and Juvik 1999). Kurilich and others (1999) found that total tocopherol content averaged 30.1 μg/g dry weight among 44 maize varieties, with γ-tocopherol most predominant at two-thirds of total tocopherol, α-tocopherol making up 27%, and δ-tocopherol only 4%. α-Tocotrienol and γ-tocotrienol are also found in maize, but in smaller quantities; γ-tocotrienol ranges from 2% to 28% of total tocol concentration and α-tocotrienol ranges from 4% to 21% (Weber 1984; Rocheford and others 2002).

Generally, processing of maize decreases vitamin E content, in part due to reduction in lipid content. Herting and Drury (1969) found α-tocopherol losses among processed maize products was very high, ranging from 35% loss for white maize meal to 73% to 98% for yellow maize in the form of meal, grits, and flakes or when puffed or shredded. Extrusion led to decreases in tocopherols and tocotrienols of 63% to 94% among other grains (including wheat, barley, rye, and oats) (Zielinski and others 2001). Crude maize oil contains about 210 mg vitamin E/100 g, which is decreased by more than half during the refining process (Ferrari and others 1996). During storage, about a third of vitamin E can be lost (Herting and Drury 1969), but, interestingly, the phytic acid in maize provides protection to the kernel from the pro-oxidant effects of the metals present, and it may also prevent damage to DNA and loss of lipids, particularly γ-tocopherol (Doria and others 2009).

Iron

The RDA for iron is 8 mg/d for men and 18 mg/d for nonpregnant, nonlactating women. Dry maize contains about 2.7 mg nonheme iron/100 g. The endosperm contains the majority of the iron in the kernel; Bressani and others (2002), for example, found that the endosperm contains 76% of kernel iron while the germ contains about 18%.

Nixtamalization may slightly decrease levels of iron in whole maize; however, these decreases were found mainly to be in the endosperm, while germ iron content actually increased, although these differences were not significant (Bressani and others 2002). Bressani and others (2004) showed that iron content of whole maize kernels was not affected by alkalinity of lime solution during nixtamalization, cooking, or cooking time. Furthermore, cooking maize in lime solution or water did not affect levels; however, steeping time was associated with a significant decrease in iron (Bressani and others 2004). Relatively high amounts of calcium may also inhibit iron bioavailability (Platt and Clydesdale 1987); therefore, soaking cooked maize in water may be preferred if iron absorption is the priority, because calcium levels are lower in water-soaked maize compared with alkaline-soaked maize (Bressani and others 2004). Extruded maize products can have higher iron levels with about one-third loss, which could be due, in part, to iron "contamination" added from extrusion machines during processing (Hazell and Johnson 1989).

Iron is bound by phytic acid, a known inhibitor of iron absorption, which highly reduces bioavailability from maize (Gillooly and others 1983; Hallberg and others 1987). Methods that reduce phytic acid will improve iron bioavailability in processed maize products (Mendoza and others 1998; Hambidge and others 2005). Removal of bran and germ results in about equal losses of phytic acid and iron, that is, about one-third to quarters (Hazell and Johnson 1989; Proulx and Reddy 2007). Soaking alone may not decrease phytic acid levels enough to improve iron absorption (Egli and others 2002). However, addition of lactic acid to unfermented maize products (that is, tortillas, arepa, and porridge) was found to increase iron bioavailability (*in vitro*), perhaps due to the improved solubility in the increased acidity (Proulx and Reddy 2007). Fermentation can reduce phytic acid levels more than cooking (66% compared to 16 to 17%) (Marfo and others 1990). Furthermore, Svanberg and others (1991) found that soaking maize flour for 24 to 48 h prior to fermentation resulted in almost a 96% reduction in phytic acid. When the researchers examined the percent of soluble iron in maize gruel, fermenting and soaking increased soluble iron from about 4% to 6%; fermentation with germinated flour or fermentation plus 10 mg phytase resulted in about 9% soluble iron, but fermentation in combination with the addition of 50 mg phytase resulted in a 99% reduction of phytic acid and a 43% increase in soluble iron. The nixtamalization process reduces phytic acid by about 20%, enough to potentially improve iron absorption (Bressani and others 2004).

Vitamin K

The RDA for vitamin K_1 (phylloquinone) is 120 μg/d for men and 90 μg/d for nonpregnant, nonlactating women (Otten and others 2006). Maize contains little phylloquinone, about 0.3 μg/100 g (USDA 2011). As a fat-soluble vitamin, the vitamin K content of maize oil is higher, at about 2.9 μg/100 g; this amount is low when compared with other oils such as rapeseed and soybean oils that contain >140 μg/100 g (Ferland and Sadowski 1992).

Vitamin K in maize oil is stable during processing; although it decreases by about 15% with heat and can be rapidly destroyed by light exposure (Ferland and Sadowski 1992). Processed maize foods, such as corn bread, grits, tortillas, and tamales, show no detectable quantities of phylloquinone, with the exception of popcorn, which has 1 to 4 μg/100 g (Ferreira and others 2006; USDA 2011; Centi and others 2015). Fried maize snacks, such as corn and tortillas chips, have 6.3 to 20.9 μg vitamin K/100 g, but this content is mainly due to the oil in which they are fried (Weizmann and others 2004). Taco shells were found to have 8.6 μg vitamin K/100 g, but this was due to the addition of hydrogenated oils during processing (Ferreira and others 2006). Hydrogenation of plant oils containing vitamin K causes the transformation of phylloquinone to dihydrophylloquinone (Davidson and others 1996), which does not occur naturally. Decreasing use of hydrogenated oils in the U.S. since 2006, when the Food and Drug Association began to require *trans*-fat labeling, has generally resulted in slight decreases in dihydrophylloquinone in processed maize foods, but no increases in phylloquinine content (Centi and others 2015).

Magnesium

Maize contains about 127 mg magnesium/100 g whole kernels (USDA 2011). The RDAs for magnesium are 265 and 420 mg for men and women, respectively (Otten and others 2006). Magnesium content of maize tends to decrease with milling when the germ is removed. Degermed flour contains 18 mg magnesium/100 g, while whole-grain maize flour retains 93 mg/100 g.

Magnesium has been studied less than iron and zinc in the context of phytic acid, but it does form phytate complexes that inhibit absorption (Bohn and others 2004). One study showed

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Motif Exhibit 1150, Page 355 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

magnesium content increased slightly, but significantly, after nixtamalization and preparation into dough and tortillas, from 165 mg/100 g unprocessed maize to 180 mg/100 g in dough and tortillas (Mendoza and others 1998). The authors offered other reasons for the increase in magnesium in the dough and tortillas as contamination from the soaking solution or transfer from grinding equipment and cooking pans.

Manganese

Manganese content of whole dry maize is about 0.49 mg/100 g. The RDAs for manganese are 2.3 and 1.8 mg/d for men and women, respectively (Otten and others 2006). Manganese content decreases with maize processing, especially with removal of the germ. Degermed maize flour contains only 0.06 mg manganese/100 g, whereas whole-grain maize flour contains 0.46 mg (USDA 2011). As with other metal minerals, reduction in phytic acid may also modestly improve absorption of manganese (Davidsson and others 1995; Lonnerdal 2002) as observed with soy formula, but this has not been well-studied in the context of maize and maize products.

Phosphorus

The adult RDA is 700 mg/d for phosphorus (Otten and others 2006). The phosphorus content of maize is around 210 mg/100 g, found mainly as phytic acid (FAO 1992). About 90% of the phytic acid in maize is found in the germ, while the remaining 10% occurs in the aleurone layer (Raboy and others 1990). Phytic acid chelates and reduces the bioavailability of iron and other nutrients (Gillooly and others 1983; Nävert and others 1985; Hallberg and others 1987). Thus, processing methods that reduce phytic acid will improve bioavailability of these nutrients in maize products (Mendoza and others 1998; Hambidge and others 2005).

Phytic acid can be reduced by one-quarter to one-third by physical removal of the maize bran and germ (Proulx and Reddy 2007). Different heat treatments of fresh and dried maize reduce phytic acid content; for example, one study found that boiling maize reduced phytic acid by 18%, while roasting maize decreased it by 24% to 53% (Khan and others 1991). The nixtamalization process reduces phytic acid by about 20% (Bressani and others 2004).

Soaking can activate endogenous phytase activity in many cereals, which decreases phytic acid; however, maize has low phytase activity and one study found that soaking whole maize for 16 h did not have an appreciable effect on phytic acid content (Egli and others 2002). On the other hand, soaking of unrefined white maize flour for at least 2 h did decrease phytic acid in another study, with a resulting 71% and 43% retention of zinc and phytic acid, respectively, with no further reduction for longer soaking times (Hotz and Gibson 2001).

During germination or fermentation of maize, phytase activity increases considerably. After 72 h of germination, phytase activity increased almost 6-fold and phytic acid levels decreased to 65% of the initial content (Egli and others 2002). Lactic acid fermentation of maize flour resulted in reduction of phytic acid to 88%, and even lower levels of phytic acid remained when using a starter culture (61%) or germinated flour (71%) (Hotz and Gibson 2001). These strategies can be combined for greater phytic acid reduction. For example, soaking maize flour with germinated flour for 24 to 48 h and adding a starter culture to facilitate fermentation or adding phytase can replace soaking and further reduce phytic acid (Svanberg and others 1991; Gibson and Ferguson 1998). Another study also found that soaking for 24 to 48 h prior to fermentation resulted in almost 96% reduction in phytic acid (Svanberg and others 1991).

Although phytic acid reduces the bioavailability of some nutrients, it also serves as an antioxidant (Graf and Eaton 1990). The phytic acid in maize provides protection to the kernel from the pro-oxidant effects of the metals present, and may also prevent damage to DNA and loss of lipids, particularly γ-tocopherol, during storage (Doria and others 2009). A low-phytic acid strain of maize was found to have double the level of free radicals compared with a wild-type strain after aging (Doria and others 2009). Thus, if lower levels of phytic acid are desired for the purpose of enhanced nutritive metal absorption, it may be important to consider the impact of low-phytic acid strains versus methods to reduce phytic acid in normal maize during processing.

Potassium

White and yellow maize contains 287 mg potassium/100 g, or about 6% of the RDA, which is 4700 mg/d for adults (Otten and others 2006). There is limited research on the effects of processing on potassium content in maize. According to the USDA Nutrient Database, whole-grain maize flour contains slightly higher amounts, at 315 mg potassium/100 g, while degermed flour contains only 90 mg/100g (USDA 2011). Further-processed maize products, such as tortillas, extruded chips, and flakes, contain 186, 144, and 168 g/100 g, respectively (USDA 2011).

Selenium

Whole-grain maize is a good source of selenium, containing about 15.5 μg/100 g (USDA 2011), which provides almost a third of the adult RDA for selenium at 55 μg/d (Otten and others 2006). Yellow maize has slightly higher quantities of selenium than white maize (Ferretti and Levander 1974). However, blue maize contains much less selenium compared with yellow or white maize, at 2.2 μg/100 g whole-grain flour (USDA 2011).

While whole-grain maize flour retains most of the selenium content, processing of maize into degermed flour or cornmeal reduces selenium content by 10% to 32% (Ferretti and Levander 1974; USDA 2011). Selenium content is further reduced by processing maize into tortillas or extruded corn chips, with losses of 51% to 61% (USDA 2011). In the case of sugared corn flake cereal, selenium losses of 40% to 46% occur, with some of the loss due to dilution of nutrient content by the added sugar (Ferretti and Levander 1974). However, due to the high content of selenium in whole yellow and white maize, processed products, such as degermed flour, chips, and flakes, still retain 15% of the RDA for selenium in 100 g.

Sodium

The RDA for sodium is 1500 mg/d for adults (Otten and others 2006). Maize is naturally very low in sodium, containing 35 mg/100 g. The sodium content of maize flour and grits is slightly lower per 100 g. However, many maize food products acquire sodium during processing, such as sodium hydroxide added to tortillas and hominy. Salt is often added for palatability reasons such that the sodium content is increased considerably especially in snack foods. Hominy, tortilla chips, and corn flakes contain between 310 and 729 mg sodium/100 g (USDA 2011).

Xanthophyll carotenoids: Lutein and zeaxanthin

Two additional carotenoids common in typical maize, lutein and zeaxanthin, have no vitamin A activity, but they have been associated with other health benefits including lowered risk of

Motif Exhibit 1150, Page 356 of 1599

Table 6--A summary of maize processing effects on retention of vitamin and mineral contents

Nutrient	Major location in kernel	Percent retention of nutrients after processing: Milling, degermination	Soaking, fermentation, germination	Nixtamalization	Heat (boiling, baking, frying, extrusion)
Provitamin A carotenoids	Endosperm	85% to 118% (Pillay and others, 2014)	Soaking: 90% to 93% (Li and others, 2007)	16% to 63% (De La Parra and others, 2007)	Porridge: 76% to 129% (Pillay and others, 2014) Tortillas: 9% to 33% Fried chips: ~0% (De La Parra and others, 2007)
Anthocyanins and polyphenols	Endosperm			34% to 63% (Del Pozo-Insfran and others, 2006)	Tortillas: 25% to 46% Fried chips: 19% to 25% (Del Pozo-Insfran and others, 2006) Extrusion: 43% (Mora-Rochin and others, 2010)
B vitamins	Aleurone, germ	28% (thiamin) 43% (riboflavin) 60% (niacin) 35% (B_6) 14% (biotin) 36% (folate) (Hegedüs and others, 1985)	Fermentation + germination: 250% (niacin) 450% (riboflavin) (Lay and Fields, 1981)	35% to 40% (thiamin) 48% to 68% (riboflavin) 70% (niacin) (Bressani and others, 1958) Increased niacin bioavailability (Kodicek and others, 1955)	Extrusion: 50% (thiamin) 100% (B_6) 84% (riboflavin) 75% (niacin) (Athar and others, 2006)
Calcium	Endosperm			18-fold increase[a] (Bressani and others, 2002)	
Copper	Germ	10% to 24% (USDA, 2011)			
Vitamin E	Germ	2% to 65% (Herting and Drury, 1969)			6% to 37% (among other grains) (Zielinski and others, 2001)
Iron	Endosperm	28% to 69% (Hazell and Johnson 1989; Proulx and Reddy 2007)	Improved bioavailability (43% increase) due to reduced phytic acid (Svanberg and others, 1991)	70% to 100% (Bressani et al., 2002, 2004)	Extrusion: 67% (Hazell and Johnson, 1989)
Vitamin K	Germ				85% (Ferland and Sadowski, 1992) Fried snacks: > 20-fold[a] (Weizmann and others, 2004)
Magnesium	Germ	14% (USDA, 2011)		109%[a] (Mendoza and others, 1998)	
Manganese	Germ	12% (USDA, 2011)			
Phosphorus (phytic acid)	Germ	65% to 75% (Proulx and Reddy, 2007)	Soaking + fermentation: 4% (Svanberg and others, 1991) Fermentation: 42% to 75% (Marfo and others, 1990; Proulx and Reddy, 2007)	80% (Bressani and others, 2004)	Boiling: 82% Roasting: 47% to 76% (Khan and others, 1991) Dry heat (tortillas): 82% (Proulx and Reddy, 2007)
Selenium	Germ	68% to 90% (Ferretti and Levander, 1974; USDA, 2011)			39% to 49% (USDA, 2011)
Xanthophyll carotenoids	Endosperm	91% to 136% (Pillay and others, 2014)	100% (Li and others, 2007)	33% to 14-fold increase (Gutiérrez-Uribe and others, 2014; De La Parra and others, 2007)	78% to 118% (porridge) (Pillay and others, 2014) 50% to 71% (bread, extruded puffs) (Kean and others, 2008) 27% to 116% (tortilla) (Gutiérrez-Uribe and others, 2014; De La Parra and others, 2007) 18% to 20% (fried chips) (De La Parra and others, 2007)
Zinc	Germ	20% to 30% (Gannon and others, 2014.; Pedersen and Eggum, 1983)	Soaking: 71% (Hotz and Gibson, 2001)	90% to 100% (Bressani and others, 2002; Bressani and others, 2004)	

[a]Increases may be from exogenous sources, such as machinery, cooking oils, or processing substrates.

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Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

macular degeneration and cataracts; there is no RDA but intakes of 10 to 20 mg/d of lutein have been recommended (Vishwanathan and Johnson 2013). In yellow maize, lutein and zeaxanthin make up 70% or more of the total carotenoid content (Kean and others 2008). Yellow maize is likely the highest source of zeaxanthin in the human diet.

The effects of processing on the xanthophylls can differ from that of the provitamin A carotenoids. Unlike provitamin A carotenoids, soaking and wet-milling do not result in significant losses of lutein or zeaxanthin (Li and others 2007). While heat treatment of maize will isomerize some lutein and zeaxanthin from all-*trans* into *cis* isomers, the total xanthophyll content may increase slightly; for example, canned maize had a 5% increase in lutein compared with fresh maize (Updike and Schwartz 2003). Pillay and others (2014) found a higher retention of zeaxanthin in maize meal (116% to 136%) compared with raw *samp* (91% to 112%) among varieties of provitamin A biofortified maize, and preparation of *phutu* or *samp* porridges from these products resulted in overall retention of zeaxanthin close to or slightly higher than that of the unprocessed maize (102% to 118%). A thin porridge contained only 78% to 88% that of the initial zeaxanthin content (Pillay and others 2014). Kean and others (2008) found that yellow maize meal into bread, porridge, or extruded puffs had lutein and zeaxanthin losses of 29% to 50%. Canned yellow maize heated to 126.7 °C for 12 min had slight, nonsignificant increases in lutein and zeaxanthin (2% to 3%) (Scott and Eldridge 2005). In preparation for commercial freezing, yellow maize kernels were steam-blanched at 87.8 to 93.3 °C for about 3 min and then quickly frozen at temperatures of −17.8 °C to −23.3 °C. Lutein content increased by about 9% while zeaxanthin content increased by 2% (Scott and Eldridge 2005).

The effect of nixtamalization on the xanthophylls varies depending on the methods used. De La Parra and others (2007) found that only about one-third of initial lutein and zeaxanthin content remained in masa, with further decreases in tortillas and chips. Conversely, Gutiérrez-Uribe and others (2014) found that the nixtamalization process yielded 10-fold higher lutein and zeaxanthin in the masa; however, the levels in the tortillas made from this masa were very similar to those of the original whole kernel.

Zinc

The RDA for zinc is 8 mg/d for nonpregnant, nonlactating adult women and 11 mg/d for men (Otten and others 2006). Dry yellow and white maize contain a little over 2 mg zinc/100 g (USDA 2011), the majority of which is found in the germ (Bressani and others 2002). The zinc content of whole maize and common maize products is shown in Table 1. However, maize also contains about 1 g phytic acid/100 g, concentrated in the germ, which forms insoluble complexes with zinc, reducing its bioavailability (Nävert and others 1985; Hídvégi and Lásztity 2002). One approach to enhance zinc absorption is the reduction of phytic acid content (Kivistö and others 1989), which can be accomplished through a variety of methods (described in section on phosphorus). Zinc absorption is also influenced by other nutrients found in maize and the rest of the diet.

Milling. Refined milling of maize removes the germ leaving mainly the starchy endosperm. This process removes many nutrients including zinc; thus, degermed maize contains only about 20% of the zinc content of whole maize (Pedersen and Eggum 1983). Gannon and others (2014) found that the zinc content of refined (germ and pericarp removed) white and orange maize had 21% and 30% of the zinc as that in the whole grain. On the other hand, removal of the germ reduces phytic acid, decreasing the phytate:zinc molar ratio, which enhances the bioavailability of the remaining zinc (Pedersen and Eggum 1983).

Thermal processing and extrusion. Heat-treated corn products can be subject to the Maillard reaction, evidenced by "browning," which can bind zinc, making it less bioavailable. Lykken and others (1986) compared the absorption of zinc from cornflakes (browned) and corn grits, finding higher zinc absorption from the grits, which they attributed to the Maillard reaction occurring in the cornflakes. The high heat and pressure of extrusion processing are unlikely to affect the content of zinc in maize, but they may reduce phytic acid and thus improve zinc availability (Singh and others 2007); however, Kivisto and others (1989) found that the extrusion process actually caused a decrease in zinc absorption in an extruded wheat bran product, but was ameliorated when phytate was removed before extrusion. Further work is needed to determine the effect of extrusion on phytic acid and zinc availability in maize products.

Soaking, germination, and fermentation. These methods can be used to reduce phytic acid and enhance zinc bioavailability. For example, soaking of unrefined maize flour for at least 2 h resulted in a 29% loss of zinc and 57% loss of phytic acid (Hotz and Gibson 2001). Germination and fermentation can increase phytase activity further than soaking alone (Svanberg and others 1991; Gibson and Ferguson 1998; Egli and others 2002). Combining these strategies may reduce phytic acid and further enhance zinc bioavailability.

Nixtamalization. Phytic acid is decreased during the nixtamalization process, which may improve the bioavailability of zinc, but there is no considerable increase in zinc itself in nixtamalized maize (Bressani and others 2002). Altering the amount of lime and length of cooking time can affect phytic acid levels. Bressani and others (2004) found the highest losses of phytic acid using 1.2% lime and 75 min cooking time, while zinc content was unchanged. Changes to the steeping time and the solution (alkaline cooking liquid versus water) also affected phytic acid; after cooking in lime, phytic acid was reduced 22%, and steeping for up to 8 h in either an alkaline or water medium reduced phytic acid further to a total of 28% to 29% losses from dry maize. Steeping for over 5 h resulted in small but statistically significant losses of zinc.

Nutrients that influence zinc absorption. Fiber, protein, calcium, and iron are all known to influence zinc absorption. Fiber has been implicated in poor zinc absorption, but this may be due more to the concomitant occurrence of phytic acid in high-fiber foods, and studies have shown that fiber actually has little independent effect on zinc absorption (Lonnerdal 2000). Generally, higher protein intakes modestly augment zinc absorption (Miller and others 2013), and animal protein, even in small quantities, can improve zinc absorption during a meal by inhibiting the negative effect of phytic acid (Sandström and others 1989). Individual amino acids enhance zinc absorption, including lysine, which is found in higher amounts in opaque or quality protein maize (Bänziger and Long 2000; Graham and others 2001). The potential impact of quality protein maize on lysine and tryptophan intake in Africa has been predicted by Nuss and Tanumihardjo (2011).

Previous studies have shown a decrease in the bioavailability of zinc in the presence of calcium and phytic acid due to the formation of zinc-calcium-phytate complexes in the intestine (Fordyce and others 1987). However, more recent research suggests that phytic acid will preferentially bind to calcium and iron and, thus, may actually increase the bioavailability of zinc (Miller and others 2013; Moretti and others 2014). This is particularly pertinent in the case of nixtamalized maize, which has enhanced levels of

Motif Exhibit 1150, Page 358 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

calcium due to the lime (Bressani and others 2002). More research is needed in this area to determine the effect of interactions between zinc, calcium, and phytate on zinc absorption.

Summary and Conclusions

Maize is a good source of some micronutrients, but amounts can vary by genotype, and different processing methods can have substantial effects on the nutrient content of the resulting products (summarized in Table 6). The majority of B vitamins are lost during long-term storage and milling (degermination), and additionally in soaking and cooking. Other processing methods, however, such as fermentation and nixtamalization, can increase bioavailability of riboflavin and niacin. Carotenoid content may increase through degermination because they are mostly found in the endosperm. A large proportion of the minerals in maize are lost during degermination, while mineral bioavailability can be improved by processing methods that reduce phytic acid, such as soaking, fermenting, cooking, and nixtamalization.

Losses to micronutrients during processing can be mitigated by changes in processing methods or reduction in processing, and also by encouraging consumption of whole-grain maize products over degermed, refined products. When losses cannot be mitigated and populations consuming the product are at risk of specific micronutrient deficiencies, these can be potentially reduced through fortification (Peña-Rosas and others 2014) or biofortification strategies (Tanumihardjo and others 2010).

The effects of different processing methods on nutrient content in maize, from field to plate, indicate that, generally, the fresher and less processed the maize is, the more nutrients it retains. However, this is nutrient-specific, and this trend is reversed in the case of nutrients such as niacin, which has very low bioavailability from unprocessed maize, but can be released during processing. As such, the general trends in the effects of processing on the micronutrient content of maize products can be used to guide food producers, food analysis and dietary nutrient evaluations. However, due to the high variability in baseline nutrient content among maize varieties, combined with additional variability in processing effects, for the most accurate data it will be necessary to analyze the actual nutrient content of the maize product. Furthermore, attention should be placed on not only the nutrient content but also the potential *in vivo* bioavailability.

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Author Contributions

D. Suri prepared the manuscript. S. Tanumihardjo conceptualized, reviewed, and edited the manuscript.

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Motif Exhibit 1150, Page 359 of 1599

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Motif Exhibit 1150, Page 360 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

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Motif Exhibit 1150, Page 361 of 1599

EXHIBIT F

DONALD DANFORTH
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Proteomics & Mass Spectrometry Facility

January 12, 2017

Name: Davis Chris
Affiliation: Industry
Sample Information:
Date In: October 5, 2016
Handler: Jia Li
No. of samples: 3
Requested Procedure(s): Free Amino Acid Analysis (FAA)

Results Summary:

The samples were weighed, they were extracted as follows: add 600µL of water: chloroform: methanol (3:5:12 v/v) extraction solvent, collect the supernatant, the residue was re-extracted with another 600µL of water: chloroform: methanol extraction solvent. Supernatants were combined then 300µL of chloroform and 450µL of water were added. The upper phase was collected and dried. The pellets were resuspended with 100 µL of 20 mM HCl. During derivatization, the samples were diluted ten times; 10 µL was used for a reaction volume totaling 100 µL, 1 µL was injected of the reaction. Further dilutions or concentrations were made to bring the signal within the linear range of the assay; these factors are noted in the reports attached. The percent of each amino acid found in the sample are tabulated below. The lower limit of quantitation for this data was 0.00050%; values lower than are below the lowest calibration point and may be inaccurate. The concentrations in pmol/µL for each amino acid detected in each sample are found in the attached data with the summary report. The concentrations are calculated using a series of standard dilutions run before the samples.

Sample Name	weight(mg)
Flour_2	98.9
Flour_3	101.9
Flour_4	100.1



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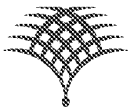
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	Percent in sample		
	Flour_2	Flour_3	Flour_4
His	0.00091	0.00074	0.00082
Asn	0.00946	0.00824	0.00871
Tau	0.00000	0.00000	0.00000
Ser	0.00098	0.00089	0.00089
Gln	0.00083	0.00071	0.00078
Arg	0.00156	0.00138	0.00140
Gly	0.00134	0.00120	0.00122
Asp	0.00326	0.00293	0.00310
Glu	0.00280	0.00250	0.00276
Thr	0.00091	0.00081	0.00092
Ala	0.00623	0.00565	0.00582
GABA	0.00344	0.00314	0.00323
Pro	0.03382	0.03038	0.03151
Cys	0.00000	0.00000	0.00000
Cya	0.00000	0.00000	0.00000
Cys-Cys	0.00000	0.00000	0.00000
Lys	0.00131	0.00118	0.00123
Tyr	0.00200	0.00175	0.00187
Met	0.00041	0.00037	0.00040
MetS	0.00000	0.00000	0.00000
Val	0.00111	0.00100	0.00104
NVa	0.00000	0.00000	0.00000
Ile	0.00047	0.00046	0.00063
Leu	0.00086	0.00060	0.00078
Phe	0.00080	0.00075	0.00095
Trp	0.00081	0.00110	0.00131

Publication acknowledgement: If any results from this method are being used for publication or grants, it is greatly appreciated to acknowledge the Proteomics & Mass Spectrometry Facility at the Danforth Plant Science Center for its contribution.

Thank you for your patronage! Please contact us if you have any questions regarding your results.

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http://www.danforthcenter.org/scientists-research/core-technologies/proteomics-mass-spectrometry

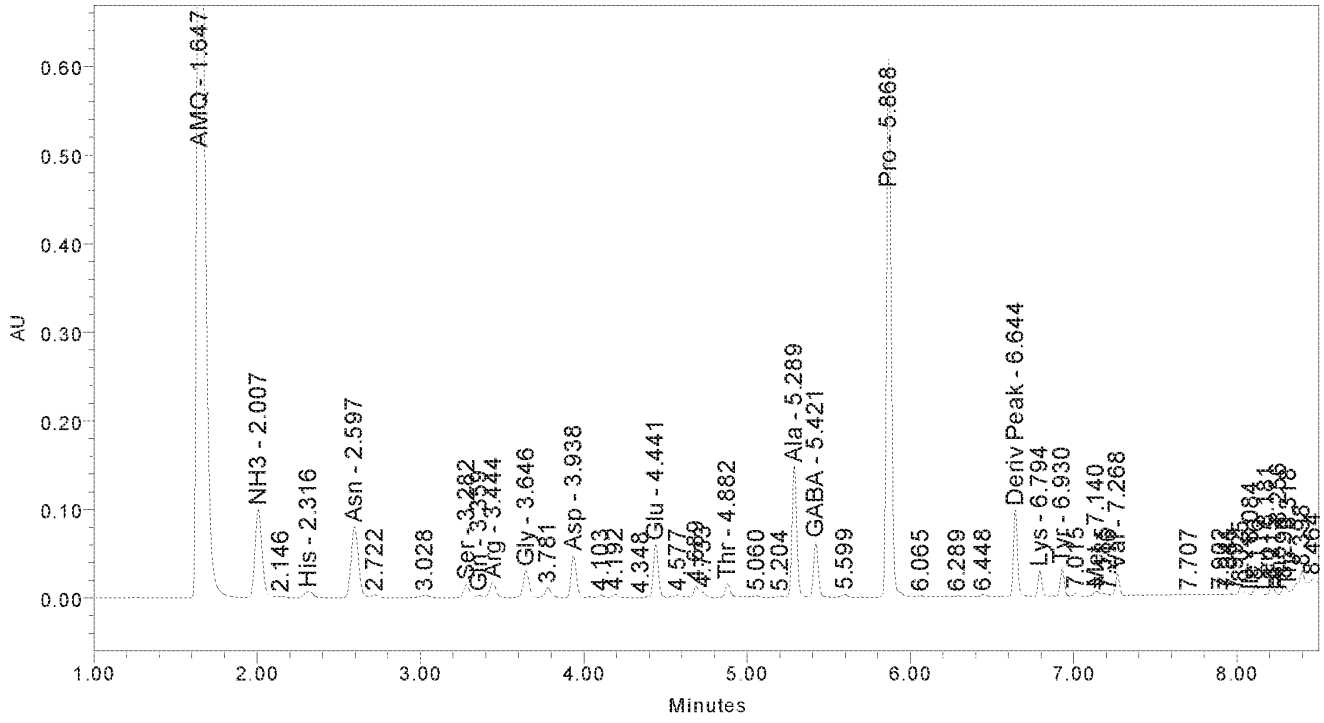
Brad Evans, Ph.D.	Director	bevans@danforthcenter.org	314-587-1464
Shin-Cheng Tzeng, Ph.D.	Staff Scientist	stzeng@danforthcenter.org	314-587-1415
Anita Durairaj, Ph.D	Staff Scientist	adurairaj@danforthcenter.org	314-587-1431
Jia Li	Senior Lab Tech	jli@danforthcenter.org	314-587-1623

AccQ-Tag Ultra Sample Information

SampleName Flour-2
MW 0
Matrix None
Vial 1:A,7
Injection 1
Injection Volume 1.00 ul
Project Name AccQTag_Ultra_Template
Acq Method Set AccQTag Cell Culture 2996
Processing Method 20150701_FAA
Report Method Name Mole Percent 2_Free AA

Sample Type Unknown
Run Time 9.5 Minutes
Dilution 1.00000
Channel ACQ-2996 260.0nm-4.8
Date Acquired 1/9/2017 1:14:55 PM CST
Date Processed 1/12/2017 12:06:46 PM CST
Current Date 1/12/2017

Auto-Scaled Chromatogram



AccQ•Tag Ultra

UPLC® Amino Acid Analysis

Peak Results

	Amino Acid	RT	pmole	Mole%
1	AMQ	1.647		
2	NH3	2.007		
3		2.146		
4	His	2.316	5.824	0.95
5	Asn	2.597	70.869	11.57
6		2.722		
7	Tau	2.902		
8		3.028		
9	Ser	3.282	9.202	1.50
10	Gln	3.359	5.654	0.92
11	Arg	3.444	8.881	1.45
12	Gly	3.646	17.678	2.89
13		3.781		
14	Asp	3.938	24.267	3.96
15		4.103		
16		4.192		
17		4.348		
18	Glu	4.441	18.823	3.07
19		4.577		
20		4.689		
21		4.733		
22	Thr	4.882	7.602	1.24
23		5.060		
24		5.204		
25	Ala	5.289	69.197	11.30
26	GABA	5.421	32.998	5.39
27		5.599		
28	Pro	5.868	290.833	47.49
29		6.065		
30		6.289		
31		6.448		
32	Deriv Peak	6.644		
33	Cys	6.700		

AccQ•Tag Ultra

UPLC® Amino Acid Analysis

Peak Results

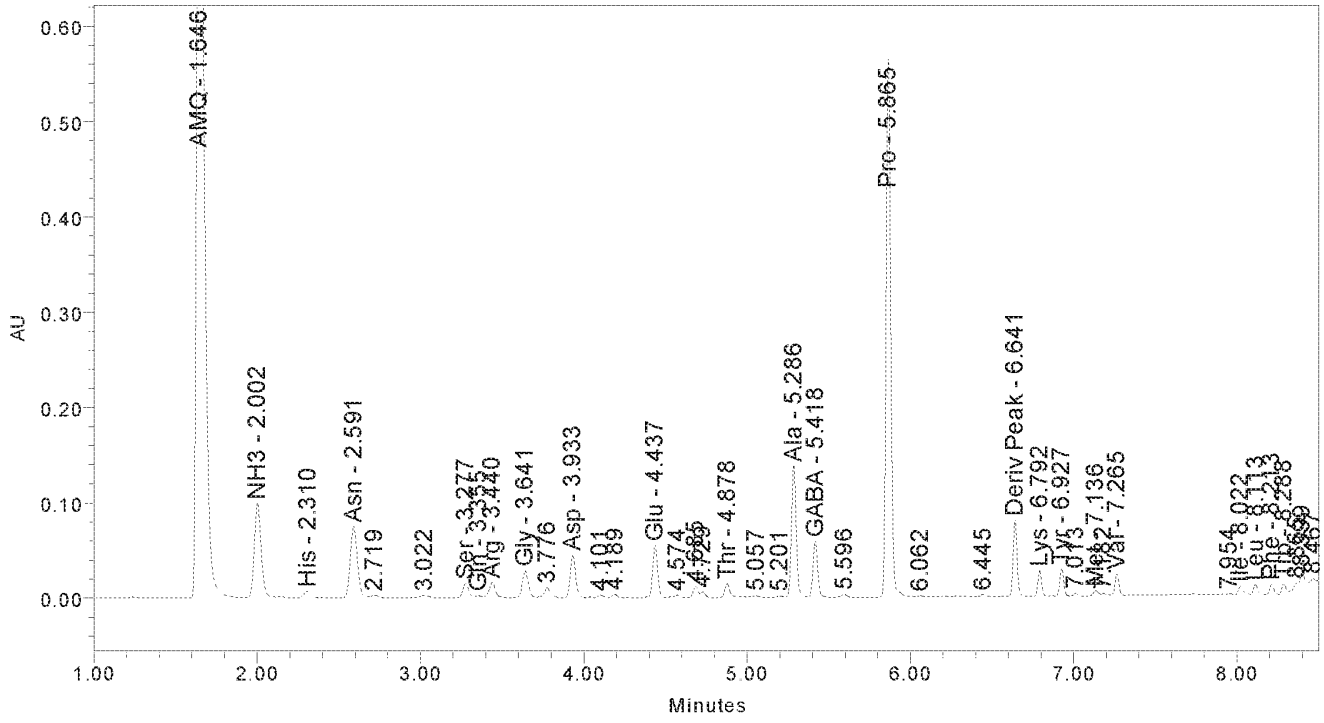
	Amino Acid	RT	pmole	Mole%
34	Lys	6.794	8.892	1.45
35	Tyr	6.930	10.918	1.78
36		7.015		
37	Met	7.140	2.689	0.44
38		7.185		
39		7.216		
40	Val	7.268	9.407	1.54
41	NVa	7.396		
42		7.707		
43		7.902		
44		7.941		
45		7.992		
46		8.025		
47	Ile	8.084	3.561	0.58
48		8.116		
49	Leu	8.181	6.479	1.06
50		8.215		
51	Phe	8.256	4.775	0.78
52		8.290		
53	Trp	8.318	3.909	0.64
54		8.398		
55		8.464		
56		8.523		
57		8.570		
58		8.806		
59		9.099		
60		9.282		

AccQ-Tag Ultra Sample Information

SampleName Flour-3
MW 0
Matrix None
Vial 1:A,8
Injection 1
Injection Volume 1.00 ul
Project Name AccQTag_Ultra_Template
Acq Method Set AccQTag Cell Culture 2996
Processing Method 20150701_FAA
Report Method Name Mole Percent 2_Free AA

Sample Type Unknown
Run Time 9.5 Minutes
Dilution 1.00000
Channel ACQ-2996 260.0nm-4.8
Date Acquired 1/9/2017 1:26:02 PM CST
Date Processed 1/9/2017 3:24:58 PM CST
Current Date 1/9/2017

Auto-Scaled Chromatogram



AccQ•Tag Ultra

UPLC Amino Acid Analysis

Peak Results

	Amino Acid	RT	pmole	Mole%
1	AMQ	1.646		
2	NH3	2.002		
3	His	2.310	4.854	0.86
4	Asn	2.591	63.635	11.25
5		2.719		
6	Tau	2.909		
7		3.022		
8	Ser	3.277	8.596	1.52
9	Gln	3.355	4.977	0.88
10	Arg	3.440	8.087	1.43
11	Gly	3.641	16.294	2.88
12		3.776		
13	Asp	3.933	22.439	3.97
14		4.101		
15		4.189		
16	Glu	4.437	17.322	3.06
17		4.574		
18		4.685		
19		4.729		
20	Thr	4.878	6.944	1.23
21		5.057		
22		5.201		
23	Ala	5.286	64.669	11.43
24	GABA	5.418	31.033	5.49
25		5.596		
26	Pro	5.865	269.158	47.57
27		6.062		
28		6.445		
29	Deriv Peak	6.641		
30	Cys	6.704		
31	Lys	6.792	8.231	1.45
32	Tyr	6.927	9.831	1.74
33		7.013		

AccQ•Tag Ultra

Peak Results

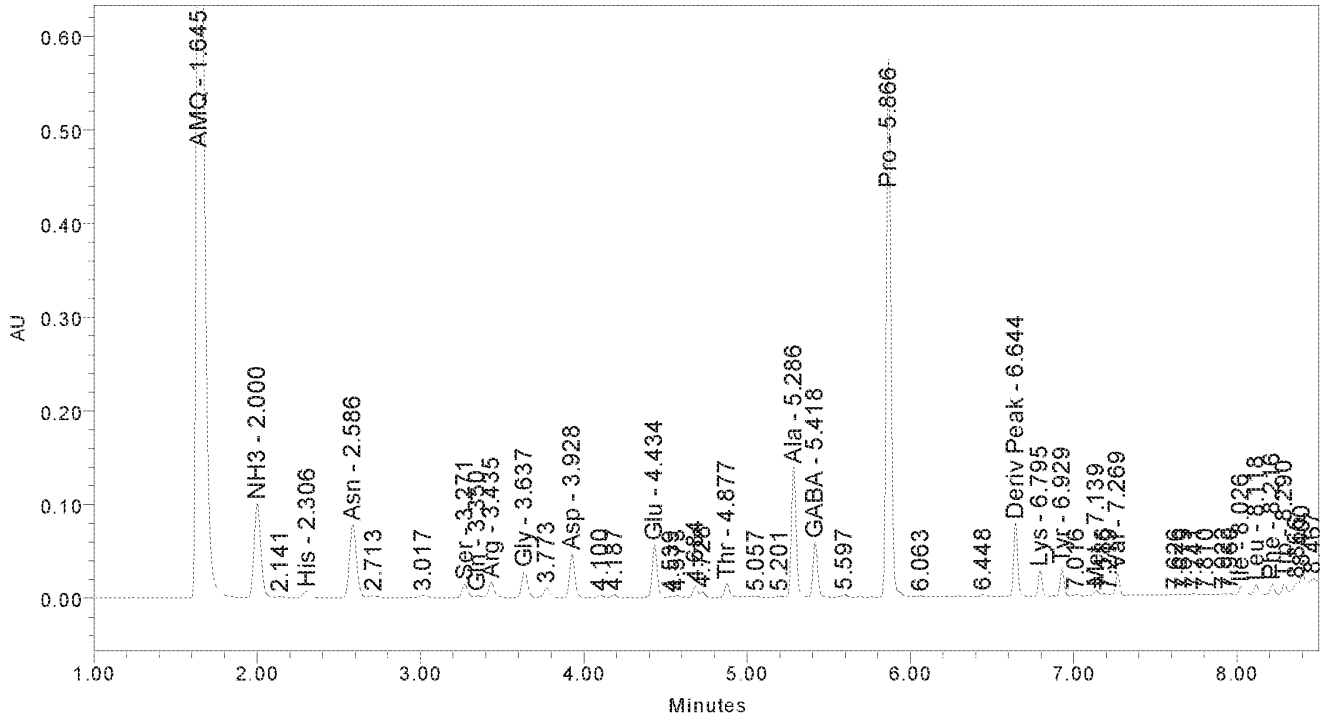
	Amino Acid	RT	pmole	Mole%
34	Met	7.136	2.527	0.45
35		7.182		
36	Val	7.265	8.746	1.55
37	NVa	7.403		
38		7.954		
39	Ile	8.022	3.609	0.64
40	Leu	8.113	4.671	0.83
41	Phe	8.213	4.624	0.82
42	Trp	8.288	5.514	0.97
43		8.365		
44		8.399		
45		8.467		
46		8.527		
47		8.572		
48		8.681		
49		8.715		
50		8.802		
51		8.860		
52		8.904		
53		8.949		
54		8.984		
55		9.101		

AccQ-Tag Ultra Sample Information

SampleName Flour-4
MW 0
Matrix None
Vial 1:B,1
Injection 1
Injection Volume 1.00 ul
Project Name AccQTag_Ultra_Template
Acq Method Set AccQTag Cell Culture 2996
Processing Method 20150701_FAA
Report Method Name Mole Percent 2_Free AA

Sample Type Unknown
Run Time 9.5 Minutes
Dilution 1.00000
Channel ACQ-2996 260.0nm-4.8
Date Acquired 1/9/2017 1:37:09 PM CST
Date Processed 1/9/2017 3:24:58 PM CST
Current Date 1/9/2017

Auto-Scaled Chromatogram



AccQ•Tag Ultra

UPLC® Amino Acid Analysis

Peak Results

	Amino Acid	RT	pmole	Mole%
1	AMQ	1.645		
2	NH3	2.000		
3		2.141		
4	His	2.306	5.284	0.91
5	Asn	2.586	66.039	11.31
6		2.713		
7	Tau	2.909		
8		3.017		
9	Ser	3.271	8.464	1.45
10	Gln	3.350	5.356	0.92
11	Arg	3.435	8.038	1.38
12	Gly	3.637	16.335	2.80
13		3.773		
14	Asp	3.928	23.316	3.99
15		4.100		
16		4.187		
17	Glu	4.434	18.791	3.22
18		4.539		
19		4.573		
20		4.684		
21		4.728		
22	Thr	4.877	7.708	1.32
23		5.057		
24		5.201		
25	Ala	5.286	65.453	11.21
26	GABA	5.418	31.375	5.38
27		5.597		
28	Pro	5.866	274.247	46.99
29		6.063		
30		6.448		
31	Deriv Peak	6.644		
32	Cys	6.704		
33	Lys	6.795	8.424	1.44

AccQ•Tag Ultra

Peak Results

	Amino Acid	RT	pmole	Mole%
34	Tyr	6.929	10.331	1.77
35		7.016		
36	Met	7.139	2.659	0.46
37		7.186		
38		7.219		
39	Val	7.269	8.867	1.52
40	NVa	7.403		
41		7.626		
42		7.679		
43		7.741		
44		7.810		
45		7.922		
46		7.960		
47	Ile	8.026	4.822	0.83
48	Leu	8.118	5.994	1.03
49	Phe	8.216	5.745	0.98
50	Trp	8.290	6.430	1.10
51		8.366		
52		8.400		
53		8.467		
54		8.527		
55		8.572		
56		8.681		
57		8.715		
58		8.803		
59		8.862		
60		8.907		
61		8.950		
62		8.983		
63		9.101		

Attorney Docket No.: 38767-0015002 / Client Ref: Meat3CON

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Rachel Fraser	Art Unit	:	1791
Serial No.	:	15/398,479	Examiner	:	Elizabeth A. Gwartney
Filed	:	January 4, 2017	Conf. No.	:	2358
Title	:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 35 U.S.C. § 1.132 OF DAVID J. LIPMAN M.D.

I, Dr. David J. Lipman, hereby declare as follows:

1. I am currently Chief Scientific Officer of Impossible Foods, the assignee of U.S. Application Serial No. 15/398,479 ("the '479 application"). I have held this position since May 2017, and am responsible for research and development and information technology. I am submitting this Declaration in support of the '479 application.

2. It is my understanding that the Office has rejected the pending claims in the '479 application as being anticipated and/or obvious over a number of references, including Tong et al. ("Blood Composition of Different Beef Breed Types", Can. J. Anim. Sci, 1986;66: 915-924); Shimbayashi et al. ("Free Amino Acids and Phosphorylethanolamine in Milk Whey of Cow", Agr. Biol. Chem, Vol. 29, No. 1, 1965;13-19); Koutsidis et al. ("Water-soluble precursors of beef flavor: I. Effect of diet and breed", Meat Science, 79, 2008;79:124-130); Field et al. ("Heme Pigment Content of Bovine Hemopoietic Marrow and Muscle", J. Food Sci., 1980;45:1109-1112); and Jamieson ("Iroquois Stew With Beef, Chicken and Pork", Bon Appetit, November 1995, http://w ww.epicurious.conilrecipesifoodiviewsiiroquois-stewwith-beef-chicken-and-pork-865). I have read the '479 application, the Office Action, and the cited art. I have also read the Declarations of Dr. Keith R. Cadwallader, Dr. Devin Grant Peterson, Dr. S. Christopher Davis and Dr. Celeste Holz-Schietinger, the cited art, and prior Office Action Responses that Impossible Foods has submitted with prior responses to Office Actions, including

those submitted in U.S. Application Serial No. 14/797,006, now U.S. Patent No. 9,700,067, which is the parent application of the '479 application.

3. I have a Bachelor of Science degree conferred on me by Brown University in 1976, and a M.D. degree conferred on me in 1980 by the State University of New York at Buffalo.

4. I am a nationally and internationally recognized expert in the field of bioinformatics. I was actively involved in the development of a series of sequence similarity algorithms, including the Wilbur-Lipman algorithm in 1983, FASTA search in 1985, BLAST in 1990, and Gapped BLAST and PSI-BLAST in 1997.

5. I am an elected member of the National Academy of Sciences and the National Academy of Medicine, and a fellow of the International Society of Computational Biology, the American Academy of Arts and Sciences, and the American College of Medical Informatics. I received the Jim Gray eScience Award from Microsoft in 2013.

6. I served as Director of the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) from 1989 to 2017. NCBI is the home of GenBank, the U.S. node of the International Sequence Database Consortium, and PubMed, one of the most heavily used sites in the world for the search and retrieval of biomedical information.

7. A copy of my curriculum vitae is attached as **Exhibit A**.

8. Under my direction, a pairwise alignment was performed on 27 heme-containing protein sequences provided in the '479 application (i.e., SEQ ID NOs. 1-27 disclosed in the '479 application), representing a diverse range of organisms, to evaluate amino acid sequence similarity. All but three of the proteins are heme-containing globin proteins from a wide phylogenetic spread. The amino acid sequences of the three non-globin proteins are provided in SEQ ID Nos: 6-8.

9. The pairwise alignments were performed using the Needleman & Wunsch alignment algorithm [Needleman SB, Wunsch CD. "A general method applicable to the search for similarities in the amino acid sequence of two proteins", J Mol Biol. 1970 Mar;48(3):443-53, attached as **Exhibit B**] as implemented by the National Center for Biotechnology Information [see https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLA

ST_PROG_DEF=blastn&BLAST_SPEC=GlobalAln&LINK_LOC=BlastHomeLink, attached as **Exhibit C**)] and run using the default parameters.

10. The results of the pairwise alignments are shown in **Exhibit D**. The table provided in Exhibit D reports the percentage of identical amino acids shared among each pair of aligned sequences. This global alignment approach was used in order to maximize the number of shared amino acids across the entire amino acid sequence, and thus it is a highly conservative approach in this context. Despite this conservative approach, this set of proteins is so diverse that many pairs exhibit very little sequence similarity.

11. Example 9 of the '479 application describes an experiment employing Gas Chromatography-Mass Spectroscopy (GCMS) and Gas Chromatography-Olfactory (GCO) methods to evaluate the production of volatile compounds, including those associated with a meaty aroma, during thermal treatment (cooking) of four aqueous flavor reaction mixtures containing four distinct globin heme-containing proteins (SEQ ID NOs: 3-5 and 18).

12. A summary of the pairwise alignment shared among each pair of aligned sequences for SEQ ID NOs: 3-5 and 18 shown in Exhibit B is provided in Table 1 below. As one can see from Exhibit B and Table 1, these 4 globin heme-containing proteins demonstrate less than 36% pairwise identity between each pair of aligned sequences. These four globin proteins represent heme-containing proteins obtained from animals, plants, and bacteria, which have been separated by over a billion years of evolution; (see, for example, Proc Natl Acad Sci U S A. 2001 Feb 27;98(5):2497-502, attached as **Exhibit E**; and Mol Biol Evol. 2015 Apr;32(4):835-45, attached as **Exhibit F**).

Table 1

		Pairwise Identity			
		SEQ ID NO:3	SEQ ID NO:4	SEQ ID NO:5	SEQ ID NO:18
Aquifex aeolicus	SEQ ID NO:3	100%	25%	19%	21%
Leghemoglobin	SEQ ID NO:4	25%	100%	36%	19%
Barley (*Hordeum vulgare*)	SEQ ID NO:5	19%	36%	100%	20%
Bovin Myoglobin	SEQ ID NO:18	21%	18%	20%	100%

13. The first flavor reaction mixture in Example 9 contained 1% (10 mg/ml) Leghemoglobin (SEQ ID NO: 4) mixed with 10 mM Cysteine and 10 mM Ribose; the second

flavor reaction mixture contained 1% (10 mg/ml) Barley hemoglobin (*H. vulgare*, (SEQ ID NO: 5) mixed with 10 mM Cysteine and 10 mM Ribose; the third flavor reaction mixture contained 1% (10 mg/ml) *B. myoglobin* hemoglobin (SEQ ID NO: 18) mixed with 10 mM Cysteine and 10 mM Ribose; and the fourth flavor reaction mixture contained 1% (10 mg/ml) an *A. aeolicus* heme-containing protein (SEQ ID NO: 3) mixed with 10 mM Cysteine and 10 mM Ribose. All flavor reaction mixtures were adjusted to pH 6.0, heated to 150°C for 3 minutes and evaluated by GCMS to identify volatile aroma compounds produced that are associated with meaty aroma.

14. The results of the above-identified experiments are set forth in Table 9 of the '479 application, which is reproduced below. The results show that that inclusion of any of the four members of this widely diverse set of heme containing proteins, proteins that in my opinion are from perhaps the farthest branches of the tree of life, in an aqueous thermal flavor reaction with Cysteine and Ribose, produced multiple aroma compounds associated with a meaty aroma upon heating.

Table 9 of the '479 Application

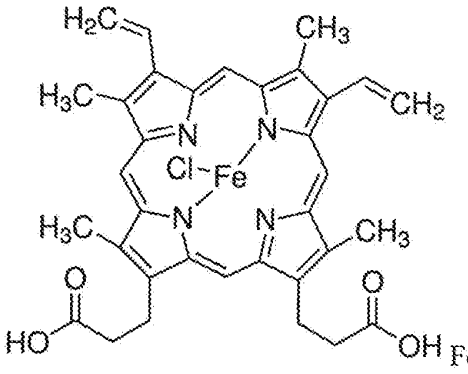
Name	LegH	Barley	*B. myoglobin*	*A. aeolicus*
Furan	x	x	x	x
Thiazole	x	x	x	x
benzaldehyde	x	x	x	x
2-acetylthiazole	x	x	x	x
2-methyl-propanal	x	x	x	x
furfural	x	x	x	x
2,3-butanedione	x	x	x	x
2-pentyl-furan	x	x	x	x
2-pentanone	x	x		
pyrazine	x	x	x	x
dimethyl trisulfide	x	x	x	x
3-methyl-butanal	x	x		x
2-methyl-thiazole	x	x	x	x
pentanal	x	x	x	x
1,3,5-cycloheptatriene	x	x	x	x
methacrolein	x	x	x	x
heptanal	x	x	x	x
2-methyl-butanal	x	x		x
isothiazole	x	x	x	x
thiophene	x	x	x	x

propanal	x	x	x	x
2-heptenal	x		x	x
methyl-pyrazine	x	x	x	x
1-octene	x		x	x
butanal	x	x	x	x
2-acetyl-propen-2-ol	x	x	x	x
pyrrole	x	x	x	x
2-methyl-furan	x	x	x	x
nonanal		x	x	x
2-propenal		x	x	x
2-decenal		x	x	x
2-nonanone		x		x
2-octanone		x	x	x
2-tridecen-1-ol,			x	x
2-octanone			x	
2-octenal			x	x
4-methyl-2-heptanone			x	x
octanal			x	x
2-undecenal				x
butyrolactone				x
1-octen-3-one				x
3-methylheptyl acetate				x
2-pentyl-thiophene				x

15. Example 13 of the '479 application describes an experiment employing GCMS methods to evaluate the production of volatile aroma compounds associated with a meaty aroma during cooking of reaction mixtures containing either myoglobin free in solution or hemin (heme unbound to protein) coupled to a solid support. A first reaction mixture included heme protein (equine myoglobin -Sigma) at 0.35% in a phosphate buffer (20 mM) at pH 6.0 with 100 mM NaCl; this was then mixed with Magic Mix (see Table 13 of the '479 application). A second reaction mixture was created with immobilized hemin (bovine hemin (Sigma Aldrich)) at 0.35% in a phosphate buffer (20 mM) at pH 6.0 with 100 mM NaCl; this was mixed with Magic Mix (Table 13 of the '479). The reaction mixtures were heated to 150°C for 3 minutes and evaluated by GCMS to identify volatile aroma compounds that are associated with meaty aroma.

16. Hemin is an example of a conjugated heterocyclic ring complexed to an iron ion, as described in the '479 application. More specifically, hemin is an iron-containing porphyrin

(protoporphyrin IX) containing a ferric iron ion (heme B) with a chloride ligand, having the structure of Formula I below.



Formula I

17. The results of the above-identified experiments are set forth in Table 17 of the '479 application, which is reproduced below. The results provided in Table 17 show that reaction mixtures containing the immobilized hemin and the heme-protein both produce multiple aroma compounds associated with a meaty aroma upon heating. Notably, the profiles of volatile compounds, measured by GCMS, produced by the reaction mixtures containing the immobilized hemin and the heme-protein, respectively, were very similar.

Table 17 of the '479 Application

Flavor compound	myoglobin	hemin-linker-resin
2-methyl-5-(methylthio)-thiophene	Low	
dihydro-5-propyl-2(3H)-furanone	Low	
octane	Low	
pyrrole	Low	Low
methanethiol	Low	Low
2-thiophenecarboxaldehyde	Low	Low
methyl-pyrazine	Low	Low
1-hydroxy-2-propanone	Low	Low
propanal	Low	Low
thiophene	Low	medium
pyridine	Low	Low
2-methyl-furan	Low	medium
oxalic acid, butyl propyl ester	Low	Low
pyrazine	medium	Low
oxalic acid, diallyl ester	medium	medium
2-butenal	medium	large
furfural	medium	medium

nonanal	medium	medium
2-ethyl-furan	medium	Low
ethanol	medium	very large
tert-butanol	medium	
3,3'-dithiobis[2-methyl]-furan	medium	medium
m-aminophenylacetylene	medium	medium
2,5-dihydro-3,4-dimethyl-furan	medium	medium
2-acetylthiazole	medium	medium
cyclohexane	medium	
ethyl tert-butyl ether	medium	
carbon disulfide	medium	medium
thiazole	medium	medium
acetonitrile	medium	large
2-pentyl-furan	medium	Low
3-thiophenecarboxaldehyde	medium	medium
2-methyl-butanal	medium	medium
thiazole	medium	large
2-methyl-3-furanthiol	large	large
2-propenal	large	large
3-methyl-2-butenal	large	medium
2-methyl-3-(methylthio) furan	large	large
ethyl acetate	large	medium
methacrolein	large	medium
methyl-thiirane	large	large
methional	large	large
methyl alcohol	large	medium
2-butanone	large	Low
2,3-butanedione	large	medium
acetone	large	large
furan	large	medium
benzaldehyde	large	medium
methyl thiolacetate	large	medium
acetaldehyde	very large	very large
2-methyl-propanal	very large	very large
dimethyl trisulfide	very large	very large
3-methyl-butanal	very large	very large
propyl-cyclopropane		medium
(E)-2-octenal		medium
2-n-propylaziridine		medium

thiirane		medium
ethyl formate		medium
methyl vinyl ketone		medium
2-propenoic acid, ethyl ester		medium
1-nonanol		large
1-octene		large
1-heptanol		large
1-dodecene		large
phorone		very large

18. In my opinion, the experimental data provided in Example 9 of the '479 application relating to the surprising ability of four very distinct members of the set of heme containing proteins to generate meat-associated volatile aroma compounds upon heating, when coupled with the experimental data provided in Example 13 of the '479 application, demonstrating that even heme unbound to protein (i.e., hemin), also shared such meat aroma and flavor production properties when heated in a reaction mixture, make it reasonable to conclude that these functional properties are not based on protein sequence similarity, but are instead based on the commonality among the proteins and the hemin experiments, namely, the inclusion of the heme co-factor.

19. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: July 11, 2017

David J. Lipman, M.D.

EXHIBIT A

July 2017

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Personal Information

Born in Rochester, New York, April 27, 1954
Married, three children

Education

B.A. Honors in Biology, 1976
Brown University, Providence, Rhode Island

M.D., 1980
State University of New York at Buffalo

Internship (Internal Medicine), 1981
University of Arizona at Tucson

Maryland State License
National Board of Medical Examiners Part III, 1981

Professional Appointments

June 2017 - Present
Chief Scientist, Impossible Foods

March 1989 – May 2017
Director, National Center for Biotechnology Information,
National Library of Medicine, NIH

April 1984 – December 2003
Senior Surgeon, Commissioned Corps, Public Health Service

July 1981 – 1989
Medical Staff Fellow, Mathematical Research Branch, NIDDK, NIH

Honors and Awards

- Elected Fellow, International Society of Computational Biology, 2009
- Elected Fellow, American Academy of Arts and Sciences, 2008

- National Institutes of Health NIH Director's Award (Public Access), 2008
- National Institutes of Health NIH Director's Award (Genome-Wide Association Studies), 2008
- Finalist, Service to America Medals, 2008
- American Society for Microbiology Promega Biotechnology Research Award, 2008
- National Institute of Allergy and Infectious Diseases NIH Merit Award, 2006
- U.S. Dept. of Health & Human Services Secretary's Award for Distinguished Service, 2004
- International Society for Computational Biology Senior Scientist Accomplishment Award, 2004
- Elected Member, National Academy of Sciences, 2003
- Priscilla M. Mayden Lecturer, University of Utah, 2002
- Elected Fellow, American College of Medical Informatics, 2001
- Elected Member, Institute of Medicine, 2000
- National Institutes of Health NIH Director's Award, Summer 1998
- Public Health Service Meritorious Service Medal, Summer 1997
- Association of Biomolecular Resource Facilities' Award, 1996
- NLM Director's Honor Award, 1996
- Public Health Service Meritorious Service Medal, Spring 1993
- Public Health Service Outstanding Service Medal, Spring 1991

Memberships

- American Academy of Arts and Sciences, Elected Fellow, April 2008 – Present
- National Academy of Sciences, Elected Member, April 2003 – Present
- American College of Medical Informatics, Elected Fellow, September 2001 – Present
- Institute of Medicine, Elected Member, October 2000 - Present
- American Association of Physicians, UCSD Department of Medicine, May 2000 - Present

Publications *[> 107,000 citations as of 10/2015, h-index=60]*

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Case No.: IPR2023-00208
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A General Method Applicable to the Search for Similarities in the Amino Acid Sequence of Two Proteins

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A computer adaptable method for finding similarities in the amino acid sequences of two proteins has been developed. From these findings it is possible to determine whether significant homology exists between the proteins. This information is used to trace their possible evolutionary development.

The maximum match is a number dependent upon the similarity of the sequences. One of its definitions is the largest number of amino acids of one protein that can be matched with those of a second protein allowing for all possible interruptions in either of the sequences. While the interruptions give rise to a very large number of comparisons, the method efficiently excludes from consideration those comparisons that cannot contribute to the maximum match.

Comparisons are made from the smallest unit of significance, a pair of amino acids, one from each protein. All possible pairs are represented by a two-dimensional array, and all possible comparisons are represented by pathways through the array. For this maximum match only certain of the possible pathways must be evaluated. A numerical value, one in this case, is assigned to every cell in the array representing like amino acids. The maximum match is the largest number that would result from summing the cell values of every pathway.

1. Introduction

The amino acid sequences of a number of proteins have been compared to determine whether the relationships existing between them could have occurred by chance. Generally, these sequences are from proteins having closely related functions and are so similar that simple visual comparisons can reveal sequence coincidence. Because the method of visual comparison is tedious and because the determination of the significance of a given result usually is left to intuitive rationalization, computer-based statistical approaches have been proposed (Fitch, 1966; Needleman & Blair, 1969).

Direct comparison of two sequences, based on the presence in both of corresponding amino acids in an identical array, is insufficient to establish the full genetic relationships between the two proteins. Allowance for gaps (Braunitzer, 1965) greatly multiplies the number of comparisons that can be made but introduces unnecessary and partial comparisons.

2. A General Method for Sequence Comparison

The smallest unit of comparison is a pair of amino acids, one from each protein. The maximum match can be defined as the largest number of amino acids of one protein that can be matched with those of another protein while allowing for all possible deletions.

Motif Exhibit 1150, Page 393 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

The maximum match can be determined by representing in a two-dimensional array, all possible pair combinations that can be constructed from the amino acid sequences of the proteins, A and B, being compared. If the amino acids are numbered from the N-terminal end, Aj is the jth amino acid of protein A and Bi is the ith amino acid of protein B. The Aj represent the columns and the Bi the rows of the two-dimensional array, MAT. Then the cell, MATij, represents a pair combination that contains Aj and Bi.

Every possible comparison can now be represented by pathways through the array. An i or j can occur only once in a pathway because a particular amino acid cannot occupy more than one position at one time. Furthermore, if MATmn is part of a pathway including MATij, the only permissible relationships of their indices are $m > i$, $n > j$ or $m < i$, $n < j$. Any other relationships represent permutations of one or both amino acid sequences which cannot be allowed since this destroys the significance of a sequence. Then any pathway can be represented by MATab . . . MATyz, where $a \geqslant 1$, $b \geqslant 1$, the i and j of all subsequent cells of MAT are larger than the running indices of the previous cell and $y \leqslant K$, $z \leqslant M$, the total number of amino acids comprising the sequences of proteins A and B, respectively. A pathway is signified by a line connecting cells of the array. Complete diagonals of the array contain no gaps. When MATij and MATmn are part of a pathway, $i - m \neq j - n$ is a sufficient, but not necessary condition for a gap to occur. A necessary pathway through MAT is defined as one which begins at a cell in the first column or the first row. Both i and j must increase in value; either i or j must increase by only one but the other index may increase by one or more. This leads to the next cell in a MAT pathway. This procedure is repeated until either i or j, or both, equal their limiting values, K and M, respectively. Every partial or unnecessary pathway will be contained in at least one necessary pathway.

In the simplest method, MATij is assigned the value, one, if Aj is the same kind of amino acid as Bi; if they are different amino acids, MATij is assigned the value, zero. The sophistication of the comparison is increased if, instead of zero or one, each cell value is made a function of the composition of the proteins, the genetic code triplets representing the amino acids, the neighboring cells in the array, or any theory concerned with the significance of a pair of amino acids. A penalty factor, a number subtracted for every gap made, may be assessed as a barrier to allowing the gap. The penalty factor could be a function of the size and/or direction of the gap. No gap would be allowed in the operation unless the benefit from allowing that gap would exceed the barrier. The maximum-match pathway then, is that pathway for which the sum of the assigned cell values (less any penalty factors) is largest. MAT can be broken up into subsections operated upon independently. The method also can be expanded to allow simultaneous comparison of several proteins using the amino acid sequences of n proteins to generate an n-dimensional array whose cells represent all possible combinations of n amino acids, one from each protein.

The maximum-match pathway can be obtained by beginning at the terminals of the sequences ($i = y, j = z$) and proceeding toward the origins, first by adding to the value of each cell possessing indices $i = y - 1$ and/or $j = z - 1$, the maximum value from among all the cells which lie on a pathway to it. The process is repeated for indices $i = y - 2$ and/or $j = z - 2$. This increment in the indices is continued until all cells in the matrix have been operated upon. Each cell in this outer row or column will contain the maximum number of matches that can be obtained by originating

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

any pathway at that cell and the largest number in that row or column is equal to the maximum match; the maximum-match pathway in any row or column must begin at this number. The operation of successive summations of cell values is illustrated in Figures 1 and 2.

	A	B	C	N	J	R	Q	C	L	C	R	P	M
A	1												
J					1								
C			1					1		1			
J					1								
N				1									
R						1	4	3	3	2	2	0	0
C	3	3	4	3	3	3	3	4	3	3	1	0	0
K	3	3	3	3	3	3	3	3	3	2	1	0	0
C	2	2	3	2	2	2	2	3	2	3	1	0	0
R	2	1	1	1	1	2	1	1	1	1	2	0	0
B	1	2	1	1	1	1	1	1	1	1	1	0	0
P	0	0	0	0	0	0	0	0	0	0	0	1	0

FIG. 1. The maximum-match operation for necessary pathways.
The number contained in each cell of the array is the largest number of identical pairs that can be found if that cell is the origin for a pathway which proceeds with increases in running indices. Identical pairs of amino acids were given the value of one. Blank cells which represent non-identical pairs have the value, zero. The operation of successive summations was begun at the last row of the array and proceeded row-by-row towards the first row. The operation has been partially completed in the R row. The enclosed cell in this row is the site of the cell operation which consists of a search along the subrow and subcolumn indicated by borders for the largest value, 4 in subrow C. This value is added to the cell from which the search began.

	A	B	C	N	J	R	Q	C	L	C	R	P	M
A	8	7	6	6	5	4	4	3	3	2	1	0	0
J	7	7	6	6	6	4	4	3	3	2	1	0	0
C	6	6	7	6	5	4	4	4	3	3	1	0	0
J	6	6	6	5	6	4	4	3	3	2	1	0	0
N	5	5	5	6	5	4	4	3	3	2	1	0	0
R	4	4	4	4	4	5	4	3	3	2	2	0	0
C	3	3	4	3	3	3	3	4	3	3	1	0	0
K	3	3	3	3	3	3	3	3	3	2	1	0	0
C	2	2	3	2	2	2	2	3	2	3	1	0	0
R	2	1	1	1	1	2	1	1	1	1	2	0	0
B	1	2	1	1	1	1	1	1	1	1	1	0	0
P	0	0	0	0	0	0	0	0	0	0	0	1	0

FIG. 2. Contributors to the maximum match in the completed array.
The alternative pathways that could form the maximum match are illustrated. The maximum match terminates at the largest number in the first row or first column, 8 in this case.

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

It is apparent that the above array operation can begin at any of a number of points along the borders of the array, which is equivalent to a comparison of N-terminal residues or C-terminal residues only. As long as the appropriate rules for pathways are followed, the maximum match will be the same. The cells of the array which contributed to the maximum match, may be determined by recording the origin of the number that was added to each cell when the array was operated upon.

3. Evaluating the Significance of the Maximum Match

A given maximum match may represent the maximum number of amino acids matched, or it may just be a number that is a complex function of the relationship between sequences. It will, however, always be a function of both the amino acid compositions of the proteins and the relationship between their sequences. One may ask whether a particular result found differs significantly from a fortuitous match between two random sequences. Ideally,one would prefer to know the exact probability of obtaining the result found from a pair of random sequences and what fraction of the total possibilities are less probable, but that is prohibitively difficult, especially if a complex function were used for assigning a value to the cells.

As an alternative to determining the exact probabilities, it is possible to estimate the probabilities experimentally. To accomplish the estimate one can construct two sets of random sequences, a set from the amino acid composition of each of the proteins compared. Pairs of random sequences can then be formed by randomly drawing one member from each set. Determining the maximum match for each pair selected will yield a set of random values. If the value found for the real proteins is significantly different from the values found for the random sequences, the difference is a function of the sequences alone and not of the compositions. Alternatively, one can construct random sequences from only one of the proteins and compare them with the other to determine a set of random values. The two procedures measure different probabilities. The first procedure determines whether a significant relationship exists *between* the real sequences. The second procedure determines whether the relationship *of* the protein used to form the random sequences *to* the other proteins is significant. It bears reiterating that the integral amino acid composition of each random sequence must be equal to that of the protein it represents.

The amino acid sequence of each protein compared belongs to a set of sequences which are permutations. Sequences drawn randomly from one or both of these sets are used to establish a distribution of random maximum-match values which would include all possible values if enough comparisons were made. The null hypothesis, that any sequence relationship manifested by the two proteins is a random one, is tested. If the distribution of random values indicates a small probability that a maximum match equal to, or greater than, that found for the two proteins could be drawn from the random set, the hypothesis is rejected.

4. Cell Values and Weighting Factors

To provide a theoretical framework for experiments, amino acid pairs may be classified into two broad types, identical and non-identical pairs. From 20 different amino acids one can construct 180 possible non-identical pairs. Of these, 75 pairs of amino acids have codons (Marshall, Caskey & Nirenberg, 1967) whose bases differ at only one position (Eck & Dayhoff, 1966). Each change is presumably the result of a

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

single-point mutation. The majority of non-identical pairs have a maximum of only one or zero corresponding bases. Due to the degeneracy of the genetic code, pair differences representing amino acids with no possible corresponding bases are uncommon even in randomly selected pairs. If cells are weighted in accordance with the maximum number of corresponding bases in codons of the represented amino acids, the maximum match will be a function of identical and non-identical pairs. For comparisons in general, the cell weights can be chosen on any basis.

If every possible sequence gap is allowed in forming the maximum match, the significance of the maximum match is enhanced by decreasing the weight of those pathways containing a large number of gaps. A simple way to accomplish this is to assign a penalty factor, a number which is subtracted from the maximum match for each gap used to form it. The penalty is assigned before the maximum match is formed. Thus the pathways will be weighted according to the number of gaps they contain, but the nature of the contributors to the maximum match will be affected as well. In proceeding from one cell to the next in a maximum-match pathway, it is necessary that the difference between each cell value and the penalty, be greater than the value for a cell in a pathway that contains no gap. If the value of the penalty were zero, all possible gaps could be allowed. If the value were equal to the theoretical value for the maximum match between two proteins, it would be impossible to allow a gap and the maximum match would be the largest of the values found by simply summing along the diagonals of the array; this is the simple frame-shift method.

5. Application of the Method

To illustrate the role of weighting factors in evaluating a maximum match, two proteins expected to show homology, whale myoglobin (Edmundson, 1965) and human β-hemoglobin (Konigsberg, Goldstein & Hill, 1963), and two proteins not expected to exhibit homology, bovine pancreatic ribonuclease (Smyth, Stein & Moore, 1963) and hen's egg lysozyme (Canfield, 1963) were chosen for comparisons.

The FORTRAN programs used in this study were written for the CDC3400 computer. The operations employed in forming the maximum match are those for the special case when none of the cells of the array have a value less than zero. Four types of amino acid pairs were distinguished and variable sets consisting of values to be assigned to each type of pair and a value for the penalty were established. The pair types are as follows:

Type 3. Identical pairs: those having a maximum of three corresponding bases in their codons.

Type 2. Pairs having a maximum of two corresponding bases in their codons.

Type 1. Pairs having a maximum of one corresponding base in their codons.

Type 0. Pairs having no possible corresponding base in their codons.

The value for type 3 pairs was 1·0 and the value for type 0 pairs was zero for all variable sets.

At program execution time, the amino acids (coded by two-digit numbers) of the sequences to be compared were read into the computer, and were followed by a twenty-by-twenty symmetrical array, the maximum correspondence array, analogous to one used by Fitch (1966), that contained all possible pairs of amino acids and identified each pair as to type. The RNA codons for amino acids used to construct the maximum-correspondence array were taken from a single Table (Marshall *et al.*,

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

1967). The UGA, UAA and UAG codons were not used, but UUG was used as a codon for leucine. The subsequent data cards indicated the numerical values for a variable set.

The two-dimensional comparison array was generated row-by-row. The amino acid code numbers for Ai and Bj referenced the correspondence array to determine the type of amino acid pair constituted by Ai and Bj. The type number referenced a short array, the variable set, containing the type values, and the appropriate value from that set was assigned to the appropriate cell of the comparison array. The maximum match was then determined by the procedure of successive summations.

Following the determination of the maximum match for the real proteins, the amino acid sequence of only one member of the protein pair was randomized and the match was repeated. The sequences of β-hemoglobin and ribonuclease were the ones randomized. The randomization procedure was a sequence shuffling routine based on computer-generated random numbers. A cycle of sequence randomization–maximum-match determination was repeated ten times in all of the experiments in this report, giving the random values used for comparison with the real maximum-match. The average and standard deviation for the random values of each variable set was estimated.

6. Results and Discussion

The use of a small random sample size (ten) was necessary to hold the computer time to a reasonable level. The maximum probable error in a standard deviation estimate for a sample this small is quite large and the results should be judged with this fact in mind. For each set of variables, it was assumed that the random values would be distributed in the fashion of the normal-error curve; therefore, the values of the first six random sets in the β-hemoglobin–myoglobin comparison were converted to standard measure, five was added to the result, and these values were plotted as *one* group against their calculated probit. The results of the plot are shown in Figure 3. The fit is good indicating the probable adequacy of the measured standard deviations for these variable sets in estimating distribution functions for random values through two standard deviations. The above fit indicates no bias in the randomization procedure. In other words, randomization of the sequence was complete before the maximum match was determined for any sequence in a random set.

The results obtained in the comparison of β-hemoglobin with myoglobin are summarized in Table 1 and the results for the ribonuclease–lysozyme comparison are in Table 2. These Tables indicate the values assigned to the pair types, the penalty factor used in forming each of the maximum matches, and the statistical results obtained. The number of gaps roughly characterizes the nature of the pathway that formed the maximum match. A large number is indicative of a devious pathway through the array. One gap means that all of the pathway may be found on only two partial diagonals of the array.

The most important information is obtained from the standardized value of the maximum match for the real proteins, the difference from the mean in standard-deviation units. For this sample size all deviations greater than 3·0 were assumed to include less than 1% of the true random population and to indicate a significant difference. As might be expected, all matches of myoglobin and β-hemoglobin show a significant deviation. Among the sets of variables, set 1, which results in a search for identical amino acid pairs while allowing for all deletions, indicates that 63

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

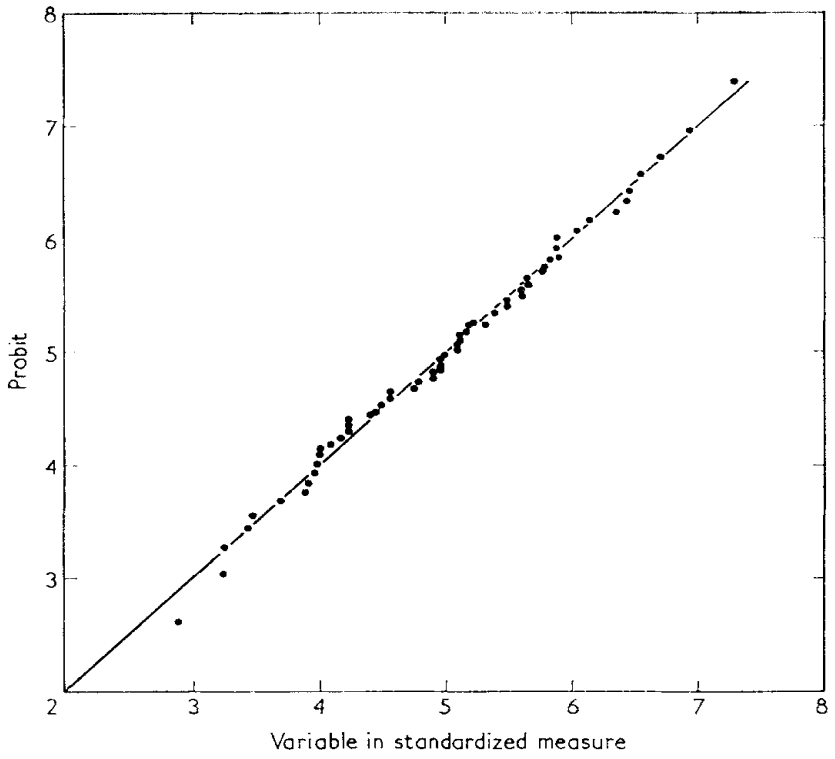


FIG. 3. Probit plot for six grouped random samples.
The solid line indicates the plot that would result from a probit analysis on an infinite number of samples from a normally-distributed population. The points represent the results of probit calculations on 60 random maximum match values that were assumed to have come from one population.

TABLE 1

β-Hemoglobin–myoglobin maximum matches

Variable set	Match values for pair types 2	Match values for pair types 1	Penalty	Maximum-match value sum Real	Maximum-match value sum Random†	s	Real X	Minimum deletions Real	Minimum deletions Random†
1	0	0	0	63·00	55·60	1·80	4·11	35	36·2
2	0	0	1·00	38·00	27·80	2·09	4·88	4	5·5
3	0·67	0·33	0	97·00	91·47	1·55	3·57	18	24·3
4	0·67	0·33	1·03	89·63	80·25	1·11	8·46	1	3·6
5	0·25	0·05	0	71·55	64·78	1·59	4·27	46	45·0
6	0·25	0·05	1·05	51·95	40·54	1·46	7·80	3	7·5
7	0·25	0·05	25	47·30	33·80	1·52	8·87	0	0

s is the estimated standard deviation; X, the standardized value, (real − random)/s, of the maximum match of the real proteins. The values for type 3 and type 0 pairs were 1·0 and 0, respectively, in each variable set.

† An average value from 10 samples.

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

TABLE 2

Ribonuclease–lysozyme maximum matches

Variable set	Match values for pair types		Penalty	Maximum-match value sum		s	Real X	Minimum deletions	
	2	1		Real	Random†			Real	Random†
1	0	0	0	48·00	44·20	2·56	1·48	34	29·2
2	0	0	1·00	23·00	22·00	1·73	0·58	5	5·2
3	0·67	0·33	0	78·33	76·17	0·82	2·64	21	18·8
4	0·67	0·33	1·03	67·93	67·37	1·27	0·43	2	2·2
5	0·25	0·05	0	56·00	52·26	2·12	1·77	35	35·5
6	0·25	0·05	1·05	33·70	33·02	1·66	0·41	8	6·8
7	0·25	0·05	25	28·15	27·67	1·75	0·22	0	0

s is the estimated standard deviation; X, the standardized value, (real − random)/s, of the maximum match of the real proteins. The values for type 3 and type 0 pairs were 1·0 and 0, respectively in each variable set.

† An average value from 10 samples.

amino acids in β-hemoglobin and myoglobin can be matched. To attain this match, however, it is necessary to permit at least 35 gaps. In contrast, when two gaps are allowed according to Braunitzer (1965), it is possible to match only 37 of the amino acids. Curiously, when this variable set was used for comparing *human* myoglobin (Hill, personal communication) with human β-hemoglobin, the maximum match obtained was not significant. Differences between real and random values were highly significant, however, when other variable sets were used.

Variable set 2 attaches a penalty equal to the value of one identical amino acid pair to the search for identical amino acid pairs. This penalty will exclude from consideration any possible pathway that leaves and returns to a principal diagonal, thereby needing two gaps, in order to add only one or two amino acids to the maximum match. This set results in a total of $30 + 4 = 42$ amino acids matched (the maximum-match value plus the number of gaps is reduced to four) and the significance of the result relative to set 1 appears to be increased. Braunitzer's comparison would have a value of $37 - 2 = 35$ using this variable set, hence it was not selected by the method.

Variable sets 3 and 4 have an interesting property. Their maximum-match values can be related to the minimum number of mutations needed to convert the selected parts of one amino acid sequence into the selected parts of the other. The minimum number of mutations concept in protein comparisons was first advanced by Fitch (1966). If the type values for these sets are multiplied by three, they become equal to their pair type and directly represent the maximum number of corresponding bases in the codons for a given amino acid pair. Thus the maximum match and penalty factors may be multiplied by three, making it possible to calculate the maximum number of bases matched in the combination of amino acid pairs selected by the maximum-match operation.

β-Hemoglobin, the smaller of the two proteins, contains 146 amino acids; consequently the highest possible maximum match (disregarding integral amino-acid composition data) with myoglobin is $146 \times 3 = 438$. Insufficient data are available

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

to analyze the result from set 3 on the basis of mutations. If it is assumed that the gap in set 4 does not exclude any part of β-hemoglobin from the comparison, this set has a maximum of 3(89·63 + 1·03) = 272 bases matched, indicating a minimum of 438 − 272 = 166 point mutations in this combination. Using this variable set and placing gaps according to Braunitzer, a score of 88·6 was obtained, thus his match was not selected. Again it may be observed that the penalty greatly enhanced the significance of the maximum match.

Variable sets 5 and 6 have no intrinsic meaning and were chosen because the weight attached to type 2 and type 1 pairs is intermediate in value with respect to sets 1 and 2 and sets 3 and 4. The maximum match for set 6 is seen to have a highly significant value.

The data of set 7 are results that would be obtained from using the frame-shift method to select a maximum match; the penalty was large enough to prevent any gaps in the comparisons. The slight differences in significance found among the maximum-match values of β-hemoglobin and myoglobin resulting from use of sets 4, 6 and 7 are probably meaningless due to small sample size and errors introduced by the assumptions about the distribution functions of random values. Finding a value in set 7 that is approximately equal to those from sets 4 and 6 in significance is not surprising. A larger penalty factor would have increased the difference from the mean in sets 4 and 6 because almost every random value in each set was the result of more gaps than were required to form the real maximum match. Further, the gaps that are allowed are at the N-terminal ends so that about 85% of the comparison can be made without gaps. If an actual gap were present near the middle of one of the sequences, it would have caused a sharp reduction in the significance of the frame-shift type of match.

Set 3 is the only variable set in Table 2 that shows a possible difference. Assuming the value is accurate, other than chance, there is no simple explanation for the difference. A small but meaningful difference in any comparison could represent evolutionary divergence or convergence. It is generally accepted that the primary structure of proteins is the chief determinant of the tertiary structure. Because certain features of tertiary structure are common to proteins, it is reasonable to suppose that proteins will exhibit similarities in their sequences, and that these similarities will be sufficient to cause a significant difference between most protein pairs and their corresponding randomized sequences, being an example of submolecular evolutionary convergence. Further, the interactions of the protein backbone, side chains, and the solvent that determine tertiary structure are, in large measure, forces arising from the polarity and steric nature of the protein side-chains. There are conspicuous correlations in the polarity and steric nature of type 2 pairs. Heavy weighting of these pairs would be expected to enhance the significance of real maximum-match values if common structural features are present in proteins that are compared. The presence of sequence similarities does not always imply common ancestry in proteins. More experimentation will be required before a choice among the possibilities suggested for the result from set 3 can be made. If several short sequences of amino acids are common to all proteins, it seems remarkable that the relationship of ribonuclease to lysozyme in six of the seven variable sets appears to be truly a random one. It should be noted, however, that the standard value of the real maximum-match is positive in each variable set in this comparison.

This method was designed for the purpose of detecting homology and defining its nature when it can be shown to exist. Its usefulness for the above purposes depends in

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

part upon assumptions related to the genetic events that could have occurred in the evolution of proteins. Starting with the assumption that homologous proteins are the result of gene duplication and subsequent mutations, it is possible to construct several hypothetical amino-acid sequences that would be expected to show homology. If one assumes that following the duplication, point mutations occur at a constant or variable rate, but randomly, along the genes of the two proteins, after a relatively short period of time the protein pairs will have nearly identical sequences. Detection of the high degree of homology present can be accomplished by several means. The use of values for non-identical pairs will do little to improve the significance of the results. If no, or very few, deletions (insertions) have occurred, one could expect to enhance the significance of the match by assigning a relatively high penalty for gaps. Later on in time the hypothetical proteins may have a sizable fraction of their codons changed by point mutations, the result being that an attempt to increase the significance of the maximum match will probably require attaching substantial weight to those pairs representing amino acids still having two of the three original bases in their codons. Further, if a few more gaps have occurred, the penalty should be reduced to a small enough value to allow areas of homology to be linked to one another. At a still later date in time more emphasis must be placed on non-identical pairs, and perhaps a very small or even negative penalty factor must be assessed. Eventually, it will be impossible to detect the remaining homology in the hypothetical example by using the approach detailed here.

From consideration of this simple model of protein evolution one may deduce that the variables which maximize the significance of the difference between real and random proteins gives an indication of the nature of the homology. In the comparison of human β-hemoglobin to whale myoglobin, the assignment of some weight to type 2 pairs considerably enhances the significance of the result, indicating substantial evolutionary divergence. Further, few deletions (additions) have apparently occurred.

It is known that the evolutionary divergence manifested by cytochrome (Margoliash, Needleman & Stewart, 1963) and other heme proteins (Zuckerkandl & Pauling, 1965) did not follow the sample model outlined above. Their divergence is the result of *non-random* mutations along the genes. The degree and type of homology can be expected to differ between protein pairs. As a consequence of the difference there is no *a priori* best set of cell and operation values for maximizing the significance of a maximum-match value of homologous proteins, and as a corollary to this fact, there is no best set of values for the purpose of detecting only slight homology. This is an important consideration, because whether the sequence relationship between proteins is significant depends solely upon the cell and operation values chosen. If it is found that the divergence of proteins follows one or two simple models, it may be possible to derive a set of values that will be most useful in detecting and defining homology.

The most common method for determining the degree of homology between protein pairs has been to count the number of non-identical pairs (amino acid replacements) in the homologous comparison and to use this number as a measure of evolutionary distance between the amino acid sequences. A second, more recent concept has been to count the minimum number of mutations represented by the non-identical pairs. This number is probably a more adequate measure of evolutionary distance because it utilizes more of the available information and theory to give some measure of the number of genetic events that have occurred in the evolution of the proteins. The approach outlined in this paper supplies either of these numbers.

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

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Designation	Organism/Strain
H6WZ70	H6WZ70_VIGRA Non-symbiotic hemoglobin OS=Vigna radiata GN=NSH PE=2 SV=1
B3DUZ7	B3DUZ7_METI4 Hemoglobin-like flavoprotein OS=Methylacidiphilum infernorum (isolate V4) GN=hmp PE=1 SV=1
O66586	Y211_AQUAE Uncharacterized globin-like protein aq_211 OS=Aquifex aeolicus (strain VF5) GN=aq_211 PE=3 SV=1
P02236	LGB2_SOYBN Leghemoglobin C2 OS=Glycine max PE=1 SV=2
Q42831	HBL_HORVU Non-symbiotic hemoglobin OS=Hordeum vulgare GN=HB PE=1 SV=1
G4N2X9	G4N2X9_MAGO7 Heme peroxidase OS=Magnaporthe oryzae (strain 70-15 / ATCC MYA-4617 / FGSC 8958) GN=MGG_10859 PE=4 SV=1
N4UFN7	N4UFN7_FUSC1 Peroxidase OS=Fusarium oxysporum f. sp. cubense (strain race 1) GN=FOC1_g10007184 PE=3 SV=1
Q4ING3	CCPR_GIBZE Cytochrome c peroxidase mitochondrial OS=Gibberella zeae (strain PH-1 / ATCC MYA-4620 / FGSC 9075 / NRRL 31084) GN=CCP1 PE=3 SV=1
Q08753	TRHN1_CHLMO Group 1 truncated hemoglobin LI637 OS=Chlamydomonas moewusii GN=LI637 PE=1 SV=1
P17724	LGB2_SOYBN Leghemoglobin C2 OS=Glycine max PE=1 SV=2
P15160	TRHBN_PARCA Group 1 truncated hemoglobin trHbN OS=Paramecium caudatum PE=1 SV=2
Q5DVM4	Q5DVM4_ASPNG Putative haemoglobin OS=Aspergillus niger GN=hmb1 PE=3 SV=1
Q9FY42	HBL_MAIZE Non-symbiotic hemoglobin OS=Zea mays GN=HB PE=2 SV=1
O04985	HBL2_ORYSJ Non-symbiotic hemoglobin 2 OS=Oryza sativa subsp. japonica GN=HB2 PE=2 SV=1
O24520	HBL1_ARATH Non-symbiotic hemoglobin 1 OS=Arabidopsis thaliana GN=AHB1 PE=1 SV=1
O48668	LGB2_PEA Leghemoglobin Lb5-10 OS=Pisum sativum PE=2 SV=3
Q43236	LGB1_VIGUN Leghemoglobin-1 OS=Vigna unguiculata GN=LBI PE=2 SV=1
P02192	MYG_BOVIN Myoglobin OS=Bos taurus GN=MB PE=1 SV=3
P02189	MYG_PIG Myoglobin OS=Sus scrofa GN=MB PE=1 SV=2
P68082	MYG_HORSE Myoglobin OS=Equus caballus GN=MB PE=1 SV=2
Nicotiana_benthamiana	Nicotiana_benthamiana non symbiotic hemoglobin
A0A063XGS7	A0A063XGS7_BACIU Group 2 truncated hemoglobin YjbI OS=Bacillus subtilis GN=yjbI PE=4 SV=1
Q8NMW6	Q8NMW6_CORGL Hemoglobin-like proteins OS=Corynebacterium glutamicum (strain ATCC 13032 / DSM 20300 / JCM 1318 / LMG 3730 / NCIMB 10025) GN=Cgl2448 PE=4 SV=1
P73925	TRHBN_SYNY3 Group 1 truncated hemoglobin GlbN OS=Synechocystis sp. (strain PCC 6803 / Kazusa) GN=glbN PE=1 SV=1
B4WLV9	B4WLV9_SYNS7 Globin domain protein OS=Synechococcus sp. (strain ATCC 29403 / PCC 7335) GN=S7335_2344 PE=3 SV=1
Q00812	TRHBN_NOSCO Group 1 truncated hemoglobin GlbN OS=Nostoc commune GN=glbN PE=3 SV=1
G2RXE0	G2RXE0_BACME Hemoglobin-like protein OS=Bacillus megaterium WSH-002 GN=BMWSH_4530 PE=4 SV=1

	SEQ ID NO:
H6WZ70	SEQ ID NO:1 *Vigna radiata*
B3DUZ7	SEQ ID NO:2 *Methylacidiphilum infernorum*
O66586	SEQ ID NO:3 *Aquifex aeolicus*
P02236	SEQ ID NO:4 *Glycine max*
Q42831	SEQ ID NO:5 *Hordeum vulgare*
G4N2X9	SEQ ID NO:6 *Magnaporthe oryzae*
N4UFN7	SEQ ID NO:7 *Fusarium oxysporum*
Q4ING3	SEQ ID NO:8 *Fusarium graminearum*
Q08753	SEQ ID NO:9 *Chlamydomonas eugametos*
P17724	SEQ ID NO:10 *Tetrahymena pyriformis*
P15160	SEQ ID NO:11 *Paramecium caudatum*
Q5DVM4	SEQ ID NO:12 *Aspergillus niger*
Q9FY42	SEQ ID NO:13 *Zea mays*
O04985	SEQ ID NO:14 *Oryza sativa subsp. japonica*
O24520	SEQ ID NO:15 *Arabidopsis thaliana*
O48668	SEQ ID NO:16 *Pisum sativum*
Q43236	SEQ ID NO:17 *Vigna unguiculata*
P02192	SEQ ID NO:18 *Bos taurus*
P02189	SEQ ID NO:19 *Sus scrofa*
P68082	SEQ ID NO:20 *Equus caballus*
Nicotiana_benthamiana	SEQ ID NO:21 *Nicotiana benthamiana*
A0A063XGS7	SEQ ID NO:22 *Bacillus subtilis*
Q8NMW6	SEQ ID NO:23 *Corynebacterium glutamicum*
P73925	SEQ ID NO:24 *Synechocystis* PCC6803
B4WLV9	SEQ ID NO:25 *Synechococcus* sp. PCC 7335
Q00812	SEQ ID NO:26 *Nostoc commune*
G2RXE0	SEQ ID NO:27 *Bacillus megaterium*

	H6WZ70	B3DUZ7	O66586	P02236	Q42831	G4N2X9	N4UFN7	Q4ING3	Q08753	P17724	P15160	Q5DVM4	Q9FY42	O04985
H6WZ70	100%	21%	23%	40%	70%	5%	11%	13%	18%	14%	16%	18%	70%	68%
B3DUZ7	21%	100%	30%	24%	24%	5%	9%	11%	18%	19%	19%	22%	21%	22%
O66586	23%	28%	100%	25%	19%	5%	8%	11%	16%	15%	16%	37%	19%	17%
P02236	40%	24%	25%	100%	36%	6%	10%	11%	19%	21%	18%	23%	37%	38%
Q42831	70%	24%	19%	36%	100%	5%	10%	13%	18%	15%	19%	15%	84%	80%
G4N2X9	5%	5%	5%	6%	5%	100%	13%	11%	6%	5%	5%	6%	5%	6%
N4UFN7	11%	9%	8%	10%	10%	13%	100%	15%	9%	8%	9%	9%	10%	11%
Q4ING3	13%	11%	11%	11%	13%	11%	15%	100%	13%	12%	12%	11%	14%	13%
Q08753	18%	18%	16%	19%	18%	6%	9%	13%	100%	32%	28%	18%	19%	17%
P17724	14%	19%	15%	21%	14%	5%	8%	12%	32%	100%	35%	19%	15%	14%
P15160	16%	19%	16%	18%	19%	5%	9%	12%	28%	35%	100%	16%	17%	18%
Q5DVM4	18%	22%	37%	23%	15%	6%	9%	11%	18%	19%	16%	100%	17%	16%
Q9FY42	70%	21%	19%	37%	84%	5%	10%	14%	19%	15%	17%	17%	100%	80%
O04985	68%	22%	17%	38%	80%	6%	11%	13%	17%	14%	18%	16%	80%	100%
O24520	73%	27%	22%	40%	68%	6%	10%	10%	16%	14%	14%	21%	67%	65%
O48668	44%	27%	27%	65%	38%	5%	9%	12%	17%	19%	17%	17%	39%	38%
Q43236	37%	22%	22%	79%	36%	4%	10%	12%	19%	22%	23%	20%	36%	39%
P02192	20%	15%	21%	18%	20%	6%	10%	10%	20%	23%	17%	24%	21%	19%
P02189	18%	17%	20%	19%	21%	5%	9%	10%	19%	22%	18%	25%	22%	22%
P68082	17%	17%	22%	18%	21%	5%	10%	12%	21%	21%	19%	25%	22%	20%
Nicotiana_benthamiana	78%	24%	23%	41%	67%	5%	10%	11%	18%	17%	17%	21%	67%	66%
A0A063XGS7	18%	17%	13%	19%	19%	5%	8%	12%	18%	24%	19%	17%	17%	14%
Q8NMW6	21%	15%	18%	16%	18%	5%	8%	10%	16%	19%	20%	16%	18%	17%
P73925	21%	19%	17%	24%	18%	5%	7%	10%	34%	46%	33%	16%	20%	17%
B4WLV9	19%	24%	22%	19%	19%	6%	10%	12%	17%	14%	19%	21%	20%	18%
Q00812	14%	17%	16%	17%	15%	4%	8%	11%	24%	31%	28%	20%	17%	17%
G2RXE0	17%	18%	18%	18%	17%	5%	8%	10%	18%	23%	17%	21%	15%	13%

	O24520	O48668	Q43236	P02192	P02189	P68082	Nicotiana_benthamiana	A0A063XGS7	Q8NMW6	P73925	B4WLV9	Q00812	G2RXE0
H6WZ70	73%	44%	37%	20%	18%	17%	78%	18%	21%	21%	18%	14%	17%
B3DUZ7	27%	27%	22%	15%	17%	17%	24%	17%	15%	19%	24%	17%	18%
O66586	22%	27%	22%	21%	20%	22%	23%	13%	18%	17%	22%	16%	17%
P02236	40%	65%	79%	19%	20%	19%	41%	19%	16%	24%	19%	17%	18%
Q42831	68%	38%	36%	20%	21%	21%	67%	19%	18%	18%	19%	15%	17%
G4N2X9	6%	5%	4%	6%	5%	5%	5%	5%	5%	5%	6%	4%	5%
N4UFN7	10%	9%	10%	10%	9%	10%	10%	8%	8%	7%	10%	8%	8%
Q4ING3	10%	12%	12%	10%	10%	12%	11%	12%	10%	10%	12%	11%	10%
Q08753	16%	17%	19%	20%	19%	21%	18%	18%	16%	34%	17%	24%	18%
P17724	14%	19%	22%	23%	22%	21%	17%	24%	19%	46%	14%	31%	23%
P15160	14%	17%	23%	17%	18%	19%	17%	19%	20%	33%	19%	28%	17%
Q5DVM4	21%	17%	20%	23%	25%	25%	21%	16%	16%	16%	21%	20%	21%
Q9FY42	67%	39%	36%	21%	22%	22%	67%	17%	18%	20%	20%	17%	15%
O04985	65%	38%	39%	19%	22%	20%	66%	14%	17%	17%	18%	17%	13%
O24520	100%	43%	39%	20%	20%	23%	71%	17%	19%	21%	17%	15%	17%
O48668	43%	100%	62%	19%	17%	18%	45%	21%	21%	20%	19%	17%	17%
Q43236	39%	62%	100%	18%	19%	20%	39%	18%	15%	22%	18%	18%	14%
P02192	20%	19%	18%	100%	88%	88%	19%	20%	17%	20%	18%	19%	20%
P02189	20%	17%	19%	88%	100%	91%	19%	18%	17%	18%	16%	18%	21%
P68082	23%	18%	20%	88%	91%	100%	22%	18%	15%	18%	19%	19%	21%
Nicotiana_benthamiana	71%	45%	39%	19%	19%	22%	100%	19%	19%	21%	19%	20%	15%
A0A063XGS7	17%	21%	18%	20%	18%	18%	19%	100%	39%	24%	18%	23%	63%
Q8NMW6	19%	21%	14%	17%	17%	15%	19%	39%	100%	25%	16%	23%	33%
P73925	21%	20%	22%	20%	18%	18%	21%	24%	25%	100%	16%	36%	23%
B4WLV9	17%	19%	18%	18%	16%	19%	19%	18%	16%	16%	100%	17%	19%
Q00812	15%	17%	18%	19%	18%	19%	20%	23%	23%	36%	17%	100%	23%
G2RXE0	17%	17%	14%	20%	21%	21%	15%	63%	33%	23%	19%	23%	100%

Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organisms

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When many protein sequences are available for estimating the time of divergence between two species, it is customary to estimate the time for each protein separately and then use the average for all proteins as the final estimate. However, it can be shown that this estimate generally has an upward bias, and that an unbiased estimate is obtained by using distances based on concatenated sequences. We have shown that two concatenation-based distances, i.e., average gamma distance weighted with sequence length (d_2) and multiprotein gamma distance (d_3), generally give more satisfactory results than other concatenation-based distances. Using these two distance measures for 104 protein sequences, we estimated the time of divergence between mice and rats to be approximately 33 million years ago. Similarly, the time of divergence between humans and rodents was estimated to be approximately 96 million years ago. We also investigated the dependency of time estimates on statistical methods and various assumptions made by using sequence data from eubacteria, protists, plants, fungi, and animals. Our best estimates of the times of divergence between eubacteria and eukaryotes, between protists and other eukaryotes, and between plants, fungi, and animals were 3, 1.7, and 1.3 billion years ago, respectively. However, estimates of ancient divergence times are subject to a substantial amount of error caused by uncertainty of the molecular clock, horizontal gene transfer, errors in sequence alignments, etc.

The molecular clock hypothesis asserts that the number of amino acid substitutions in a protein is roughly proportional to the time since divergence of the two species compared (1, 2). Strictly speaking, no gene or protein would evolve at a constant rate for a long evolutionary time, because gene function is likely to change over time, and mutational and DNA repair mechanisms appear to vary among different groups of organisms (3). For this reason, the molecular clock has been controversial for several decades (3–6). However, even if the substitution rate is not strictly constant, it is still possible to obtain rough estimates of divergence times, and these estimates are very useful when there is no reliable fossil record (6, 7). Furthermore, if a gene evolves excessively fast or slow in a few evolutionary lineages, one can eliminate these lineages and then estimate divergence times for the rest of the species (8). The accuracy of time estimates is expected to increase as the number of genes or proteins used increases, and in recent years, many authors have used multiple genes or proteins for this purpose (9–11).

There are several statistical methods for estimating divergence times, but the theoretical basis of the methods is not well understood when multiple genes are used. For this reason, different authors obtained widely different estimates for the same set of species by using different methods (10, 12–16). We have therefore examined the reliability of different methods for estimating divergence times and have developed new methods that are likely to give more reasonable estimates than previous ones. The purpose of this paper is to discuss statistical problems related to this subject and to present new methods. These new methods will then be used to estimate the divergence times between mice and rats and between humans and rodents, which have been controversial for the last few decades. We also consider the divergence times of animals, plants, fungi, protists, and bacteria to show the dependency of time estimates on the assumptions made and the statistical methods used. In this paper, we consider only protein sequences, because they usually give more satisfactory results than DNA sequences when long-term evolution is considered. We also consider only distance methods of time estimation, because most recent time estimates have been obtained by these methods.

Theoretical Basis of Estimation of Divergence Times

Individual Protein (IP) Approach. In the past, most investigators have used a method that may be called the IP approach (11, 13, 14, 17, 18). In this approach, the estimate of divergence time is computed for each protein, and the average of the estimates over all proteins is used as the final estimate. Consider Fig. 1*A*, in which a phylogenetic tree for five species is given. Here, *a–f* stand for the least-squares estimates of branch lengths (numbers of amino acid substitutions) obtained from a pairwise distance matrix for a protein. Species 5 is used as an outgroup to determine the root of the tree for the remaining sequences, and therefore the branch length estimate for this branch is not given. Here we assume that the topology of the tree for the five species has been established from other information. To estimate divergence times between species, it is convenient to construct a linearized tree (8), in which the branch lengths are reestimated under the assumption of a constant-rate evolution (Fig. 1*B*). When this linearized tree is constructed, a timescale for the tree is produced to estimate divergence times (t_1 and t_2). This timescale can be obtained by computing the rate of amino acid substitution per year (r) by using the known divergence time and the corresponding branch-length estimate for a pair of species or species clusters.

For example, if T is the calibration point in Fig. 1*B*, the rate of amino acid substitution can be estimated by $\hat{r} = \hat{b}_3/T$, where $\hat{b}_3$ is the branch-length estimate for species 4 after divergence from species 1, 2, and 3 in the linearized tree (Fig. 1*B*). When this rate is obtained, we can estimate t_1 by

$$\hat{t}_1 = \hat{b}_1/\hat{r} = (\hat{b}_1/\hat{b}_3)T. \qquad [1]$$

Here, the estimates $\hat{b}_1$, $\hat{b}_2$, and $\hat{b}_3$ can be obtained from pairwise distances by Takezaki *et al.*'s (8) method. Similarly, the estimate of t_2 is given by $\hat{t}_2 = \hat{b}_2/\hat{r} = (\hat{b}_2/\hat{b}_3)T$. The variances of $\hat{t}_1$ or $\hat{t}_2$ can be obtained by Takezaki *et al.*'s method or by the bootstrap method (19). For certain data sets (18, 20), it is possible to use several calibration points (paleontological dates for different pairs of species). In this case, r may be estimated by fitting a regression line to the relationship between branch length estimates and paleontological dates.

Before the construction of a linearized tree, it is customary to conduct a statistical test of the molecular clock and eliminate

Abbreviations: IP, individual protein approach; CD, concatenation-based distance; MY, million years; PC, Poisson correction; NJ, neighbor joining.

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EVOLUTION

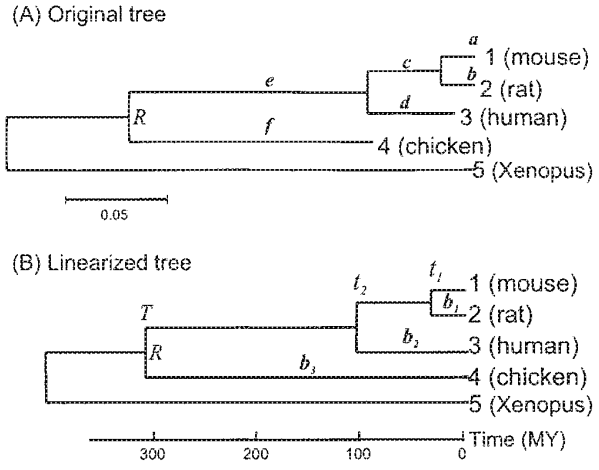


Fig. 1. Phylogeny of the five species used. (*A*) NJ tree constructed by using distance d_2 for 104 protein sequences. (*B*) Linearized tree of the above NJ tree. *R*, root of the four species under consideration.

species that evolved excessively fast or slow, although this is not always necessary (see below). A number of authors (7, 11) have used the relative rate tests (21–23) for this purpose. In these tests, three sequences (or three sequence clusters) are used, and the equality of evolutionary rate for two evolutionary lineages is tested by using a third sequence as an outgroup. For example, the equality of the expectations of branch lengths a (for mice) and b (for rats) in Fig. 1*A* can be tested by using the chicken sequence as the outgroup. Theoretically, however, this test is not appropriate for estimating the divergence time t_1, because it does not test the equal rates for sequences 1, 2, and 3. For this purpose, we should test the null hypotheses $E(a) = E(b)$ and $E(a/2 + b/2 + c + e) = E(f)$, where E is the expectation operator. These null hypotheses can be tested by Takezaki *et al.*'s (8) U statistic, which approximately follows the χ^2 distribution and tests the equality of the branch lengths from the root (R) to tips (1, 2, 3, and 4) for all species. In this test, at least four sequences are necessary.

When there are data from many different proteins, the divergence time t_1 is often estimated by the simple average of $\hat{t}_1$s for all the proteins used, as mentioned above. That is,

$$\hat{t}_1 = \sum_{i=1}^{k} \hat{t}_{1i}/k, \qquad [2]$$

where $\hat{t}_{1i}$ is the estimate of t_1 obtained by the ith protein, and k is the total number of proteins used. Theoretically, however, $\hat{t}_1$ obtained in this way is not an unbiased estimator of t_1, even if the branch length estimates (numbers of amino acid substitutions) for each protein are unbiased. Let $\hat{b}_{1i}$ and $\hat{b}_{3i}$ be unbiased estimates of b_1 and b_3 for the ith protein, so that $\hat{t}_{1i} = (\hat{b}_{1i}/\hat{b}_{3i})T$. In this case, an unbiased estimator of t_1 is given by $[(\Sigma_i \hat{b}_{1i})/(\Sigma_i \hat{b}_{3i})]T$ rather than by $\hat{t}_1 = \Sigma_i(\hat{b}_{1i}/\hat{b}_{3i})T/k$. If we assume that $\hat{b}_1$ and $\hat{b}_3$ are random variables (subscript i dropped), the expectation of $\hat{b}_1/\hat{b}_3$ is approximately given by

$$E(\hat{b}_1/\hat{b}_3) = \frac{E(\hat{b}_1)}{E(\hat{b}_3)} - \frac{\mathrm{Cov}(\hat{b}_1, \hat{b}_3)}{E^2(\hat{b}_3)} + \frac{E(\hat{b}_1)V(\hat{b}_3)}{E^3(\hat{b}_3)}, \qquad [3]$$

where $V(\hat{b}_3)$ is the variance of $\hat{b}_3$, and $\mathrm{Cov}(\hat{b}_1, \hat{b}_3)$ is the covariance of $\hat{b}_1$ and $\hat{b}_3$ (24). Numerical evaluation of the second and third terms in Eq. **3** suggests that $\hat{t}_1$ in Eq. **2** often gives overestimates of divergence times. This is particularly so when the calibration point is smaller than the divergence time to be estimated. Suppose we know t_1 instead of T in Fig. 1*B* and want to estimate T by $\hat{T} = \Sigma_i(\hat{b}_{3i}/\hat{b}_{1i})t_1/k$. In this case, $\hat{T}_i \equiv (\hat{b}_{3i}/\hat{b}_{1i})t_1$ may become very large if $\hat{b}_{1i}$ happens to be close to 0 by chance (the upper limit being infinity). If $\hat{b}_{1i}$ happens to be large relative to $\hat{b}_{3i}$, $\hat{T}_i$ becomes small but never smaller than t_1. Therefore, $\hat{T}$ tends to be an overestimate when $\hat{b}_{1i}$ varies extensively. To avoid this overestimation, we should use concatenation-based distances (CDs) mentioned below.

Distance Measures to Be Used. When all protein sequences used are closely related, the Poisson correction (PC) distance appears to give sufficiently accurate estimates of divergence times (25). This distance is given by $d = -\ln(1 - p)$, where p is the proportion of sites at which the amino acids of the two sequences compared are different. However, the PC distance is obtained under the assumption that the rate of amino acid substitution per year (r) is the same for all amino acid sites. In practice, this assumption rarely holds, and empirical data have suggested that the rate varies from site to site approximately following the gamma distribution (26). In this case, the evolutionary distance between two sequences can be measured by the following PC gamma distance

$$d = a[(1 - p)^{-1/a} - 1], \qquad [4]$$

where a is the shape parameter of the gamma distribution (gamma parameter) and decreases as the variation of r among sites increases (27, 28).

If we assume that a is a constant, the variance of d is given by $V_1(d) = p[(1 - p)^{-(1+2/a)}]/n$ (28), but if we take into account the sampling variance $[V(\hat{a})]$ of the estimate ($\hat{a}$) of a, the total variance is approximately given by

$$V(d) = V_1(d) + V(\hat{a})\left[(1-p)^{-1/a}\left\{1 + \frac{1}{a}\ln(1-p)\right\} - 1\right]^2, \qquad [5]$$

where $V(\hat{a}) = [2a(a + 1)(p + a)^2]/(np^2)$, and n is the number of amino acids used. This equation was obtained by the delta method by using Anscombe's (29) formula for $V(\hat{a})$.

One might question the applicability of Eq. **4** to actual data, because it does not take into account higher rates of substitution between similar amino acids than between dissimilar amino acids (30). Grishin (31) developed a complex distance measure by taking into account variation in substitution rate among different amino acid sites as well as among different pairs of amino acids. However, this distance can also be approximated very well by a PC gamma distance with $a = 0.65$ (32). Therefore, for most practical purposes, we may use PC gamma distance.

CD Approach. Previously, we mentioned that to obtain an unbiased estimate of t_1, pairwise CDs for all proteins should be computed and b_1 and b_3 be estimated from these distances. There are several ways of concatenating pairwise distances (ds) for different proteins to obtain unbiased estimates of b_1 and b_3.

(i) Simple average distance (d_1). In this method, a PC or PC gamma distance is computed for each protein, and the simple average of the distances for all proteins is used.

(ii) Average distance weighted by sequence length (d_2). One disadvantage of distance d_1 is that the average of ds over loci is computed without regard to the number of amino acids (sequence length). Because a protein distance based on many amino acids would be more reliable than d_1, it would be better to use the average distance weighted with sequence length.

(iii) Multiprotein gamma distance (d_3). As mentioned earlier, PC gamma distance is very flexible and can be applied to most amino acid sequence data (32). However, the gamma parameter a is expected to vary from protein to protein, and it has been shown that the rate of amino acid substitution per protein

roughly follows the gamma distribution (25). This suggests that if we consider many protein sequences simultaneously, the rate of amino acid substitution per site approximately follows the gamma distribution when the entire set of amino acids for all proteins is considered. We can therefore estimate the gamma parameter a for the entire set of amino acids and compute the gamma distance using Eq. **4**. We call this distance the *multiprotein gamma distance* in this paper. The standard errors of the estimates ($\hat{d}_1$, $\hat{d}_2$, and $\hat{d}_3$) of the above three distances may be computed by the bootstrap or the jackknife method by using individual proteins as units of resampling. For distance estimate $\hat{d}_3$, the variance can also be computed by Eq. **5**, but the jackknife variance appears to be more appropriate, because the unit of evolution is a gene or protein rather than an amino acid.

A standard way of concatenating different statistical estimators is to use the inverse of variance as the weight. Lynch (15) suggested that the average of the variances of all pairwise distances for each protein be used as the weight. Although the CD obtained in this way does not give unbiased estimates of b_1 and b_2, it may be useful for the estimation of divergence times. The CD obtained by this method will be denoted by d_L. We used Eqs. **1** and **2** in his paper to compute the (gamma) distance and its variance (with $a = 0.5$) for each protein, following his suggestion.

Divergence Times Between Mice and Rats and Between Humans and Rodents

A large number of authors have estimated the times of divergence between different groups of mammals by using molecular data (11, 33, 34), but the results obtained are conflicting and controversial (35–37). Of special interest in this regard are the divergence times between mice and rats and between humans and rodents. Molecular estimates of these divergence times have been controversial, because the fossil record is poor (38, 39), and rodent genes appear to have evolved faster than primate genes (22). In this paper, we therefore focus our attention primarily on these divergence times. We use five vertebrate species, i.e., mice, rats, humans, chickens, and *Xenopus laevis*, of which the evolutionary relationships are well established and for which many shared protein sequences are available. *Xenopus* is used as an outgroup species (Fig. 1).

Protein Sequence Data. We obtained protein sequence data from the December 1999 edition of the HOVERGEN database (40) and used only sequences that are available for all five species. In this database, orthologous and paralogous genes are not always distinguished, and we attempted to exclude paralogous genes as much as possible by eliminating multigene families such as major histocompatibility complex and immunoglobulin genes. We also constructed a neighbor-joining (NJ) tree using p distance (41) for each gene and eliminated all genes that produced a topology different from the known tree for the five species.

Using the above procedure, we obtained 104 putative orthologous proteins (see Table 3, which is published as supplemental data at www.pnas.org). We used both PC and PC gamma distances in this paper. The gamma parameter a was estimated by Gu and Zhang's (42) method (the computer program available from the web site http://mep.bio.psu.edu) for each protein separately. Fig. 2 shows the estimates ($\hat{a}$) of a for the 104 proteins in relation to the extents of sequence divergence (average root-to-tip branch length; b_R). The $\hat{a}$ value varies extensively from protein to protein, and it is positively correlated with b_R (43) or the proportion (p_v) of variable sites among the five sequences. Because PC gamma distance is disproportionately large compared with PC distance when $\hat{a}$ is small, the relationships in Fig. 2 suggest that the extent of sequence divergence as measured by PC gamma is less heterogeneous among proteins than that obtained by PC. As mentioned earlier, the multiprotein gamma distance is computed by using the $\hat{a}$ value obtained from the entire set of amino acids. This value was 0.57, whereas the average ($\bar{a}$) of $\hat{a}$s for all proteins was 0.76.

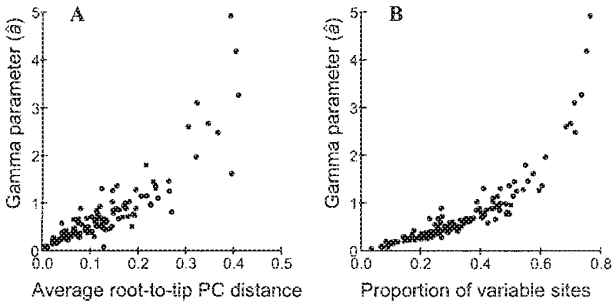


Fig. 2. (*A*) Relationship between estimated gamma parameter ($\hat{a}$) and the average root-to-tip distance (b_R) for 104 nuclear proteins from the five species used. (*B*) Relationships between $\hat{a}$ and the proportion of variable sites (p_v) among the five sequences.

Time Estimation. Theoretically, it is better to eliminate sequence data that do not pass the molecular clock test. In practice, however, proteins that violate the molecular clock hypothesis do not necessarily give unreasonable estimates of divergence times (11). We therefore examined the relationships between U statistic values and time estimates for individual proteins (Fig. 3). In the case of PC distance, 25 proteins did not pass the molecular clock test and showed a U value of 11.3 (1% significance level of χ^2 with 3 degrees of freedoms) or higher. However, the estimates of the mouse-rat and the human-rodent divergence times were nearly the same whether these 25 deviant proteins were included or not. In the case

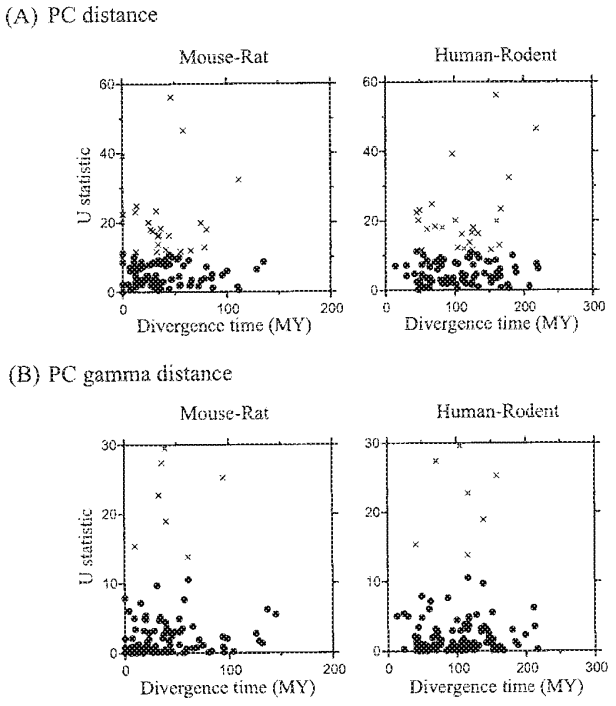


Fig. 3. Relationships between estimated divergence times and U statistic values for each of 104 proteins. The molecular clock hypothesis was rejected for the proteins indicated with the × symbol.

Table 1. Estimates (± standard errors) of divergence times (MY) between mice and rats and between humans and rodents

Method	Mouse-rat (t_1)		Human-rodent (t_2)	
	PC	PC Gamma	PC	PC Gamma
	IP approach			
	44.0 ± 3.4	38.5 ± 3.2	113.4 ± 5.0	102.9 ± 5.0
	CD approach			
d_1 ($\bar{a}$ = 0.76)	40.7 ± 3.0	34.4 ± 2.8	112.3 ± 5.4	99.9 ± 5.3
d_2 ($\bar{a}$ = 0.76)	39.1 ± 2.3	33.0 ± 2.0	110.0 ± 4.4	97.6 ± 4.4
d_3 (a = 0.57)		32.9 ± 2.3		95.5 ± 4.2
d_3 (a = 0.28)		(25.2 ± 2.0)		(82.0 ± 4.0)
d_L (a = 0.5)		32.0 ± 5.0		90.0 ± 10.0

d_1, unweighted average distance; d_2, average distance weighted with sequence length; d_3, multiprotein gamma distance; d_L, Lynch's distance. The standard errors for the CD approach were computed by the jackknife method. The standard errors of $\hat{t}_1$ and $\hat{t}_2$ obtained by using Eq. 5 for d_3 with $a = 0.57$ were 2.0 and 4.0, respectively.

of PC gamma distance, only 7 proteins did not pass the molecular clock test, and the mean estimates of divergence times were again virtually unaffected by inclusion or exclusion of these proteins. For this reason, we used the average time estimates for all proteins as the final estimates disregarding the U values in the independent protein (IP) approach. However, note that the extent of variation in $\hat{t}_1$ and $\hat{t}_2$ is so enormous that the average estimates based on a few proteins are quite unreliable. In this paper, we used $T = 310$ million year (MY) (divergence time between birds and mammals) as the calibration point (11, 38).

In the CD approach, CDs with a large number of amino acids (48,092 in the present case) are used, so that even small differences in evolutionary rate among species become statistically significant. For example, the tree in Fig. 1*A* shows the branch length estimates obtained when distance d_2 was used. The average branch length (0.053) for the mouse and rat lineages after their separation from the human lineage is about 1.2 times longer than that (0.043) of the human lineage, and the difference is highly significant (at the 0.01% level). Similarly, the branch length (0.17) for the rodent lineage after separation from the chicken lineage is 1.3 times greater than that (0.12) of the chicken lineage, and the difference is again significant at the 0.01% level.

However, this extent of variation in evolutionary rate among lineages does not seem to affect time estimates seriously (32). In Fig. 1*A*, it is unclear whether the evolutionary rate was accelerated in the rodent lineage compared with the chicken lineage or was decelerated in the chicken lineage. In either case, however, it is possible to estimate the divergence times t_1 and t_2 by considering the times of separation of chicken and humans from the rodent lineage. For example, t_1 and t_2 can be estimated by $[(a + b)/(a + b + 2c + 2e)]T$ and $[(a + b + 2c)/(a + b + 2c + 2e)]T$, respectively, where $T = 310$ MY. The estimates of t_1 and t_2 obtained in this way are 31 MY and 97 MY, respectively. These estimates are close to those obtained by the linearized tree method (Table 1). For this reason, we decided to use all 104 protein data in all the methods of the CD approach.

Estimates of divergence times between mice and rats and between humans and rodents (t_1 and t_2 in Fig. 1*B*) obtained by all methods are presented in Table 1. When the IP approach is used, the estimates of t_1 and t_2 obtained with PC distance are 44 and 113 MY, respectively. When PC gamma distances are used, the estimates of t_1 and t_2 are both considerably smaller than those obtained by using PC distances.

In the case of the CD approach, PC distances d_1 and d_2 with $a = \infty$ give similar estimates of t_1 (39~41 MY) and t_2 (110~112 MY). When PC gamma is used, the time estimates are somewhat smaller than those obtained by the IP approach for both t_1 and t_2. In general, distance d_2 with $\bar{a} = 0.76$ and d_3 with $a = 0.57$ give very close estimates. Distance d_L gives $\hat{t}_1 = 32$ MY, which is similar to $\hat{t}_1$ obtained by d_3, but $\hat{t}_2$ obtained by d_L is considerably smaller than the values obtained by the other methods. Note also that the standard errors of the estimates obtained by d_L are greater than those obtained by other methods.

Previously we mentioned that the IP approach tends to give overestimates of divergence times, particularly when the times are estimated from recent calibration points. Assuming that t_1 is known to be 33 MY but T is unknown, we can estimate T for the present data set. In this case, we obtain $\hat{T} = \infty$, because there are four proteins for which $\hat{b}_{1i}$ is 0. If we eliminate these four proteins, the average estimate is still 450 MY. By contrast, if we use d_3 with $a = 0.57$, we obtain $\hat{T} = 310$ MY, as expected. If we use t_2 (=96 MY) as the calibration point, the IP approach gives $\hat{T} = 353$ MY, but the CD with d_3 gives $\hat{T} = 310$ MY.

Estimates of Gamma Parameter *a* and Divergence Times. In the above computation of divergence times for humans and rodents, we estimated gamma parameter a from the entire set of species. In practice, the estimate of a tends to be smaller when closely related species are used than when distantly related species are used (43). This occurs because multiple substitutions at the same amino acid sites can be detected more easily in the former group of species than in the latter. Because the estimate obtained from closely related species should be closer to the true a value in the absence of sampling bias (43), one might argue that this estimate should be used for computing pairwise distances for all species. We therefore estimated the a value for d_3 using the mammalian sequences only and obtained $\hat{a} = 0.28$. When this estimate was used, we obtained $\hat{t}_1 = 25$ MY and $\hat{t}_2 = 82$ MY, respectively (Table 1).

However, our computer simulation (G.G. and M.N., unpublished data) has shown that when the a value estimated from closely related species is used for computing pairwise distances, divergence times smaller than the calibration point tend to be underestimated, whereas divergence times greater than the calibration point tend to be overestimated. This happens because for distantly related species the amino acid sites that evolve very fast have little effect on overall sequence divergence and further divergence is primarily determined by slowly evolving sites, which show a rather large a value. Therefore, if we use a small a value obtained from closely related species, it will give unduly large pairwise distances for distantly related species and consequently give overestimates of divergence times for them but may give underestimates for closely related species. Therefore, time estimates of 82 and 25 MY for the human-rodent and the mouse-rat divergence time appear to be underestimates. In practice, this problem is rather complex, and detailed aspects will be discussed elsewhere.

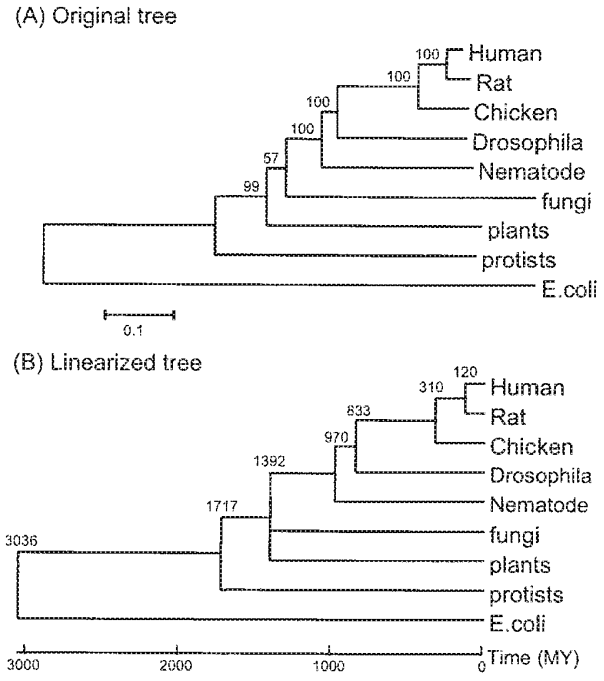


Fig. 4. (*A*) NJ tree constructed by using distance d_3 with $a = 1.24$ for 11 protein sequences. The numbers given for this tree stand for the bootstrap values (500 replications). (*B*) Linearized tree of the above NJ tree. The numbers given for this tree represent the estimates of divergent times.

Estimation of Ancient Divergence Times

Estimation of early divergence times such as those between animals, fungi, and plants is much more difficult than that of divergence times of mammalian species, because the timescale for a linearized tree for these species has to be produced from the fossil record for vertebrates (16, 18, 33), and there is no assurance of constant-rate evolution for a long evolutionary time (12, 44). Furthermore, different statistical methods often give different time estimates even if the same calibration time is used. Here we would like to examine only statistical problems considering protein sequence data from eubacteria (mostly *Escherichia coli*), protists (mostly *Plasmodium*), plants (*Arabidopsis*), fungi (yeast), and five species of animals (nematode, *Drosophila*, chicken, rat, and human). Although a large number of genes have been sequenced in some of these organisms, we could find only 11 orthologous genes that are shared by the above nine species and show relatively few alignment gaps (see supplemental Table 4 at www.pnas.org). All of these proteins were considered to be of eubacterial rather than archebacterial origin (45).

We tested the molecular clock hypothesis for each protein using PC gamma distance, but none of the proteins except one violated the clock hypothesis. This hypothesis was not rejected even when the multiprotein gamma distance for all proteins (3,310 amino acids) was used. The a value obtained for the latter set of proteins was 1.24, and the NJ and the linearized trees constructed from the multiprotein gamma distances are presented in Fig. 4. The timescale for this tree was obtained by using the calibration point of 310 MY between chicken and mammals. The time estimates obtained by this and other methods are presented in Table 2. The divergence time between the *E. coli* genes and their homologues from the eukaryotes used here was obtained under the assumption of a molecular clock, because there was no outgroup for this species group.

CDs d_2 with $\bar{a} = 1.53$ and d_3 with $a = 1.24$ gave essentially the same estimates for all the divergence times considered here (Table 2). Distance d_1 gave slightly smaller estimates than those obtained by d_2 and d_3. Table 2 includes the estimates obtained by d_3 with $a = 0.54$, which was obtained by using only animal sequences (five species). This distance again gives a smaller estimate (115 MY) for the human-rat divergence, which is below the calibration point (310 MY). However, it gives rather high estimates for divergence times earlier than the calibration point. In particular, the estimate of the *E. coli*-eukaryote divergence is unrealistic, because it is older than the age of Earth (ca. 4,500 MY).

Table 2 also includes the time estimates obtained by the IP approach. These estimates are similar to those obtained by Wang *et al.* (16) with a similar method. However, they are considerably higher than the estimates obtained by d_2 and d_3 with $a = 1.24$. Because the IP approach is expected to give overestimates, the values obtained by d_2 and d_3 with $a = 1.24$ appear to be more reliable than those obtained by the IP approach. Unlike the case of mammalian data, Lynch's distance (d_L) gives the smallest time estimates (62 MY) for the human-rat divergence but give large estimates for ancient divergence times. However, the standard errors of these estimates are very large.

Our estimate of divergence time (about 3,000 MY) between eubacteria and eukaryotes based on d_2 and d_3 with $a = 1.24$ is younger than the age (ca. 3,500 MY) of some old microfossils reported (46). If these microfossils are genuine and if the molecular clock hypothesis holds up to ancient bacterial evolution, the difference can be explained by (*i*) the large standard error of our estimate, (*ii*) horizontal gene transfer that might have occurred between the ancestors of current eubacteria and eukaryotes, and/or (*iii*) the possibility that the ancient microfossils reported do not represent the ancestors of current eubacteria and/or eukaryotes.

Table 2. Estimates (± standard errors) of divergence times (MY) of various organisms from the human lineage

Method	Rats	Chicken	*Drosophila*	Nematodes	Fungi	Plants	Protists	Eubacteria
				IP approach				
PC gamma	124 ± 28	310	962 ± 132	1,225 ± 211	1,768 ± 311	1,715 ± 257	2,282 ± 557	3,557 ± 649
				CD approach				
d_1 ($\bar{a} = 1.53$)	113 ± 38	310	745 ± 196	930 ± 274	1,229 ± 402	1,343 ± 394	1,578 ± 485	2,600 ± 568
d_2 ($\bar{a} = 1.53$)	128 ± 38	310	798 ± 121	951 ± 168	1,372 ± 275	1,372 ± 272	1,707 ± 379	3,000 ± 476
d_3 ($a = 1.24$)	120 ± 36	310	833 ± 114	970 ± 160	1,392 ± 256	1,392 ± 252	1,717 ± 349	3,036 ± 470
d_3 ($a = 0.54$)	115 ± 35	310	931 ± 153	1,115 ± 229	1,740 ± 422	1,740 ± 424	2,276 ± 667	5,010 ± 1,060
d_L ($a = 0.50$)	62 ± 10	310	798 ± 274	881 ± 354	1,779 ± 651	1,557 ± 970	1,834 ± 1,034	6,468 ± 5045

d_1, unweighted average PC gamma; d_2, average PC gamma weighted with sequence length; d_3, multiprotein gamma; d_L, Lynch's distance. The divergence time (310 MY) between mammals and birds was used as the calibration point. Evolutionary relationships among animals, fungi, and plants varied with distance measure.

EVOLUTION

Discussion

We have examined various methods of estimating divergence times and have shown that the IP approach is expected to give biased estimates, which are usually greater than those obtained by the CD approach. In the latter approach, distances d_2 and d_3 are expected to give more reliable estimates than distance d_1, although the difference is usually small unless ancient divergence times are considered. Distances d_2 and d_3 usually give similar estimates, but it is easier to compute d_3 than d_2.

We have seen that molecular estimates of divergence times depend on a number of assumptions, and they are generally very crude. Nevertheless, if we use a large number of protein sequences, the estimates appear to be reasonably good (11, 17, 18). Our estimate (96 MY) of the time of human-rodent divergence from d_3 is somewhat smaller than a recent estimate (112 MY) obtained by Kumar and Hedges (11). This difference occurred primarily because we used the CD approach with multiprotein gamma distance, whereas Kumar and Hedges used the IP approach with PC distance. In the case of the mouse-rat divergence, the difference between our estimate (33 MY) and Kumar and Hedges' (41 MY) is substantial.

In the present paper, we did not consider the uncertainty of the calibration point used. In general, the degree of this uncertainty is quite high (12), so that we should always keep in mind that molecular time estimates are very crude, and that the standard errors attached to them merely represent the statistical error associated with molecular data under the substitution model used. Therefore, small standard errors do not necessarily mean a high accuracy of estimates. If we consider uncertainty of the calibration point, the reliability of time estimates is reduced considerably. For example, Lee (12) states that the divergence between birds and mammals probably occurred 288–310 MY ago. In our study, we used $T = 310$ MY, because the true divergence time is likely to be at the higher end of paleontological estimates. However, if we use 288 MY, all the time estimates in Tables 1 and 2 will be lowered by 7.6%.

Molecular time estimates are usually greater than paleontological estimates. Molecular evolutionists tend to argue that this is mainly caused by incomplete fossil records and that molecular estimates are more accurate (4, 39). By contrast, paleontologists and other critics (12, 36, 37) often ascribe this difference to the inaccuracy of the molecular approach of dating. It is not easy to settle this controversy at this stage. Fortunately, molecular data are now rapidly increasing thanks to the recent genome-sequencing projects for many different organisms, and when more data become available, we will be able to make more reliable phylogenetic trees and more reliable estimates of divergence times. If we can construct consistent phylogenetic trees with time estimates for many species and for many genes, we should be able to reconstruct a reasonably good evolutionary history of different organisms at the molecular level. This history can then be compared with paleontological data to develop a unified view of the tree of life. At the present time, the amount of molecular data used for phylogenetic inference and time estimation are often too small to give reliable results.

Of course, it is important to as much as possible use genes or proteins whose evolution follows the molecular clock hypothesis. In recent years, a number of authors have used mitochondrial genes or proteins for estimating divergence times (47, 48). However, these data appear to be inappropriate for time estimation when different orders or classes of vertebrates are considered, because the evolutionary rate varies extensively from species group to species group. For example, the evolutionary rate appears to be more than two times lower in fish than in mammals (49) and more than two times lower in artiodactyls than in primates (47, 50). In these cases, the linearized tree method would not give reliable time estimates.

Another problem is the absence of reliable fossil records to calibrate ancient divergence times. At present, it is customary to use vertebrate fossil records to infer ancient divergence times such as early metazoan divergence and the divergence between animals, fungi, and plants (e.g., refs. 14, 16, 18). Estimation of ancient divergence times by using recent calibration dates is more error prone than that of recent divergence times. In this case, erroneous sequence alignment often causes a serious problem, and small differences in the gamma parameter value influence the estimates substantially. In the case of bacterial evolution, horizontal gene transfer also plays an important role (51), and this would introduce another source of errors in inferring phylogenies and divergence times. Great caution is necessary in the estimation of ancient evolutionary times.

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Table 3. 104 protein sequence data from five vertebrate species (data from Hovergen)

	Protein	Estimate (a) of gamma parameter	Number of amino acids used	Accession number*
1	Insulin-like growth factor I	0.588	112	FAM00007
2	Prepro-insulin	0.811	106	FAM00008
3	Heparin cofactor II	1.139	453	FAM000037
4	Muscarinic acetylcholine receptor M1	0.454	459	FAM000059d
5	Angiotensin II receptor type I	0.994	354	FAM000084
6	Alcohol dehydrogenase-1 (Adh-1)	0.938	228	FAM000111
7	Gastric H+, K+ ATPase beta-subunit	1.144	281	FAM000133
8	Na+, K+ ATPase beta-subunit	0.718	301	FAM000135
9	Acetylcholine receptor delta-subunit	0.886	508	FAM000152a
10	Acetylcholine gamma-subunit	1.307	500	FAM000152b
11	Acetylcholine receptor alpha-subunit	0.409	456	FAM000152e
12	Serum albumin	4.916	596	FAM000192
13	Gap junction protein connexin-4	0.23	377	FAM000242
14	Alpha-A-crystallin	0.887	163	FAM000248a
15	Cu-Zn superoxide dismutase (SOD1)	0.853	150	FAM000256
16	Neural cadherin (N-cadherin)	0.355	900	FAM000258
17	Basic fibroblast growth factor (bFGF)	0.369	152	FAM000274
18	Preproglucagon	1.358	131	FAM000286
19	Bone morphogenetic protein-4 (BMP-4)	0.392	394	FAM000306a
20	Bone morphogenetic protein-2 (BMP-2)	0.581	351	FAM000306b
21	Tissue inhibitor of metalloproteinase-3 (TIMP-3)	0.512	209	FAM000330b
22	Aldehyde dehydrogenase I (ALDH-1)	0.595	499	FAM000337
23	Ornithine decarboxylase (ODC)	0.489	448	FAM000348
24	Osteonectin (Sparc)	0.367	292	FAM000350
25	Poly (ADP-ribose) synthetase	0.634	990	FAM000356
26	Phosphatase 2A-beta (PP2A-beta)	0.039	309	FAM000362
27	Preprosomatostatin	0.398	115	FAM000371
28	ABC transporter (TAP2)	2.661	656	FAM000385
29	Tryptophan hydroxylase (Tph)	0.668	437	FAM000388b
30	Iron binding protein (ferretin) heavy chain	0.403	176	FAM000533
31	Glutamine synthetase	0.46	368	FAM000534
32	Retinol-binding protein (RBP)	0.924	189	FAM000555
33	Type IIA activin receptor	0.286	513	FAM000561a
34	Alpha-enolase (2-phospho-D-glycerate hydrolase)	0.252	434	FAM000627
35	Cyclin D1	0.711	290	FAM000646a
36	Complement component C	1.96	1556	FAM000664

37	Zona pellucida glycoprotein-3 (ZP3)	3.254	397	FAM000667
38	Helix-loop-helix protein (Id-2)	0.243	133	FAM000769
39	Cardiac troponin	0.74	162	FAM000868a
40	Hepatic nuclear factor 1-alpha unit	0.608	585	FAM001004b
41	Retinal degeneration slow (RDS) protein	0.602	343	FAM001091
42	Hepatocyte growth factor (HGF)	0.883	706	FAM001247a
43	Hepatocyte growth factor-like protein	1.243	692	FAM001247c
44	Vimentin (intermediate filament)	0.365	455	FAM001327a
45	Middle weight neurofilament (NF-M)	0.838	748	FAM001328b
46	Cardiac calsequestrin	1.301	380	FAM001355
47	Steroidogenic factor-1 SF-1	0.505	451	FAM001365a
48	Thyroid hormone receptor alpha (c-erbA)	0.288	408	FAM001369a
49	Thyroid hormone receptor beta subunit	0.192	368	FAM001369b
50	Very low density lipoprotein receptor (VLDLR)	0.398	830	FAM001390b
51	Domain transcription factor LIM-1	0.187	401	FAM001396
52	Cyclin-dependent kinase (CDC2)	0.422	296	FAM001432b
53	Neural cell-adhesion molecule (N-CAM)	0.576	716	FAM001444
54	Neural cell surface protein F	0.665	991	FAM001446a
55	Focal adhesion kinase (FAK)	0.182	1051	FAM001463
56	Protein-tyrosine (src) kinase (CSK)	0.224	450	FAM001467
57	Neural receptor protein-tyrosine kinase (trkB)	0.508	797	FAM001471b
58	Insulin-like growth factor I receptor	0.434	1301	FAM001471e
59	c-met oncogene	0.881	1366	FAM001472
60	c-kit receptor tyrosine kinase	0.957	924	FAM001478b
61	Oocyte maturation factor Mos (c-mos)	1.456	324	FAM001493
62	Fibronectin (FN1)	0.839	1069	FAM001519
63	Zinc finger protein slug (SLUG)	0.665	148	FAM001601
64	Wilms tumor protein	0.35	375	FAM001607
65	Integrin beta-1 subunit	0.376	789	FAM001621
66	Muscle regulatory factor (MRF4)	0.38	239	FAM001622c
67	Achaete-scute homologue	0.293	189	FAM001624
68	Collagenase-3 (matrix metalloproteinase-13)	1.02	378	FAM001631c
69	Fos-related antigen-2 (fra-2)	0.336	313	FAM001679a
70	Follistatin (activin antagonist)	0.354	315	FAM001730
71	Structure-specific recognition protein 1 (SSRP1)	0.305	529	FAM001987
72	Gonadotrophin-releasing hormone (GnRH)	3.093	87	FAM002205
73	Alpha 2,8-sialyltransferase-1 (pst-1)	0.577	353	FAM002490
74	Syndecan-3 (Syn-3)	2.598	317	FAM002852
75	Distal-less 3 (Dlx-5) homeobox	0.331	278	FAM002888a
76	Homeobox protein Nkx-2.5 subunit	1.097	285	FAM002890b
77	Corticotrophin-releasing factor type1 receptor	0.375	410	FAM003058b

78	T-cell leukemia (LERK-2)	0.439	326	FAM003670
79	Delta-1 protein	0.584	705	FAM003932a
80	Conductin/axin (AXIN)	0.667	818	FAM006148
81	Transforming growth factor – beta3	0.653	389	FAM000027†
82	Na, K-ATPase beta-2 subunit (ATP1B2)	1.61	271	FAM000134†
83	Embryonic beta-type globin	1.364	137	FAM000214†
84	Alpha globin	1.277	142	FAM000215†
85	Heat shock protein	0.138	650	FAM000300†
86	Lactate dehydrogenase-A	0.616	330	FAM000364a†
87	Histone H2A	0.149	129	FAM000427†
88	bcl-x regulator of cell death	0.764	200	FAM000506†
89	c-ets-1 (p54) protein	0.202	437	FAM000514†
90	Glutamine synthetase	0.471	368	FAM000534a†
91	Bone morphogenetic receptor	0.28	522	FAM000306d†
92	Cyclin (D2, D3)	0.291	288	FAM000646†
93	Furin	0.522	768	FAM000747†
94	Hepatocyte growth factor (HGF)	1.261	686	FAM001247†
95	Liver glyceraldehyde-3-phosphate dehydrogenase	0.243	332	FAM001297†
96	Nucleolin	1.793	605	FAM001318†
97	Vimentin	0.361	454	FAM001327†
98	Lamin B1	0.675	578	FAM001331†
99	Nucleolar phosphoprotein B23 (NPM1)	1.022	285	FAM001359†
100	N-myc protein	0.698	398	FAM001383†
101	CDC2 involved in cell cycle control	0.332	296	FAM001432†
102	Protein tyrosine phosphatase (PTPase-alpha)	0.280	778	FAM001447†
103	Phosphotyrosil protein phosphatase	0.084	591	FAM001456†
104	M-sam fibroblast growth factor	0.314	807	FAM001479†

*Gene family names from the Hovergen database (pbil.univ-lyon1.fr/search/query.html).

†Entries 81--104 from Hovergen 1996 Release.

Tree of Life Reveals Clock-Like Speciation and Diversification

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Abstract

Genomic data are rapidly resolving the tree of living species calibrated to time, the timetree of life, which will provide a framework for research in diverse fields of science. Previous analyses of taxonomically restricted timetrees have found a decline in the rate of diversification in many groups of organisms, often attributed to ecological interactions among species. Here, we have synthesized a global timetree of life from 2,274 studies representing 50,632 species and examined the pattern and rate of diversification as well as the timing of speciation. We found that species diversity has been mostly expanding overall and in many smaller groups of species, and that the rate of diversification in eukaryotes has been mostly constant. We also identified, and avoided, potential biases that may have influenced previous analyses of diversification including low levels of taxon sampling, small clade size, and the inclusion of stem branches in clade analyses. We found consistency in time-to-speciation among plants and animals, ~2 My, as measured by intervals of crown and stem species times. Together, this clock-like change at different levels suggests that speciation and diversification are processes dominated by random events and that adaptive change is largely a separate process.

Key words: **biodiversity, diversification, speciation, timetree, tree of life.**

Introduction

The evolutionary timetree of life (TTOL) is needed for understanding and exploring the origin and diversity of life (Hedges and Kumar 2009; Nei 2013). For this reason, scientists have been leveraging the genomics revolution and major statistical advances in molecular dating techniques to generate divergence times between populations and species. Collectively, tens of thousands of species have been timed, and new divergence time estimates are appearing in hundreds of publications each year (supplementary fig. S1, Supplementary Material online). A global synthesis of these results will allow direct comparison of the TTOL with the fossil record and Earth history and provide new opportunities for discovery of patterns and processes that operated in the past. The TTOL is also essential for studying the multidimensional nature of biodiversity and predicting how anthropogenic changes in our environment will impact the distribution and composition of biodiversity in the future (Hoffmann et al. 2010). A robust TTOL will provide a framework for research in diverse fields of science and medicine and a stimulus for science education. Data now exist for building a synthetic species-level TTOL of substantial size from the growing knowledge (supplementary fig. S1, Supplementary Material online).

There are challenges in synthesizing a global TTOL. The most common approach for constructing a large timetree using a sequence alignment or super alignment is possible (Smith and O'Meara 2012; Tamura et al. 2012), but not generally practical because of data matrix sparseness. For example, genes appropriate for closely related species are unalignable at higher levels, and those appropriate for higher levels are too conserved for resolving relationships of species. Disproportionate attention to some species, such as model organisms and groups of general interest (e.g., mammals and birds), also results in an uneven distribution of knowledge. In addition, computational limits are reached for Bayesian timing methods involving more than a few hundred species (Battistuzzi et al. 2011; Jetz et al. 2012).

Here, we have taken an approach to build a global TTOL by means of a data-driven synthesis of published timetrees into a large hierarchy. We have synthesized timetrees and related information in 2,274 molecular studies, which we collected and curated in a knowledgebase (Hedges et al. 2006) (supplementary Materials and Methods, Supplementary Material online). We mapped timetrees and divergence data from those studies on a robust and conservative guidetree based on community consensus (National Center for Biotechnology Information 2013) and used those times to resolve polytomies and derive nodal times in the TTOL (supplementary fig. S2, Supplementary Material online). We present this synthesis here, for use by the community, and explore how it bears on evolutionary hypotheses and mechanisms of speciation and diversification.

Results

A Global Timetree of Species

Our TTOL contains 50,632 species (fig. 1). Nearly all (~99.5%) of the 1.9 million described species of living organisms are

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Motif Exhibit 1150, Page 420 of 1599

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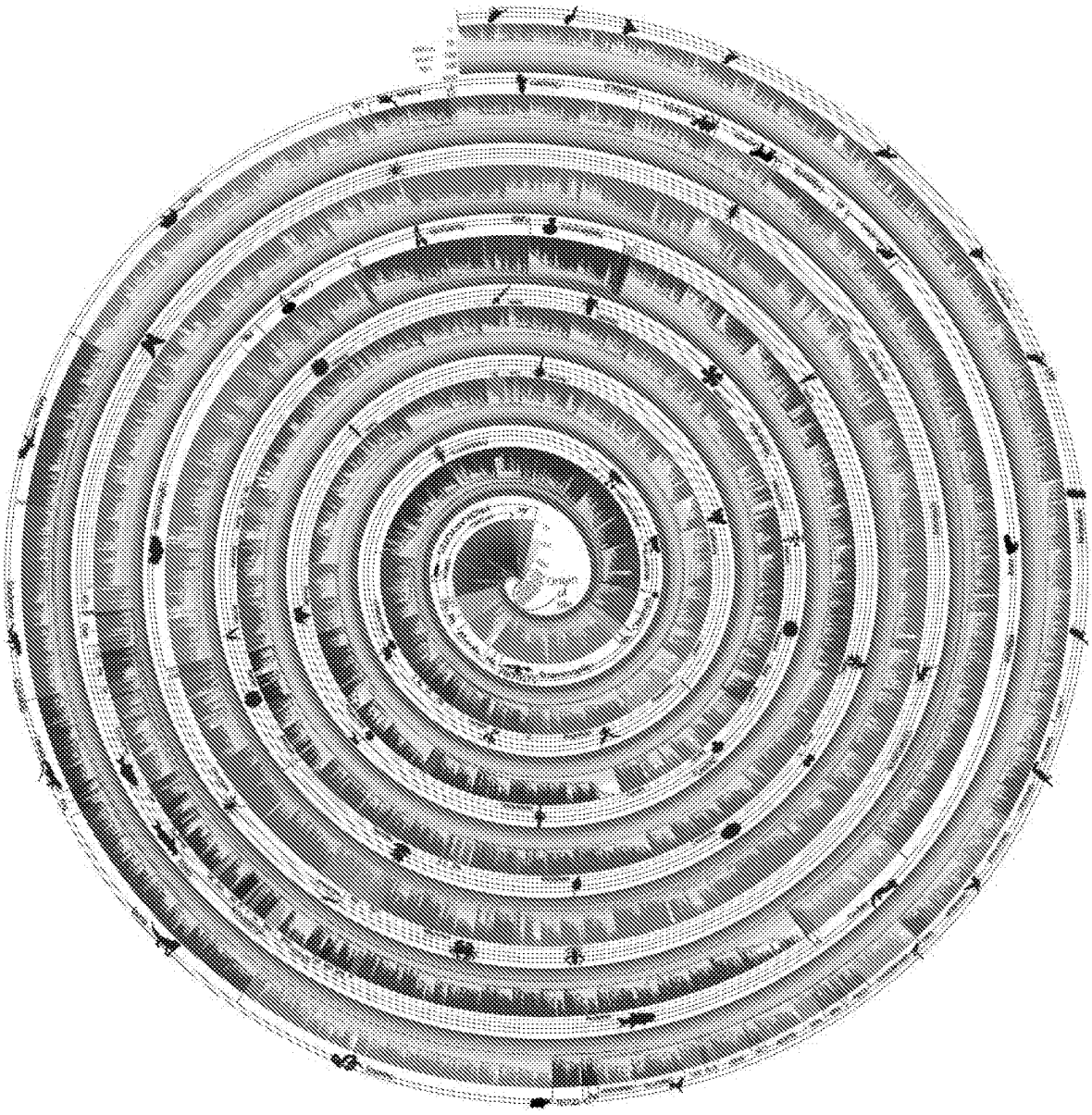


FIG. 1. (2 columns) A timetree of 50,632 species synthesized from times of divergence published in 2,274 studies. Evolutionary history is compressed into a narrow strip and then arranged in a spiral with one end in the middle and the other on the outside. Therefore, time progresses across the width of the strip at all places, rather than along the spiral. Time is shown in billions of years on a log scale and indicated throughout by bands of gray. Major taxonomic groups are labeled and the different color ranges correspond to the main taxonomic divisions of our tree.

eukaryotes (Costello et al. 2013), and the proportion is similar (99.7%) in our TTOL (fig. 1). The naturally unbalanced shape of the TTOL, for example, with more eukaryote than prokaryote species, allowed us to present it in a unique spiral format to accommodate its large size. A "timeline" can be envisioned for each species in the TTOL, based on its sequence of branching events back in time to the origin of life. For example, a timeline from humans (fig. 2) captures evolutionary events that have received the most queries (74%) by users of the TimeTree knowledgebase (Hedges et al. 2006) (supplementary Materials and Methods, Supplementary Material online).

Linnaean taxonomic ranks exhibit temporal inconsistencies (fig. 3) as has been suggested in smaller surveys (Hedges and Kumar 2009; Avise and Liu 2011). For example, a class (higher rank) of angiosperm averages younger than an order (lower rank) of fungus, and an order of animal averages younger than a genus (low rank) of basidiomycete fungus. Ranks for prokaryotes are all older than the corresponding ranks of eukaryotes, with a genus of eubacteria averaging 715.7 ± 139.4 Ma compared with 12.6 ± 1.2 Ma for a genus of eukaryotes (fig. 3 and supplementary Materials and Methods, Supplementary Material online). These inconsistencies will cause difficulties in any scientific study comparing

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

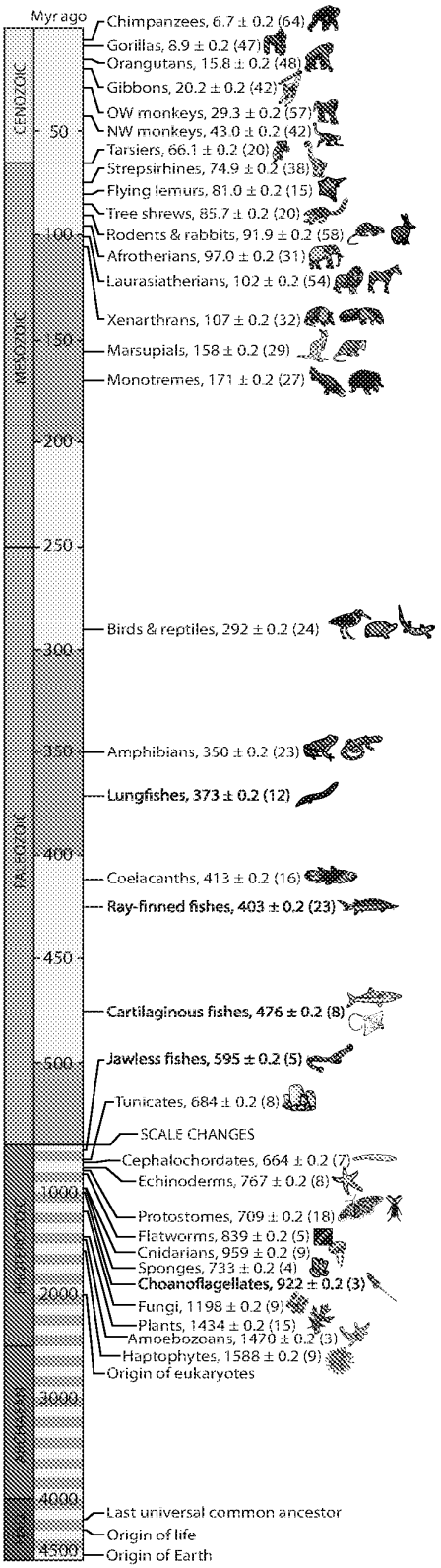


FIG. 2. (1 column) A timeline from the perspective of humans, showing divergences with other groups of organisms. In each case, mean ± standard error (among studies) is shown, with number of studies in parentheses. Also shown are the times for the origin of life, eukaryotes, and last universal common ancestor (Hedges and Kumar 2009).

different types of organisms, where Linnaean rank is assumed to have a temporal equivalence.

Diversification

The large TTOL afforded us the opportunity to examine patterns of lineage splitting across the diversity of eukaryotes (we omit prokaryotes in our TTOL analyses because they have an arbitrary species definition). Under models of "expansion," diversity will continue to expand, either at an increasing diversification rate (hyper-expansion), the same rate (constant expansion), or decreasing rate (hypo-expansion). Saturation, on the other hand, refers to a drop in rate to zero as diversity reaches a plateau (equilibrium), possibly because of density-dependent biotic factors such as species interactions (Morlon 2014). Most recent analyses, but not all (Venditti et al. 2010; Jetz et al. 2012), have suggested that hypo-expansion is the predominant pattern in the tree of life, although there has been considerable debate as to the importance of timescales, biotic or abiotic factors, and potential biases in the analyses (Sepkoski 1984; Benton 2009; Morlon et al. 2010; Rabosky et al. 2012; Cornell 2013; Rabosky 2013).

Instead, we find that constant expansion and hyper-expansion are the dominant patterns of lineage diversification in the TTOL (fig. 4*a*). Our result was statistically significant irrespective of the method used, including diversification rate tests and simulations, a coalescent approach (not conducted for the TTOL), gamma tests, a clade age-size relationship (see Materials and Methods), and branch length distribution analysis (supplementary Materials and Methods, Supplementary Material online). The rate of diversification did not decrease over the history of eukaryotes (fig. 4). The same results were retrieved with another method (supplementary fig. S3, Supplementary Material online); we did not interpret the data before 1,000 Ma because only 37 lineages were present at this time, also explaining the large confidence intervals for this period. The observed diversification closely matched a simulated pure birth–death (BD) model, increasing slightly during the last 200 My (fig. 4*a* and *b*). The terminal drop in rate at ~1 Ma is a normal characteristic of diversification plots (Etienne and Rosindell 2012) related to the taxonomic level selected for the study, in this case species. This "taxonomic bias" (rate drop) occurs at different times (Hedges and Kumar 2009) when genera, families, or other taxa are selected for study, because lower level clades (in this case, populations destined to become species) are omitted.

The TTOL was partitioned into 58 diverse Linnaean clades (Actinopterygii, Afrotheria, *Amazona*, *Ambystoma*, Amphibia, Amphibolurinae, Amphisbaenia, angiosperms, Anguimorpha, *Anolis*, Anseriformes, birds, Boidae, *Bolitoglossa*, Canidae, Cetardiodactyla, *Chamaeleo*, Chondrichthyes, Ciconiiformes, Columbiformes, Cracidae, Cuculidae, *Eleutherodactylus*, Erinaceidae, *Falco*, Feliformia, Gymnophiona, gymnosperms, *Hylarana*, Hyloidea, *Hynobius*, *Hyperolius*, Iguanidae, Kinyongia, Lacertidae, *Liolaemus*, mammals, Megapodiidae, Metatheria, Moniliformopses, Musophagidae, Palaeognathae, Pelobatoidea, Perissodactyla, Phaeophyceae, Phasianidae, *Plethodon*, Podicipedidae,

Motif Exhibit 1150, Page 422 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

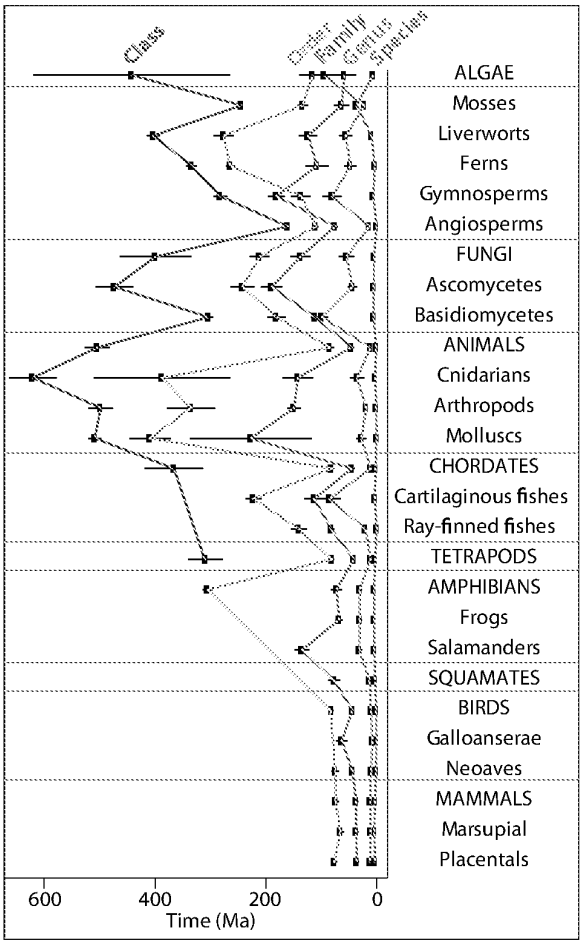


FIG. 3. (1 column) Temporal relationships of Linnaean ranks of eukaryotes, showing mode and 95% confidence intervals. Prokaryotes are not shown because of large differences in scale (supplementary Materials and Methods, Supplementary Material online).

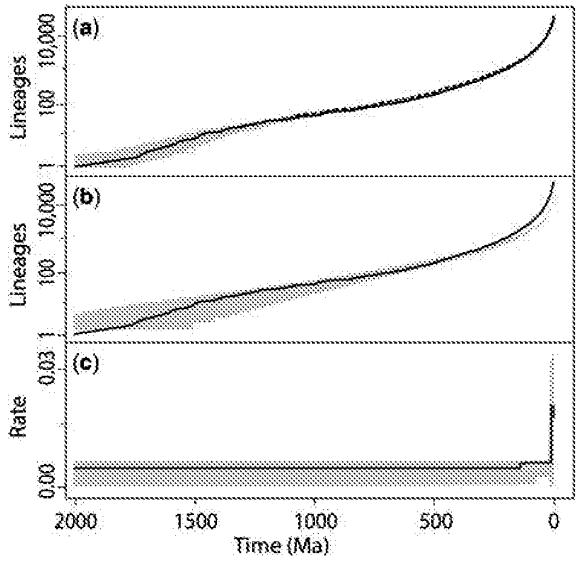


FIG. 4. (1 column) Patterns of lineage diversification. (*a*) Cumulative lineages-through-time (LTT) curve for eukaryotes (50,455 sp.), in black, showing the number of lineages through time (unsmoothed, dashed; smoothed, solid) and variance (red, 500 replicates). (*b*) Same LTT curve (black line), but compared with a simulated constant-expansion LTT curve (λ (speciation rate) = 0.073 and μ (extinction rate) = 0.070) shown as ± 99% confidence intervals (red). (*c*) Diversification rate plot of same data showing only significant changes in rate as determined in maximum-likelihood tests; variance (red, 500 replicates) shown as ± 99% intervals.

Primates, Ranoidea, Squamata, *Suncus*, Thamnophis, Tupaiidae, Ursidae, *Varanus*, Xenarthra, and *Xenopus*) for coalescence and gamma tests encompassing 44,958 total species (supplementary Materials and Methods, Supplementary Material online). The clades were chosen to represent taxonomic diversity and a range of clade size, clade age, and taxonomic sampling. Species sampling levels were high, with a median clade size of 66% of known species and 84% of the clades having 40% or more of known species. Among the 10 largest clades (Actinopterygii, Amphibia, angiosperms, birds, Ciconiiformes, Hyloidea, mammals, Moniliformopses, Ranoidea, Squamata), only one (angiosperms) showed hypo-expansion using the coalescent method, while the others were exclusively hyper-expanding. Low levels of sampling are known to bias toward hypo-expansion (Cusimano and Renner 2010; Moen and Morlon 2014), and the same clade (angiosperms) was also the most poorly sampled (5%). Also, there was no support in those clades for a decline in diversification rate using the gamma test. Although some methods can account for incomplete random sampling, the nonrandom sampling typically used by systematists (e.g., selecting for deeply branching lineages) is not accounted for by current methods and will bias results in favor of hypo-expansion (Cusimano and Renner 2010).

Focusing on the 48 smaller clades (e.g., genera and families) of tetrapods, we found that these likewise favored the expanding models (84% of clades showing significant results) rather than the saturation model (see Materials and Methods). Of those, most (87%) were either expanding or hyper-expanding, with only 13% hypo-expanding. Concerning the gamma test, 67% of these smaller clades did not show a decline in diversification rate through time, as was true for eukaryotes as a whole (fig. 4; gamma statistic = 136.4, $P = 1$). Hyper-expanding clades were significantly larger (fig. 5*a*; $x^{\text{Pleasecheckthispezium}}$, 831 vs. 91 species; $P < 0.05$) and older (fig. 5*b*; $x^{\text{Pleasecheckthispezium}}$, 104 vs. 57 Ma; $P < 0.001$) than other clades (see Materials and Methods). We also found that TTOL branch-length distribution fits an exponential distribution significantly better than other models (see Materials and Methods), agreeing with an earlier study (Venditti et al. 2010) using a small data set and approach.

Diversification by expansion predicts a significant correlation between clade age and clade size (see Materials and Methods). Therefore, we evaluate the expansion hypothesis in two densely sampled groups: Mammals (5,363 species) and birds (9,879 species). We also tested the effect of stem versus crown age (fig. 5*c*) on clade sizes (supplementary Materials and Methods, Supplementary Material online); stem age

Motif Exhibit 1150, Page 423 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

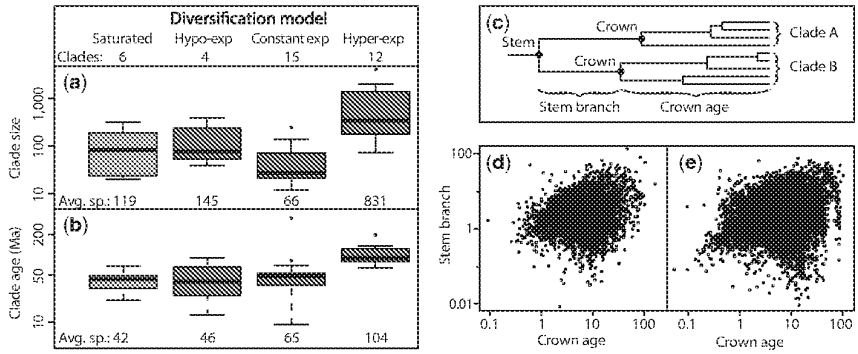


Fig. 5. (1 column) Diversification analyses of major Linnaean clades in the timetree of life. (*a*–*b*) Results of coalescent analyses testing models of diversification. Of 48 tetrapod clades, 37 showed significant model selected and they were used in these analyses. (*a*) Effect of clade size (number of described species). (*b*) Effect of clade age. (*c*) Diagram illustrating difference between stem and crown age for two clades. (*d*–*e*) Relationship of stem branch and crown age in mammals (*d*; $r^2 = 0.07$) and birds (*e*; $r^2 = 0.04$).

includes the time elapsed on the branch leading to the crown. We examined 1,990 clades in two separate analyses: Families (153 and 113 nonnested clades for birds and mammals, respectively) and genera (1,115 and 609 nonnested clades for birds and mammals, respectively). In each case, the relationship was highly significant for crown age ($r = 0.43$–0.47, $P < 0.001$) but nonsignificant or weakly significant ($r = 0.02$–0.09, $P = 0.01$–0.82) using stem age (see Materials and Methods). These results demonstrate a significant relationship between clade age and clade size in major groups of vertebrates, and provide an explanation why this was not observed in past studies, as they used stem ages (crown age + stem branch time).

Our results show that it is best to avoid stem branch time, because the length of any (stem) branch in the tree should not be related to the time depth of the descendant node (crown age; fig. 5*d* and *e*). Therefore, the use of stem branch time will introduce large statistical noise and make the test extremely conservative. For example, when considering every node in a timetree of species, the coefficient of variation of stem branch length relative to crown age is over 200% in the best sampled groups, mammals (208%) and birds (224%). That noise is further weighted by the pull of the present (Nee et al. 1994), which, we determined, adds 40% time (median) to crown age at any given node (if stem age is used instead of crown age), in separate analyses of birds, mammals, and all eukaryotes. This is because the pull of the present creates longer internal branches deeper in a tree, as more lineages are pruned by extinction. Therefore, the use of stem branches in diversification analyses adds noise (variance) and gives increased weight to that noise. We believe that the stronger signal of constant expansion in our results, compared with earlier studies that have supported hypo-expansion and saturation, is in part because we have identified and avoided some biases (e.g., sampling effort, clade size, and stem age) that can impact diversification analyses.

Speciation

The rate of formation of new species is the primary input into the diversification of life over time. This process starts as two or more lineages split from an existing species at a node in the tree. To learn more about the timing of speciation, we assembled a separate data set of studies containing timetrees that included populations and species of eukaryotes (supplementary Materials and Methods, Supplementary Material online). Considering a standard model of speciation based on geographic isolation, in the absence of gene flow (Sousa and Hey 2013), we estimated the time required for speciation to occur. For a given species, the time-to-speciation (TTS), after isolation, must fall between the crown and the stem age of the species (fig. 6*a*). All lineages younger than the crown age of a species lead to populations that are still capable of interbreeding and, therefore, must be younger than the TTS. On the other hand, the stem age is the time when a species joins its closest relative and, therefore, it must be older than the TTS. A sampling omission of either a population or species could lower the crown age estimate or raise the stem age estimate, but the resulting interval would still contain the TTS. Although coalescence of alleles may lead to overestimates of time (Nei and Kumar 2000), those times were interpreted as population and species splits, not allelic splits, in the published studies reporting them. Also, calibrations can ameliorate the effect of allelic coalescence because they are usually based on population and species divergences, not coalescence events. We conducted a simulation to test this approach (supplementary Materials and Methods, Supplementary Material online) finding that the true TTS is estimated precisely with a mode because of skewness of the statistical distribution and it is robust to undersampling of lineages, either younger (populations) or older (species). We also found that the true TTS was not affected by the addition of 10% noise (intervals that do not include the TTS) and only weakly affected (2.5%) by adding a large amount (50%) of noise.

Analyses of three disparate taxonomic groups (vertebrates, arthropods, and plants) with greatly varying generation times and life histories produced similar TTS of ~2 My (fig. 6*b*). For comparison, divergence times among closely related species of prokaryotes, which are defined in practical terms (Cohan 2002), are about 50–100 times older (supplementary Materials and Methods, Supplementary Material online).

Motif Exhibit 1150, Page 424 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

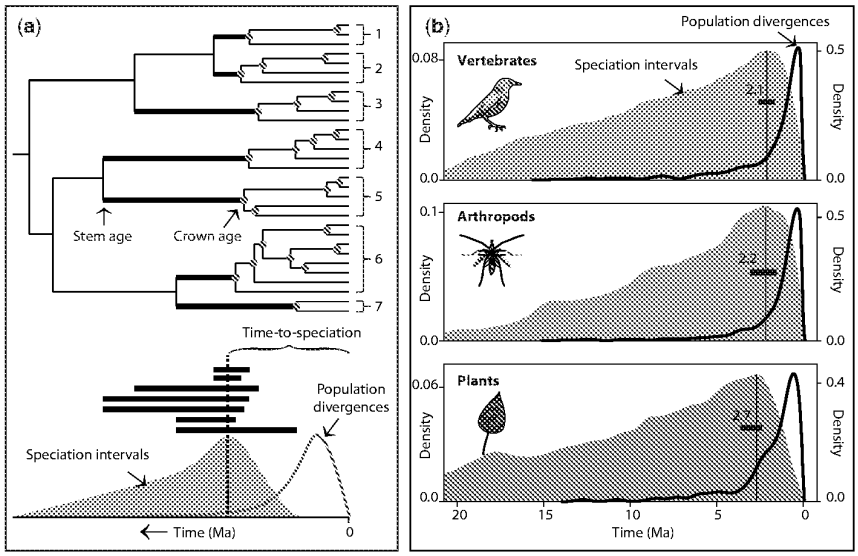


Fig. 6. (2 columns) Estimation of time-to-speciation. (*a*) Analytical design showing expected results. Intervals between stem age and crown age of seven species contain time-to-speciation (dashed line represents the modal time-to-speciation). Divergences among populations, and corresponding histogram, are in red. (*b*) Colored histograms of observed time-to-speciation, showing modes (vertical lines) and confidence intervals (bars) in vertebrates ($N = 213$, 2.1 Ma, 1.74–2.55 Ma), arthropods ($N = 85$, 2.2 Ma, 1.57–3.07 Ma), and plants ($N = 55$, 2.7 Ma, 2.37–3.63 Ma). Black curves to the right are histograms of population divergences.

The observed similarity of TTS in diverse groups of multicellular eukaryotes suggests a model of speciation that places importance on the time that two populations are isolated (fig. 7). If speciation is an outcome of the buildup of genic incompatibilities (GIs) between isolated populations (Coyne and Orr 2004; Matute et al. 2010), then the continuous fixation of selectively neutral mutations in two populations (Zuckerkandl and Pauling 1965; Kimura 1968) will accumulate with time and eventually lead to a number, or fraction, of GIs (here, "S-value") that will cause postzygotic reproductive isolation (fig. 7*c*). In essence, speciation—in the strictest sense—can be defined as this specific moment in time, a "point of no return," because reproductive incompatibility and isolation are inevitable at that point. The diverging lineages will remain independent forever or until they become extinct. If they do come back into contact (fig. 7*a*), they might hybridize briefly but would then undergo reinforcement, leading to prezygotic reproductive isolation. We focus here on the major model, geographic isolation, but time constraints should be similar with ecological speciation, and other models exist (Coyne and Orr 2004).

A speciation clock has been suggested previously (Coyne and Orr 1989), where GIs buildup linearly over time in postzygotic reproductive isolation. However, current evidence indicates a faster-than-linear ("snowball") rate of buildup of GIs in postzygotic reproductive isolation (Matute et al. 2010). The snowball pattern of increase in GIs is not a problem for a speciation clock as long as the time of attainment of the S-value is similar in different species. Our evidence from the TTS analysis (fig. 7*b*) suggests this to be the case.

Relatively few populations will remain isolated until the point of no return because environments often change rapidly in the short term, raising and lowering barriers to gene flow (fig. 7*a*). This is amplified by a preponderance of low incline landscapes (Strahler 1952), resulting in a greater impact of climate and sea level change. Therefore, the buildup of GIs will be reset many times before a successful speciation event occurs (fig. 7*c*). This resetting, which results in failed isolates, may explain "barcode gaps" (Puillandre et al. 2011), which we interpret as prespeciation loss of potential lineages. We estimate that branches between nodes in well-sampled (> 20% coverage) groups of the TTOL are, on average, 4–5 My long using a slope method (4.92 ± 0.32 My) and a coalescent method (4.21 ± 0.76 My) (Supplementary Materials and Methods, Supplementary Material online). This indicates that branch time is longer than TTS, and includes a significant "lag time," referring to any time along a branch that is not part of a successful speciation event, such as the resetting of the speciation clock by reversal of the buildup of GIs. The high variance of branch length in trees (fig. 5*d* and *e*) may reflect the stochastic nature of environmental change and isolation of populations, contributing to a "diversification clock" (fig. 4). This is consistent with the absence of a strong correlation between reproductive isolation and diversification rate in some taxa (Rabosky and Matute 2013).

In relating this speciation model to a phylogeny (fig. 7*d*), it is evident that the split of two lineages in a tree is not the speciation event but rather the moment of isolation of two or more populations that will remain isolated until the point of no return. For example, if we assume a TTS of 2 My, then two populations that only recently became species would have split ~2 Ma (fig. 7*d*, species 1–2). Yet, much deeper in a tree, where branches may be tens of millions of years long, the TTS is a relatively small fraction of total branch time, which makes

Motif Exhibit 1150, Page 425 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

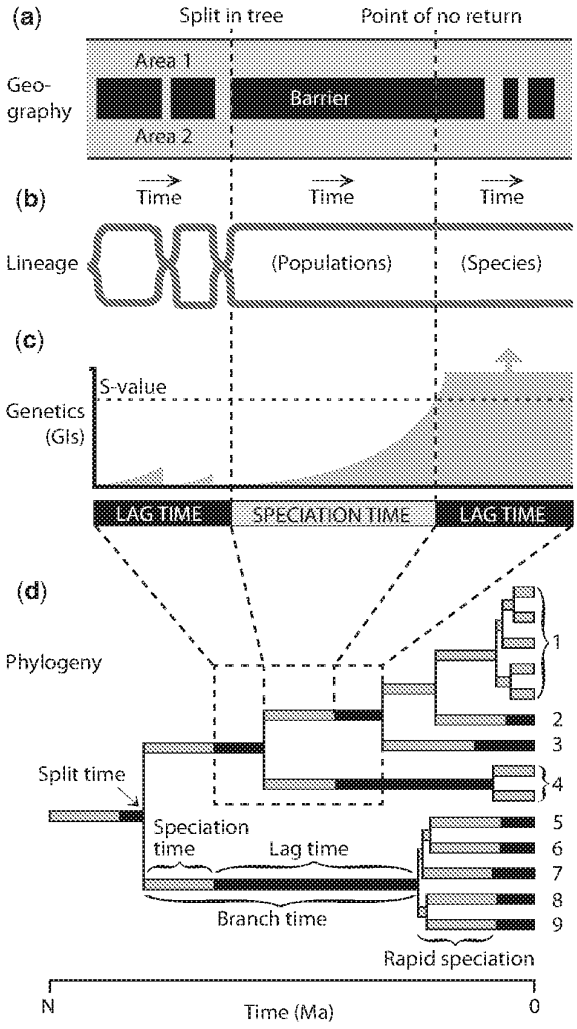


Fig. 7. (1 column) Summary model of speciation. (*a*) Biogeographic history showing the contact and isolation of areas occupied by the two populations. (*b*) Phylogenetic lineages showing times of independence (two lineages) and times of interbreeding (one lineage). (*c*) Genic incompatibilities between the two populations, showing how they accrue at a time-dependent rate during geographic isolation, reset to zero during contact (interbreeding), increase to the S-value (the number of GIs that will cause speciation, the point of no return), and continue increasing beyond the S-value despite later contact of the newly formed species. (*d*) Hypothetical phylogeny, with numbered species, illustrating parameters of speciation in (*a*–*c*) to splits and branches in a tree.

a splitting event more-or-less equivalent to the speciation event. Furthermore, branch splitting leading to eventual species formation can happen simultaneously, in many lineages, as long as the resulting populations remain isolated until the point of no return (fig. 7*d*, species 5–9).

Discussion

These results have implications for patterns of species diversification and the interpretation of timetrees. If adaptation is largely decoupled from speciation, we should not expect it to be a driver of speciation as is frequently assumed. Also, we should not expect to see major diversification rate increases following mass extinction events, even though large adaptive changes took place at those times. That expectation is realized in our analyses (fig. 4) where we see constant splitting through time across the two major Phanerozoic extinction events (251 and 66 Ma). Likewise, our diversification analyses of smaller groups, such as birds and mammals (supplementary fig. S4, Supplementary Material online), and past studies of those groups (Bininda-Emonds et al. 2007; Meredith et al. 2011; Jetz et al. 2012) have not found rate increases immediately following the end-Cretaceous mass extinction event (66 Ma). Rate decreases from the extinction events themselves are not expected because of the inability to detect, in trees, a large proportion of species extinctions that occurred (Nee and May 1997).

The consistency in TTS among groups found here suggests that the time-based acquisition of GIs, and not adaptive change, is driving reproductive isolation, almost in a neutral process. By implication, geographically isolated populations, even if morphologically different and diagnosable, are not expected to be species until they have reached the point of no return. This is because some differentiation and adaptive change should occur in isolation, and many isolates will be ephemeral (Rosenblum et al. 2012), merging with other isolates or disappearing and never becoming species that enter the tree of life. More population data are needed before it is possible to identify the point of no return with precision. Nonetheless, these data suggest that, in most cases, described species separated by only tens of thousands of years are not real species. The Linnaean rank of subspecies, which has declined in use for decades, might be appropriate for such diagnosable isolates that have not yet reached the point of no return. Oversplitting of species by taxonomists may explain the sharp peak in diversification (hyper-expansion) in the last 10 My of eukaryote history (fig. 4*b*). On the other hand, it could also result from statistical (small sample) artifacts that were published in many different studies (summarized in the TTOL). Therefore, further analysis of that unusual rate spike is warranted.

In summary, the diversification of life as a whole is expanding at a constant rate and only in some small clades (<500 species) is there evidence of decline or saturation in diversification rate. We believe these results can be explained as many different groups of organisms undergoing expansion and contraction through time, with those patterns captured in different stages (expanding, slowing, or in equilibrium). If true, the predominant pattern of expansion in large clades is expected from the law of large numbers, where such smaller, random events would average to a constant rate. Constant expansion also follows from random environmental changes leading to isolation and speciation. Rate constancy is consistent with the fossil record (Benton 2009) and does not deny the importance of biotic factors in evolution (Ricklefs 2007), but it suggests an uncoupling of speciation and adaptation. Cases where the phenotype has changed little (e.g., cryptic species) or greatly during the TTS are interpreted here as evidence of uncoupling. The lineage splitting seen in trees probably reflects, in most instances, random environmental events leading to isolation of populations,

Motif Exhibit 1150, Page 426 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

and potentially many in a short time. However, the relatively long TTS (~2 My), a process resulting from random genetic events, will limit the number of isolates that eventually become species. Under this model, diversification is the product of those two random processes, abiotic and genetic, and rate increases (bursts of speciation) are more likely to be associated with long-term changes in the physical environment (e.g., climate, sea level) causing extended isolation rather than with short-term ecological interactions. However, reductions in extinction rate could also explain increases in diversification rate. Adaptive change that characterizes the phenotypic diversity of life would appear to be a separate process from speciation. Although a full understanding of these processes remains a challenge, determining how speciation and adaptation are temporally related would be an important "next step."

Materials and Methods

Detailed methods are described in supplementary Materials and Methods, Supplementary Material online.

TTOL Data Collection

We synthesized the corpus of scientific literature where the primary research on the TTOL is published. We first identified and collected all peer-reviewed publications in molecular evolution and phylogenetics that reported estimates of time of divergence among species. These included phylogenetic trees scaled to time (timetrees) and occasionally tables of time estimates and regular text. We assembled timetree data from 2,274 studies (http://www.timetree.org/reference_list.php) that have been published between 1987 and April 2013, as well as two timetrees estimated herein (supplementary table S1, Supplementary Material online). Most (96%) of nodal times used were published in the last decade.

TTOL Analytics and Synthesis

We used a hierarchical average linkage method of estimating divergence times (T_s) of clade pairs to build a Super Timetree, along with a procedure for testing and updating topological partitions to ensure the highest degree of consistency with individual timetrees in every study. For the TTOL, uncertainty derived from individual studies is available for each node (supplementary table S2, Supplementary Material online). Branch time modes of different Linnaean categories were estimated (supplementary table S3, Supplementary Material online).

Diversification Analyses

All diversification analyses were performed in R (http://www.r-project.org/) with the APE package (Paradis et al. 2013). The gamma statistic was employed to detect decrease in speciation rate over the history of the tree. To test if diversification is saturated or expanding, we used a coalescent method as well as the relationship between the ages of different clades and their sizes (numbers of species). The number and location of shifts in diversification rates were also tested. We also estimated among-study uncertainty in both the lineages-through-time (LTT) curve and rate test by producing 500 replicates of the TTOL, sampling actual study times at each node randomly. These replicates were then used in the analysis of rate shifts to estimate and test rate change. Results of all diversification analyses were summarized (supplementary tables S4–S10, Supplementary Material online).

Gamma Test

The gamma statistic (Pybus and Harvey 2000) was employed on 59 clades (all eukaryotes plus 58 clades listed in supplementary table S5, Supplementary Material online) to detect decrease in speciation rate over the history of the tree (LASER package; Rabosky and Schliep 2013). These 58 groups were chosen to have an overview of the diversification processes on a diversity of clades across life, in terms of size, sampling effort (above 5%; median 66%, with 84% of the clades having more than 40% coverage), and clade age. Included in the 58 clades are 10 nonnested groups over the major Linnaean groups, which allowed us to draw conclusions about high-level and low-level clades. We further selected 10 nonnested clades from within each of the four well-sampled groups of tetrapods (amphibians, birds, mammals, and squamates). We also analyzed separately the Eukaryote clade because all the other groups are nested within this one.

A negative gamma value reveals a concentration of branching times near the root, meaning a decelerated diversification. Incomplete lineage sampling can affect the result of this test because some tips will be pruned, thus lengthening the branch length in the more recent past (Nee et al. 1994). An incomplete sampling could therefore lead to an erroneous detection of slowdown. To avoid this bias, the Monte Carlo constant rate test (MCCR test) (Pybus and Harvey 2000) was also performed on clades with a significant negative gamma value (LASER package). Here, the critical value for rejecting a constant rate ($\alpha = 0.05$) was calculated by examining the distribution of gamma for simulated trees with the same incomplete lineage sampling proportion as the observed tree. We conducted 5,000 Monte Carlo simulations for each MCCR test. If the MCCR test finds that the detection of slowdown is erroneous, then there is no support for decline in speciation rate for this clade.

Models of Diversification

To test if diversification is saturated or expanding, we used a coalescent method (Morlon et al. 2010) on 58 clades (supplementary table S5, Supplementary Material online). We defined two sets of models: Saturated diversity (models 1 and 2) and expanded diversity (models 3, 4a, 4b, 4c, 4d, 5, and 6). As recommended by the author of the method, we first selected the model with the lowest second-order Akaike's Information Criterion in each subset (saturated vs. expanding models). We then evaluated the relative probability of these two models based of their Akaike weights. The relative probabilities of two models (l and k) were calculated as $\frac{wl}{wl+wk}$ and $\frac{wk}{wl+wk}$, with wl (the model's l Akaike weight) as

Motif Exhibit 1150, Page 427 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

$wI = \frac{\exp(-\triangle I/2)}{\sum_{r=1}^{R}\exp(-\triangle r/2)}$ and R the number of candidate models. This method can account for missing species. We therefore used the number of described species (supplementary table S5, Supplementary Material online) as the number of tips of the phylogenies (N_0). When neither of the two relative probabilities obtained exceeded 0.6, we did not draw a conclusion on this specific clade (referred as "nonsignificant" in supplementary table S5, Supplementary Material online) and the mean λ (between the best expanding and saturating model) was used (supplementary table S4, Supplementary Material online). Statistical tests were performed (supplementary table S6, Supplementary Material online) to compare the four models, saturated (sat), hyper-expanding (exp+), expanding (exp), and hypo-expanding (exp-), at the same time (Kruskall–Wallis rank-sum test) and by pair (Wilcoxon rank-sum test). Another test was performed (*t*-test) to compare the means (described number of species and crown age) between the clades under hyper-expansion and the others (saturated, hypo-expanding, and expanding). We confirmed for all clades that the BD polytomy resolution did not change the results, although a different model was selected for 3 of the 58 clades (5%). This method was computationally unfeasible with the entire TTOL.

Clade Age and Clade Size Analyses

To test, in another way, for expansion (hyper-, constant-, and hypo-) versus saturation of the diversification curve, we evaluated the relationship between the ages of different clades and their sizes (numbers of species) (Cornell 2013). Under the saturated diversity model, no relationship should be observed, in contrast to a positive relationship under models of expanding diversity. For this test, we chose the two best sampled groups, birds and mammals, and performed comparative analyses on clades (all nonnested) of genera (1,115 and 609 for birds and mammals respectively) and families (153 and 113 for birds and mammals, respectively), accounting for phylogeny (phylogenetic generalized linear models) with the CAPER package (Orme et al. 2013). In one set of analyses, we used stem age and in another set, crown age (supplementary table S7, Supplementary Material online) was used.

Detection of Shifts in Diversification Rates

The TREEPAR package (Stadler 2013) was used to estimate the number of changes in diversification rate under a BD model and to obtain estimates of the corresponding diversification rate ($\lambda-\mu$) and the extinction fraction (μ/λ). To analyze our eukaryote timetree, as well as the individual component timetrees of birds and mammals, we used the greedy approach, as described in Stadler (2011). We estimated rates in 0.1 My steps between 2 and 70 Ma and in 10 My steps between 2 and 3,500 My for eukaryotes. The BD shift model (without mass extinction) was used and we obtained maximum-likelihood rate estimates for the different data sets for zero, one, two, three, four, five, six, and seven rate shifts. The likelihood ratio test was used to select the best model in each case at the 99% level.

For the TTOL, we estimated node time uncertainty in both the LTT curve and rate test by producing 500 replicates of the TTOL, sampling a time between the confidence intervals at each node under a uniform distribution. These replicates were then used in the TREEPAR analysis to estimate and test rate change, with the uncertainty shown in figure 4 and the significant shifts by time interval (20 My) shown in supplementary table S8, Supplementary Material online.

Our diversification results for birds are similar to an earlier analysis (Jetz et al. 2012), with a strong increase in diversification from ~45 Ma to the present. The mammal results are also generally similar to past mammal analyses in showing the absence of any rate increase immediately after the end-Cretaceous extinctions (Bininda-Emonds et al. 2007; Meredith et al. 2011). We did not find a sharp mid-Cenozoic increase in rate that was found in one other analysis (Stadler 2011), although we determined that the cause of that rate increase was from a polytomy in the mammal timetree used in that study (Stadler 2011); otherwise our results are comparable.

For the bird tree (supplementary fig. S4*a*, Supplementary Material online), the models with zero, one, two, three, four, and five rate shifts are rejected in favor of a model with six rate shifts ($P < 0.05$) (supplementary table S9, Supplementary Material online). A model with six rate shifts is not rejected in favor of a model with seven rate shifts ($P = 0.091$). The 6 shifts are detected at 1, 3.4, 14.4, 48.2, 73.3, and 84.4 Ma (supplementary table S10, Supplementary Material online). The parameters obtained for the 6 shifts model between 73.3 and 48.2 Ma ($\lambda-\mu = 0.04058$ and $\mu/\lambda = 0.0395$) were used to plot the confidence interval of the null distribution (supplementary fig. S4*a*, Supplementary Material online).

For the mammal tree (supplementary fig. S4*b*, Supplementary Material online), the models with zero, one, two, or three rate shifts are rejected in favor of a model with four rate shifts ($P < 0.05$) (supplementary table S9, Supplementary Material online). A model with four rate shifts is not rejected in favor of a model with five rate shifts ($P = 0.123$). The 4 shifts are detected, at 2.1, 9.6, 42.4 and 105 Ma (supplementary table S10, Supplementary Material online). The parameters obtained for the 4 shifts model between 105 and 42.4 Ma ($\lambda-\mu = 0.05186$ and $\mu/\lambda = 0.3528$) were used to plot the confidence interval of the null distribution (supplementary fig. S4*b*, Supplementary Material online).

For the eukaryote tree (fig. 4), the models with zero, one, two, three, four, and five rate shifts are rejected in favor of a model with six rate shifts ($P < 0.05$) (supplementary table S9, Supplementary Material online). A model with six rate shifts is not rejected in favor of a model with seven rate shifts ($P = 0.055$). Six shifts are thus detected at 1, 11, 21, 121, 14,1 and 151 Ma (supplementary table S10, Supplementary Material online). The parameters obtained for the 6 shifts model between 2,100 and 151 Ma ($\lambda-\mu = 0.00307$ and $\mu/\lambda = 0.9581$) were used to plot the confidence interval of the null distribution (fig. 4).

Motif Exhibit 1150, Page 428 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

In addition, we used the BAMMtools package (Rabosky 2014) in order to detect the diversification rate differences across lineages. The function "setBAMMpriors" was used to generate a prior block that matched the "scale" (e.g., depth of the tree) of our data. Both the speciation and the extinction rate were allowed to vary through time and across lineages, and 50,000,000 generations of MCMC simulation were performed. The sampling fraction (0.02635422) was specified by setting the "globalSamplingFraction" parameter. A burnin of 0.5 was applied and a diversification rate plot was obtained with the function "plotRateThroughTime" (supplementary fig. S3, Supplementary Material online).

Time-to-Speciation Analyses

In addition to our species-level TTOL data collection described above, we collected a separate data set on TTS from published molecular timetrees that included timed nodes among populations and closely related species of three major groups: Vertebrates, arthropods, and plants (supplementary tables S11–S13, Supplementary Material online). To test the robustness of our approach for estimating TTS, we used simulations. A BD tree was simulated using the function "sim.bd.taxa" (TreeSim in R).

Supplementary Material

Supplementary figures S1–S4, tables S1–S13, and Materials and Methods are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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Motif Exhibit 1150, Page 429 of 1599

Case No.: IPR2023-00208
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Motif Exhibit 1150, Page 430 of 1599

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES
First Named Inventor/Applicant Name:	Rachel Fraser
Filer:	M. Angela Parsons/Judith Witzel
Attorney Docket Number:	38767-0015008

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

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UTILITY EXAMINATION FEE	1311	1	760	760
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Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
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Electronic Acknowledgement Receipt

EFS ID:	31971059
Application Number:	15913090
International Application Number:	
Confirmation Number:	9878
Title of Invention:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES
First Named Inventor/Applicant Name:	Rachel Fraser
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Attorney Docket Number:	38767-0015008
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Claims	68	71
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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode – SEQ.TXT

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Application Number: 15913090 Document Date: 03/06/2018

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Form Revision Date: August 26, 2013

Attorney's Docket No.: 38767-0015008

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Rachel Fraser	Art Unit	:	Unknown
Serial No.	:	15/913,090	Examiner	:	Unknown
Filed	:	March 6, 2018	Confirm No.	:	9878
Title	:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as indicated on the following pages.

Amendments to the Claims:

Please cancel claims 1-30 without prejudice to continued examination, and please add new claims 31-53 as follows. The claims and their status are shown below.

1-30. (Cancelled)

31. (New) A meat-like food product produced by a method comprising:

providing one or more plant proteins; and

combining, with the one or more plant proteins:

a source of a heme-containing protein, wherein the source of the heme-containing protein comprises at least 60% of the heme-containing protein on a dry weight basis;

a compound selected from glucose, ribose, fructose, lactose, xylose, arabinose, glucose-6-phosphate, maltose, and galactose, and mixtures of two or more thereof;

a compound selected from cysteine, cystine, thiamine, methionine, and mixtures of two or more thereof in to a concentration of at least 1.5 mM; and

wherein cooking the meat-like food product results in the production of at least two volatile compounds which have a meat-associated aroma.

32. (New) The meat-like food product of claim 31, wherein the one or more plant proteins comprises a textured vegetable protein.

33. (New) The meat-like food product of claim 31, wherein the texture vegetable protein is textured soy protein.

34. (New) The meat-like food product of claim 31, wherein the source of the heme-containing protein comprises at least 70% of the heme-containing protein on a dry weight basis.

35. (New) The meat-like food product of claim 31, wherein the source of the heme-containing protein comprises at least 80% of the heme-containing protein on a dry weight basis.

36. (New) The meat-like food product of claim 31, wherein the heme-containing protein is a globin.

37. (New) The meat-like food product of claim 31, wherein the heme-containing protein comprises an amino acid sequence having at least 80% sequence identity to a polypeptide set forth in SEQ ID NOs. 1-26.

38. (New) The meat-like food product of claim 31, wherein the glucose, ribose, fructose, lactose, xylose, arabinose, glucose-6-phosphate, maltose, and galactose, or mixtures of two or more thereof is added to a concentration of about 5.6 to about 20 mM.

39. (New) The meat-like food product of claim 31, wherein the cysteine, cystine, thiamine, methionine, and mixtures of two or more thereof is added to a concentration of at least 10 mM.

40. (New) The meat-like food product of claim 31, wherein the cysteine is added to a concentration of about 0.8 mM to about 10 mM.

41. (New) The meat-like food product of claim 31, wherein the thiamine is added to a concentration of about 0.1 mM to about 2 mM.

42. (New) The meat-like food product of claim 31, further comprising adding one or more of inosine, inosine monophosphate (IMP), guanosine, guanosine monophosphate (GMP), and adenosine monophosphate (AMP).

43. (New) The meat-like food product of claim 31, further comprising adding one or more of beta-carotene, alpha-tocopherol, caffeic acid, propyl gallate, and epigallocatechin gallate.

44. (New) The meat-like food product of claim 31, further comprising adding one or more of a vegetable oil, an algal oil, sunflower oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, canola oil, flaxseed oil, coconut oil, and mango oil.

45. (New) The meat-like food product of claim 31, further comprising adding one or more of acetic acid, lactic acid, glycolic acid, citric acid, succinic acid, tartaric acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha linolenic acid, gamma linolenic acid, arachidic acid, arachidonic acid, behenic acid, and erucic acid.

46. (New) The meat-like food product of claim 31, wherein cooking comprises heating the meat-like food product at 150°C for about 3 to about 5 minutes.

47. (New) The meat-like food product of claim 31, wherein the meat-like food product has a pink to red color before cooking to indicate a raw or uncooked state.

48. (New) The meat-like food product of claim 31, wherein at least a portion of the meat-like food product, upon cooking, transitions in color from a pink to red color in a raw or uncooked state to a lighter pink to brown color in a partially cooked to fully cooked state.

49. (New) The meat-like food product of claim 31, wherein cooking the meat-like food product results in the production of at least five volatile compounds which have a meat-associated aroma.

50. (New) The meat-like food product of claim 31, wherein cooking the meat-like food product results in the production of at least ten volatile compounds which have a meat-associated aroma.

51. (New) The meat-like food product of claim 31, wherein cooking the meat-like food product results in the production of at least twenty volatile compounds which have a meat-associated aroma.

52. (New) The meat-like food product of claim 31, wherein the at least two volatile compounds are selected from 2-methyl-furan, bis(2-methyl-3-furyl)disulfide, 2-pentyl- furan, 3,3'-dithiobis-2-methyl-furan, 2,5-dimethyl-pyrazine, 2-methyl-3-furanthiol, dihydro-3- (2H)-thiophenone, 5-methyl-2-thiophenecarboxaldehyde, 3-methyl-2-thiophenecarboxaldehyde, 2-methyl-thiazole, dimethyl sulfide, decanal, 5-ethyldihydro-2(3H)-furanone, dihydro-5-pentyl-2(3H)-furanone, 2-octanone, 3,5-octadien-2-one, p-Cresol, and hexanoic acid.

53. (New) The meat-like food product of claim 31, wherein the at least two volatile compounds are 2-methyl-furan and bis(2-methyl-3-furyl)disulfide.

REMARKS

Claims 1-30 have been canceled without prejudice, and new claims 31-53 have been added. Support for these amendments is found throughout the original specification, based at least on the disclosure cited below in Table 1.

Table 1

Claim No.	Claim Language	Representative support in application as filed
31	"meat-like food product"	page 4, lines 9-10; page 9, lines 5-7; page 28, line 26 - page 29, line 8; page 43, line 2 - page 50 (Example 10); page 59, line 3 - page 62, line 1 (Example 12)
	"one or more plant proteins"	Example 1, page 30, lines 6-8 (e.g., Table 4)
	"a source of a heme-containing protein"	page 17, lines 14-20
	"the source of the heme-containing protein comprises at least 60% of the heme-containing protein on a dry weight basis"	page 17, lines 18-22
	"a compound selected from glucose, ribose, fructose, lactose, xylose, arabinose, glucose-6-phosphate, maltose, and galactose, and mixtures of two or more thereof"	page 11, lines 23–27; page 12 (Table 1, row 1); Example 1 (e.g., page 29, lines 15-17); Example 4 (e.g., page 35, lines 3-11); Example 5 (e.g., page 38, lines 8-10); Example 6 (e.g., page 39, lines 4-6); Example 7 (e.g., page 39, lines 28-30); Example 8 (e.g., page 40, lines 20-21); Example 9 (e.g., page 41, lines 13-17); Example 10 (e.g., page 43, lines 7-10); Example 11 (e.g., page 53, lines 12-14 and Table 13)
	"a compound selected from cysteine, cystine, thiamine, methionine, and mixtures of two or more thereof in to a concentration of at least 1.5 mM"	page 12 (Table 1, rows 4 and 6); Example 1 (e.g., page 29, lines 15-17); Example 4 (e.g., page 35, lines 3-11); Example 5 (e.g., page 38, lines 8-10); Example 6 (e.g., page 39, lines 4-6); Example 7 (e.g., page 39, lines 28-30); Example 8 (e.g., page 40,

		lines 20-21); Example 9 (e.g., page 41 lines 13-17); Example 10 (e.g., page 43, lines 7-10); Example 11 (e.g., page 53, lines 12-14 and Table 13)
	"cooking the meat-like food product results in the production of at least two volatile compounds"	page 21 – page 25, line 7; page 36-38 (Table 8)
	"which have a meat-associated aroma"	page 19, line 23 – page 20, line 1; Tables 2, 7 and 11
32	"textured vegetable protein"	page 26, line 21
33	"textured soy protein"	page 26, line 22
34	"the source of the heme-containing protein comprises at least 70% of the heme-containing protein on a dry weight basis"	page 17, lines 18-22
35	"the source of the heme-containing protein comprises at least 80% of the heme-containing protein on a dry weight basis"	page 17, lines 18-22
36	"the heme-containing protein is a globin"	page 13, lines 17-21; page 14, lines 5-10
37	"the heme-containing protein comprises an amino acid sequence having at least 80% sequence identity to a polypeptide set forth in SEQ ID NOs. 1-26"	page 15, line 10 – page 16, line 2
38	"the glucose, ribose, fructose, lactose, xylose, arabinose, glucose-6-phosphate, maltose, and galactose, and mixtures of two or more thereof is added to a concentration of about 5.6 to about 20 mM"	page 29, lines 15-17; Table 7; page 35, lines 3-11; Table 13
39	"the cysteine, cystine, thiamine, methionine, and mixtures of two or more thereof is added to a concentration of at least 10 mM"	page 12 (Table 1, rows 4 and 6); Example 1 (e.g., page 29, lines 15-17); Example 4 (e.g., page 35, lines 3-11); Example 5 (e.g., page 38, lines 8-10); Example 6 (e.g., page 39, lines 4-6); Example 7 (e.g., page 39, lines 28-30); Example 8 (e.g., page 40, lines 20-21); Example 9 (e.g., page 41 lines 13-17); Example 10 (e.g., page 43, lines 7-10); Example 11 (e.g., page 53, lines 12-14 and Table 13)
40	"the cysteine is added to a concentration of about 0.8 mM to about 10 mM"	page 29, lines 15-17; Table 7; page 42, line 9; Table 13
41	"the thiamine is added to a concentration of	page 29, lines 15-17; Table 7; page

	about 0.1 mM to about 2 mM"	42, line 10; Table 13
42	"inosine, inosine monophosphate (IMP), guanosine, guanosine monophosphate (GMP), and adenosine monophosphate (AMP)"	page 12 (Table 1, row 5)
43	"beta-carotene, alpha-tocopherol, caffeic acid, propyl gallate, and epigallocatechin gallate"	page 25, lines 29-30
44	"a vegetable oil, an algal oil, sunflower oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, canola oil, flaxseed oil, coconut oil, and mango oil"	page 3, lines 28-30
45	"acetic acid, lactic acid, glycolic acid, citric acid, succinic acid, tartaric acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha linolenic acid, gamma linolenic acid, arachidic acid, arachidonic acid, behenic acid, and erucic acid"	Page 12, Table 1 (row 3)
46	"cooking comprises heating the meat-like food product at 150°C for about 3 to about 5 minutes"	page 29, line 21; page 35, lines 3-11
47	"a pink to red color before cooking to indicate a raw or uncooked state"	page 4, lines 6-16; page 28, lines 8-12
48	"upon cooking, transitions in color from a pink to red color in a raw or uncooked state to a lighter pink to brown color in a partially cooked to fully cooked state"	page 4, lines 6-16; page 28, lines 8-12
49	"cooking the meat-like food product results in the production of at least five volatile compounds"	page 21 – page 25, line 7; page 36-38 (Table 8)
50	"cooking the meat-like food product results in the production of at least ten volatile compounds"	page 21 – page 25, line 7; page 36-38 (Table 8)
51	"cooking the meat-like food product results in the production of at least twenty volatile compounds"	page 21 – page 25, line 7; page 36-38 (Table 8)
52	"at least two volatile compounds are selected from 2-methyl-furan, bis(2-methyl-3-furyl)disulfide, 2-pentyl-furan, 3,3'-dithiobis-2-methyl-furan, 2,5-dimethyl-pyrazine, 2-methyl-3-furanthiol, dihydro-3-(2H)-thiophenone, 5-methyl-2-thiophenecarboxaldehyde, 3-methyl-2-thiophenecarboxaldehyde, 2-methyl-thiazole, dimethyl sulfide, decanal, 5-ethyldihydro-	Tables 3, 8, 9, 12 and 14

	2(3H)-furanone, dihydro-5-pentyl-2(3H)-furanone, 2-octanone, 3,5-octadien-2-one, p-Cresol, and hexanoic acid"	
53	" wherein the at least two volatile compounds are 2-methyl-furan and bis(2-methyl-3-furyl)disulfide"	Tables 3, 8, 9, 12 and 14

No new matter has been added.

A number of Declarations under 37 CFR § 1.132, which were previously filed in U.S. Application No. 14/797,006, now issued as U.S. Patent No. 9,800,067, were filed with the application as Exhibits 1-7.

Applicant asks that all claims be examined in view of the amendments herein.

The fees in the amount of $200 for excess claims fees will be paid to Deposit Account No. 06-1050. If this is incorrect, please apply any additional charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: /March 13, 2018/

/M. Angela Parsons/
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61644821.doc

Electronic Patent Application Fee Transmittal

Application Number:	15913090
Filing Date:	
Title of Invention:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES
First Named Inventor/Applicant Name:	Rachel Fraser
Filer:	M. Angela Parsons/Judith Witzel
Attorney Docket Number:	38767-0015008

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1202	2	100	200
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**200**

Electronic Acknowledgement Receipt

EFS ID:	32033116
Application Number:	15913090
International Application Number:	
Confirmation Number:	9878
Title of Invention:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES
First Named Inventor/Applicant Name:	Rachel Fraser
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0015008
Receipt Date:	13-MAR-2018
Filing Date:	
Time Stamp:	10:39:21
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$200
RAM confirmation Number	031318INTEFSW00009468061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		PreliminaryAmendment.pdf	89855 c36a77c79ab1128ae4f221c7efa8d22a2a695f43	yes	9

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1
	Claims	2	5
	Applicant Arguments/Remarks Made in an Amendment	6	9

Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30509 b3a4cb0212e4326019c6463093dfdf27adb04f7c	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	120364		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Attorney Docket No.: 38767-0015008

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Rachel Fraser	Art Unit	:	Unknown
Serial No.	:	15/913,090	Examiner	:	Unknown
Filed	:	March 06, 2018			
Title	:	METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-1449 Form. Foreign patent documents and non-patent literature are enclosed; U.S. patents and patent application publications will be provided upon request.

Under 35 U.S.C. § 120, this application relies on the earlier filing date of U.S. Application Number 15/398,479, filed on January 04, 2017. The references corresponding to Desig. ID Nos. 47, 52, 117, 118, 158, 163, 175, 213, 260, 261, 345, 359, 360,361, 404 and 417 have not been considered in the prior application and copies are enclosed herewith. All *other* references listed on the enclosed PTO-1449 were submitted to and/or cited by the Office in the earlier-filed application, and copies of those references are *not* provided in this application.

This statement is being filed before receipt of a first Office Action on the merits. Therefore, no fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /March 13, 2018/

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Sheet 1 of 21

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		38767-0015008	15/913,090
		Applicant: Impossible Foods Inc.	
		Filing Date: March 06, 2018	Group Art Unit

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	2001/0024677	09/27/2001	Bringe			
	2.	2001/0049132	12/06/2001	Kringelum et al.			
	3.	2002/0034570	03/21/2002	Krammer et al.			
	4.	2003/0198700	10/23/2003	Gruber			
	5.	2003/0212281	11/13/2003	Sinha et al.			
	6.	2003/0224476	12/04/2003	Chou			
	7.	2004/0151778	08/05/2004	Richard et al.			
	8.	2004/0161513	08/19/2004	Akashe et al.			
	9.	2005/0037111	02/17/2005	Berry			
	10.	2006/0035003	02/16/2006	McMindes et al.			
	11.	2006/0035006	02/16/2006	McMindes et al.			
	12.	2006/0204644	09/14/2006	Cavallini et al.			
	13.	2006/0233721	10/19/2006	Tamarkin et al.			
	14.	2007/0269567	11/22/2007	McMindes et al.			
	15.	2007/0269571	11/22/2007	Akita et al.			
	16.	2007/0269583	11/22/2007	McMindes et al.			
	17.	2008/0226810	09/18/2008	Passe et al.			
	18.	2008/0254168	10/16/2008	Mueller et al.			
	19.	2008/0254199	10/16/2008	Orcutt et al.			
	20.	2008/0268112	10/30/2008	Rolan et al.			
	21.	2008/0292749	11/27/2008	Goodwins et al.			
	22.	2008/0299254	12/04/2008	Kim et al.			
	23.	2009/0264520	10/22/2009	Bhagat et al.			
	24.	2009/0274817	11/05/2009	Yamaguchi et al.			
	25.	2010/0074998	03/25/2010	Vega et al.			
	26.	2010/0136201	06/03/2010	Bigeard et al.			

Examiner Signature	Date Considered

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0015008	Application No. 15/913,090
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
(37 CFR §1.98(b))		Filing Date March 06, 2018	Group Art Unit

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	27.	2010/0196575	08/05/2010	Sanchez			
	28.	2010/0233347	09/16/2010	Uhrhan			
	29.	2010/0249560	09/30/2010	Levinson et al.			
	30.	2010/0281765	11/11/2010	Schwartz			
	31.	2010/0310738	12/09/2010	Ludwig			
	32.	2010/0311950	12/09/2010	Kugitani			
	33.	2011/0008502	01/13/2011	Hosomi et al.			
	34.	2011/0064862	03/17/2011	McCready et al			
	35.	2011/0065847	03/17/2011	Miwa et al.			
	36.	2011/0081386	04/07/2011	Guth et al.			
	37.	2011/0081435	04/07/2011	Guth et al.			
	38.	2011/0117180	05/19/2011	Yan et al.			
	39.	2011/0286992	11/24/2011	Gruber et al.			
	40.	2011/0288389	11/24/2011	Levinson et al.			
	41.	2012/0059150	03/08/2012	Moloney et al.			
	42.	2012/0093994	04/19/2012	Hsieh et al.			
	43.	2013/0004617	01/03/2013	Zhang et al.			
	44.	2015/0366233	12/24/2015	Brown et al.			
	45.	2015/0296834	10/22/2015	Geistlinger et al.			
	46.	2015/0296835	10/22/2015	Anderson et al.			
	47.	2018/0027851	02/01/2018	Vrlijic et al.			
	48.	2,934,437	04/26/1960	Morton et al.			
	49.	3,394,017	07/23/1968	Giacino et al.			
	50.	3,658,550	04/25/1972	Hawley			
	51.	3,693,533	09/26/1972	Liepa			
	52.	3,719,499	03/06/1973	Hai et al.			

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0015008	Application No. 15/913,090
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date March 06, 2018	Group Art Unit

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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	54.	3,870,801	03/11/1975	Tombs			
	55.	3,973,043	08/03/1976	Lynn			
	56.	3,966,985	06/29/1976	Jonas			
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	59.	4,218,487	08/19/1980	Jaeggi			
	60.	4,435,438	03/06/1984	Lehnhardt et al.			
	61.	4,604,290	08/05/1986	Lee et al.			
	62.	4,678,676	07/07/1987	Ishizuka et al.			
	63.	4,994,285	02/19/1991	Hisano et al.			
	64.	5,039,543	08/13/1991	Lee et al.			
	65.	5,055,310	10/18/1991	Nonaka et al.			
	66.	5,264,239	11/23/1993	Cornet et al.			
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(37 CFR §1.98(b))		Filing Date March 06, 2018	Group Art Unit

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Examiner Signature	Date Considered

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Substitute Disclosure Form

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0015008	Application No. 15/913,090
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date March 06, 2018	Group Art Unit

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⑲日本国特許庁（JP）　⑪特許出願公開

⑫公開特許公報（A）　昭62－83842

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審査請求　未請求　発明の数 2　（全8頁）

⑤4発明の名称　凍結乾燥ヨーグルトとその製造方法

②1特　願　昭60－223987
②2出　願　昭60(1985)10月8日

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明細書

1．発明の名称

凍結乾燥ヨーグルトとその製造方法

2．特許請求の範囲

1. ケフイア菌による牛乳の発酵生成物であり、かつ凍結乾燥された粉体状であることを特徴とする凍結乾燥ヨーグルト。

2. 牛乳にケフイア菌スターターを接種してヨーグルトを製造し、得られたヨーグルトを凍結乾燥すること特徴とする凍結乾燥ヨーグルトの製造方法。

3．発明の詳細な説明

〔発明の技術分野〕

本発明は凍結乾燥ヨーグルトとその製造方法に関し、より詳細には長期間、安定して保存することができる粉体状のヨーグルトと、その製造方法に関する。

〔従来技術〕

ソ連や東欧諸国においては、コーカサス地方を起源とする発酵乳・ケフイア(Kefir)が古くから愛飲されており、このことは、乳業関係者の間では、以前から知られていたことである。近年、発酵乳がすぐれた健康食品であるという認識と、コーカサス地方のもつ長寿イメージとが重なり、わが国のみならず欧米においても、新しいタイプの発酵乳としてケフイアへの関心は急激に高まっている。

しかし、その高まりにもかかわらず、ヨーグルトに代表される乳酸発酵単独型の発酵乳とは異なるケフイアについては、必ずしも充分に検討されているとは言いがたい。

特にケフイアは、熟成が長びくにつれて次第にCO_2を発生して容器内圧を高めることも商品化、販売をさまたげる原因であった。

〔発明の目的〕

本発明は発酵乳・ケフイアのように保存中にCO_2の発生による容器内圧の上昇もなく、保存、取扱が容易な粉末状ヨーグルトとその製造方法を提供することを目的とするものである。

〔発明の構成〕

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

上記目的を達成する本発明の凍結乾燥ヨーグルトは、ケフイア菌による牛乳の発酵生成物であり、かつ凍結乾燥された粉体状であることを特徴とするものである。

または本発明の凍結乾燥ヨーグルトの製造方法は、牛乳にケフイア菌スターターを接種してヨーグルトを製造し、得られたヨーグルトを凍結乾燥することを特徴とするものである。

ケフイア菌は、生物学的に1つの生体としてふるまう各種微生物が天然の共生をなすものである。

ケフイア菌微生物叢の組成には、乳酸連鎖球菌及び桿菌・酵母・酢酸菌などが含まれている。

また、ケフイア・スターターの乳酸連鎖球菌群には、ケフイア菌微生物叢の一定かつ最大の活性部分をなし、発酵の始め数時間中のスターターの酸度を急速に高めるストレプトコッカス・ラクテイス(Str,lactis)及びストレプトコッカス・クレモリス(Str,cremoris)、ロイコノストック・シトロバルム(Louconostoc citrovarum)、ロイコノストック・デキストラニクム(Leuconastoc dextranicum)及びストレプトコッカス・ジアセチラクテイス(Srt,diacetilactis)型の芳香生成連鎖球菌が入っている。

ケフイア菌微生物叢にはまた、βバクテリア及びストレプトバクテリア型の中温性乳酸桿菌が属しているが、それらの量は製造されたばかりのスターター中には多くない。

微生物叢組成中にはまた、ラクトースを発酵させる酵母及びそれを発酵しない酵母が入っている。ラクトース発酵酵母は製品の品質にとってより貴重であり、ケフイア中にこの酵母及び乳酸菌があると、抗生物質の蓄積がなされ、製品を治療に役立つものとするからである。

ケフイア菌微生物叢の組成に入る酢酸菌は、その典型的な代表であるアセトバクテルアセチ(Acetobacteraceti)と異なって、鞭毛を表面全体に有し、エネルギー物質として乳酸を利用する能力をもっている。これらのバクテリアがケフイア菌微生物叢の組成に存在すると、ケフイア・スターターの活性の保持にまた製品のボディー及び味に影響を与える。

ケフイア菌の常住微生物叢にはまた、好熱性乳酸桿菌が入っている。

上述の微生物はみな、製造される製品の品質形成に主要な役割を演じている。

ケフイア・スターター1ml中の主な微生物叢群の細胞数の例を示すと下記のようである。

ホモ発酵中温性乳酸連鎖球菌…数十億または数億
ホモ発酵好熱性乳酸桿菌 …… 数十万
芳香生成菌 …… 数千万～数億
酵母 …… 数十万
酢酸菌 …… 数万～数十万

また、ケフイアが明瞭な味と香り及び良好なボディーを有するとき、その1ml中の主要微生物叢群の平均含有量は下記のとおりである。

ホモ発酵中温性連鎖球菌 …… 数十億または数億細胞
ホモ発酵好熱性乳酸桿菌 …… 数百万細胞
芳香生成菌 …… 数千万～数億細胞
酵母 …… 数十万細胞
酢酸菌 …… 数千～数万細胞

ケフイア1g当りの乳酸菌数測定結果を下記第1表に示したが、MRS寒天培地にて25℃7日間培養した値が最も高く、1g当り1.6×10^{7}であった。

（本頁以下余白）

Motif Exhibit 1150, Page 479 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

第　1　表

使用培地	培養条件	乳酸菌数／g
BL寒天	好気・37℃72時間 嫌気・37℃72時間 好気・30℃48時間 好気・25℃7日間	1,000以下 1,000以下 1,000以下 1,000以下
MRS寒天	好気・37℃72時間 嫌気・37℃72時間 好気・30℃48時間 好気・25℃7日間	1,000以下 1,000以下 1,000以下 1.6×10^{7}
BCP加プレートカウント寒天	好気・37℃72時間	1,000以下
トマトジュース寒天	好気・37℃72時間 嫌気・37℃72時間	1,000以下 1,000以下
脱脂乳寒天	好気・30℃48時間	1,000以下
Elliker寒天	好気・25℃7日間	1.6×10^{6}
グルコース・酵母エキス寒天	好気・25℃7日間	6.0×10^{6}
SI寒天	好気・25℃20日間	2.2×10^{6}

MRS寒天（分離菌a,b）、Elliker寒天（分離菌c）、グルコース・酵母エキス寒天（分離菌d）およびSI寒天（分離菌e）平板より、それぞれ1～2個の集落を釣菌し、分離菌とした後、性状試験を行ったところ、分離菌はすべてヘテロ型の乳酸菌桿菌であった。分離菌a，b，dおよびeはラクトバシルス・ブレビス(Lactobacillus brevis)と同定された。ただし、分離菌a，b，およびdについてはアラビノースあるいはマルトースの発酵性に典型的性状とは異なる点が認められた。また、分離菌cについては、リボースの発酵性が陰性ではあったが、他の性状より、ラクトバシルス・ブレビスの類縁菌と思われる。性状試験結果を第2表に示す。

なお、ケフイア菌株は、工業技術院微生物工業技術研究所に昭和60年10月7日に寄託したが技術的理由によって不受理となった。

（本頁以下余白）

第　2　表

試験項目＼分離菌	a	b	c	d	e
	試験結果				
形態＊	桿菌	桿菌	桿菌	桿菌	桿菌
グラム染色	陽性	陽性	陽性	陽性	陽性
カタラーゼ	−	−	−	−	−
生成乳酸	DL	DL	DL	DL	DL
15℃での生育	+	+	+	+	+
45℃での生育	−	−	−	−	−
グルコースよりガスの産生	+	+	+	+	+
グルコネートよりガスの産生	+	+	+	+	+
糖の発酵性					
アミグダリン	−	−	−	−	−
アラビノース	−	−	−	−	+
セロビオース	−	−	−	−	−
フラクトース	+	+	+	+	+
グルコース	+	+	+	+	+
グルコネート	+	+	+	+	+
ラクトース	+	+	+	+	+
マルトース	+	+	+	−	+
マンニトール	−	−	−	+	+
マンノース	−	−	−	−	−
メルジトース	−	−	−	−	−
メリビオース	+	+	+	+	+
ラフイノース	−	−	−	−	−
ラムノース	−	−	−	−	−
リボース	+	弱	−	+	+
ソルビトール	−	−	−	−	−
サクロース	−	−	−	−	−
トレハノース	−	−	−	−	−
キシロース	−	−	−	−	−
エクスリン	−	−	−	−	−

次に本発明の凍結乾燥ヨーグルトの製造方法について述べる。

まず、ケフイア粒を活性化する。

ケフイア粒は小豆大～小指頭大、白色、カリフラワー状の種菌で、その構成菌は休眠状態にある。

そこで構成菌が培地内で活動し、一定時間内に一定の細菌叢を作り出すように活性化する必要がある。

活性化の方法は、通常では牛乳中でケフイア粒を20～25℃で培養し、数回継代することにより達成される。

活性化の具体的1例を下記に示す。

培地……UHT殺菌牛乳(130℃2秒殺菌)

培養の温度と時間……21℃，24時間培養

ケフイア粒の保存状態による前処理と培地量に対する添加量。

凍結乾燥ケフイア粒の場合は、滅菌水または滅菌生理食塩水に約6時間浸漬後洗浄し、培地に対して、約7.5重量%添加する。

Motif Exhibit 1150, Page 480 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

また、水漬けケフイア粒の場合は、生理食塩水で洗浄し、培地に対して約7.5重量%添加する。

2回目以後の継代は両者とも同じで、培地に対して約7.5重量%のケフイア粒を添加する。約20時間で牛乳を凝固させるようになれば、活性化していると、判断される。

活性化に要する日数は凍結乾燥ケフイア粒で10日以上、水漬けケフイア粒で4～5日である。

次に活性化したケフイア粒からケフイア・スターターを調製する。

すなわち、活性化したケフイア粒を生乳30～50に対し粒1の割合で（すなわち、乳1ℓあたり粒20～30g）入れ、培養する。培養温度は通常では15～25℃であり、好ましくは夏期で15～20℃、冬期で18～22℃である。粒を乳へ入れてから15～16時間培養後に3～4分間撹拌し、その温度でさらに5～7時間培養後再度撹拌し、きれいな容器へふるいで濾過する。

ふるい下として得られた濾過液はケフイア・スターターであり、pHは4.0～4.6の範囲にある。ふるい上に残った粒は再び、新鮮な滅菌冷却した乳へ入れ、粒を濾過して得られた液（ケフイア・スターター）をケフイア製造に使用する。

このようにしてスターター調製の全過程には通常的24時間を要する。

次に得られたスターターを用いて発酵乳・ケフイアを製造する。原料には生乳あるいは必要に応じて乳製品を添加したものを用いる。生乳に乳製品を添加する場合は、たとえば粉乳または練乳などを用いる。混合比率は、通常では生乳100重量部に対して乳製品2～10重量部である。

次に、この混合物をよく撹拌しながら60～70℃に加熱し、更に均質化処理を行なう。

この均質化処理は、発酵中あるいは後述する乾燥前に脂肪が分離しないようにするためであり、一般には100～150 kg／cm²に加圧することにより行なわれる。

更に加圧下後に85～95℃に加熱して殺菌する。

この加熱時間は5～30分で良い。

殺菌後に使用するスターターの調製温度と同じ温度（15～25℃）に冷却し、撹拌しながら、前記スターターを接種する。

スターター接種量は、殺菌後の原料100重量部に対して、スターター3～5重量部である。

スターター接種後は、使用したスターターの調製温度と同じ温度で時々撹拌しながら培養する。

この培養過程においては、ヨーグルトに代表される乳酸発酵単独型と異なり、乳酸およびアルコール発酵の2つのプロセスが平行して起きる。

この培養は通常12～24時間で終了する。

培養終了後に10～15℃に冷却し、この温度で5～20時間、撹拌しながら熟成させる。

熟成終了後は、2～5℃に冷却して、発酵を停止させる。

得られた発酵乳・ケフイアの性状の一例を下記第3表に示す。

第　3　表

配　合：	
牛　乳	92.4%
乳製品	2.9%
ｹﾌｨｰﾙｽﾀｰﾀｰ	4.7%
栄養成分：	
エネルギー	68　Kcal
水　分	86.1g
蛋白質	3.9g
脂　質	3.2g
糖　質	5.9g
繊維質	0.0g
灰　分	0.9g
カルシウム	129　mg
リ　ン	116　mg
鉄	0.1mg
ナトリウム	65　mg
カリウム	199　mg
レチノール	29　ug

Motif Exhibit 1150, Page 481 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

カロチン	11 ug
A効力	111 IU
V－B1	0.05 mg
V－B2	0.19 mg
ナイアシン	0.1 mg
V－C	0 mg

このようにして得られた発酵乳ケフイアを容器たとえばトレイに収容し、－25～－35℃に冷却して凍結させる。

この凍結によって乳白色状の凍結物が得られる。

得られた凍結物を35～40℃以下に押え、真空度0.1～1.0 mmHgで凍結乾燥する。

得られた凍結乾燥ヨーグルトは、白色乳白色の粉体状で、乳状の芳香を有している。

得られた凍結乾燥ヨーグルトの成分組成の一例を第4表に示す。

（本頁以下余白）

第4表

分析試験項目	結果	検出限界	注	分析方法
水分	1.7%		1	減圧加熱乾燥法
たんぱく質	26.3%			ケルダール法
脂質	27.4%			レーゼゴットリーブ法
繊維分	0 %			ヘンネルベルグストーマン改良法
灰分	6.0%			直接灰化法
糖質	38.6%		2	
ヒ素(As_2O_3として)	検出せず	0.1ppm		DDTC-Ag吸光光度法
重金属(Pbとして)	検出せず	1 ppm		硫化ナトリウム比色法
大腸菌群	陰性		3	BGLB法
一般細菌数(生菌数)			4	
（乳酸菌を除く）	300以下/g			ポテトデキストロース(10%)寒天平板培養法
酵母数	3.7×10^2/g		5	BCP加プレートカウント寒天平板培養法
乳酸菌数	2.8×10^7/g		6	BL寒天平板嫌気培養法
乳酸菌数	8.2×10^7/g			

注1. 窒素・たんぱく質換算係数；6.38
注2. 100－(水分＋たんぱく質＋脂質＋繊維＋灰分)
注3. 食品衛生検査指針1 大腸菌群検査法に準じて試験した。
注4. 食品、添加物等の規格基準の第1食品D各条○粉末清涼飲料の乳酸菌を加えた粉末清涼飲料の細菌数の測定法（昭和34年厚生省告示第370号）によった。
注5. 乳及び乳製品の成分規格等に関する省令の別表の二の（七）乳等の成分規格の試験法（昭和54年厚生省令第17号）によった。
注6. 培養条件；25℃7日間

〔発明の効果〕

本発明の凍結乾燥ヨーグルトは、白色粉末状であり、取扱いが容易であると共に、長期間、安定に室温に保存することができる。

また、容易に顆粒状に加工することもでき、かつ乳状の芳香を有するので摂取が容易である。

従って本発明の凍結乾燥ヨーグルトは、たとえば適量に分割、包装した状態で健康食品として商品化することができる。

従来の凍結乾燥前のケフイアに見られたような、保存中に発生したCO_2による容器内圧の上昇は全く見られない。

更に注目すべきことは本発明の凍結乾燥ヨーグルトは、下記するような優れた生理活性効果を有する。

イ．抗腫瘍活性

（実験方法）

癌細胞：エーリッヒ・カルシノーマ

使用動物：JCL-ICR マウス（♀，5W，20-23g）

癌の植えつけ：エーリッヒ・カルシノーマ（5×10^6 セル／マウス）を右足筋肉内に移植（固形）

検体の投与：癌移植翌日より10日間連日1日1回経口投与した。

効果の判定：移植処置より20日めに右足（癌）の重さを測定し、左足（コントロール）の重さを差し引いて癌化の指標とした。

又、抑制率(IR:Inhibition rate)は、検体投与群の平均癌重量(T)と対照群（水のみ投与群）の平均癌重量(C)とを用いて次の式により算出した。

$$IR=(1-T/C)\times100\%$$

なお判定は

－：全く抑制しないか、抑制率(IR)が、25%以下のもの

±：25～50%のIRを示すもの

＋：50%以上の抑制を示すもの

実験結果を下記第5表に示す。

第5表から明らかなように、本発明の凍結乾燥ヨーグルトは、500mg/kgと100mg/kgの投

Motif Exhibit 1150, Page 482 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

与量において最も強い抗腫瘍性が認められる。

（本頁以下余白）

第 5 表

Drug	Medication 投与	Dose (mg/kg)	Schedule (days)	Average Tumor Wt(g) ± SE	抑制率 (%)	評価
ケフィール	経口	500	1～10	1.69±0.34	52.8	+
	経口	100	1～10	1.66±0.19	53.6	+
	経口	20	1～10	2.16±0.07	39.7	±
マイトマイシン	腹腔	1	1～10	1.53±0.19	57.3	+
コントロール	経口	—	1～10	3.58±0.78	—	−

次に比較のために、現在市販されているヨーグルトについて、500mg/kgと100mg/kgを夫々上記同様に経口投与し、抑制率を算出した。結果を第6表に示す。

第　6　表

メーカー	抑制率(%)
A　社	20.5
B　社	15
C　社	25
D　社	15

第6表から明らかなように、市販ヨーグルトのいづれにも、抗腫瘍活性は殆んど認められない。

ロ．抗血栓症作用

（方　法）

ウイスター系ラットにケフイア500mg/kg，200mg/kgを経口投与1時間後にエンドトキシン0.5 mg／kg（血栓誘発剤）を静注し、4時間後に採血し、血小板数、フイブリノーゲン量、プロトロンビン時間、フィブリン分解産物量を測定した。

（結　果）

第6表に示すように、正常群にエンドトキシンを静注すると、血栓が形成され、血小板数、フィブリノーゲン量が減少し、プロトロンビン時間が延長し、FDP量が増加した。本発明の凍結乾燥ヨーグルト投与群は有意に血液検査値の変動を抑制した。すなわち、200-500mg/kgの投与量で顕著な抗血栓症作用が認められた。実験結果を第7表に示す。

（本頁以下余白）

Motif Exhibit 1150, Page 483 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

第 7 表

治療	投与量 (mg/kg)	No. of rats	血小板数 (10^4/mm^3)	フィブリノーゲン量 (mg/dl)	プロトロンビン時間 (sec)	フィブリン分解産物 (μg/dl)
Normal		6	85±10	151±34	14.4±2.6	5.0±0.0
コントロール		9	27±3	44±5	23.9±2.0	19.0±3.1
本発明の凍結乾燥ヨーグルト	200	9	35±3*	62±10*	17.0±1.6	15.0±3.8
同上	500	9	56±8**	85±17**	15.0±1.6*	14.0±4.6

Significantry different from control, *p<0.05, **P<0.01

ハ．生体防御機構に関する検討

（初期免疫賦活効果について）

生体は、外界より異物の侵入を受けると、いちはやく排除しようと初期免疫機構が、活動する。その主役はマクロファージや細網内皮系の細胞群である。今回は、異物としてカーボンを血中へ侵入させ、その後の異物排除の能力を、血中からのカーボン消失速度を指標に把握した。この実験系は、生体防御反応を検討する際によく利用される系でフリードマン(Freedman)らによって開発されたカーボンクリアランス法である。

（実験方法）

ddy 系雄性マウス（体重20～25ｇ）を18時間絶食ケージにて絶食し（水は自由採取）、尾静脈よりカーボン浮遊液を注入しヘパリンヘマトクリット毛細管を用いて採血し、675 nmにおける吸光度から含有カーボン量を比色定量し、次式によりカーボン半減消失時間を求めた。

$$t_{1/2} = \frac{(t_2 - t_1) \times \log_{1/2} 0Dt_1}{0Dt_1 - 0Dt_2}$$

$t_{2/1}$＝半減消失時間（分）

t_1　＝5　　（分）

t_2　＝10または15　（分）

$0dt_1$＝5分後の吸光度

$0dt_2$＝10または15分後の吸光度

なお検体は、500mg/kg　100mg/kg　20mg/kgの用量に調製し（水にけん濁）、陽性コントロールにチモザン50mg/kg(i.p.) を用いて3日間、あるいはカーボン静注30分前に経口投与した。

実験結果を下記第8表に示す。

第　8　表

(A)　1回投与の場合

検体	投与量 (mg/kg)	No of mouse	Clearance rate of Carbon
コントロール	－（経口）	10	11.83±0.37
本発明の凍結乾燥ヨーグルト	500(経口)	10	11.32±0.29
同　上	100(経口)	10	11.73±0.42
同　上	20(経口)	10	10.99±0.42
チモサン	50(i.p.)	10	10.72±0.29

(B)　3日投与の場合（1日1回）

検体	投与量 (mg/kg)	No of mouse	Clearance rate of Carbon
コントロール	－（経口）	10	12.84±0.57
本発明の凍結乾燥ヨーグルト	500(経口)	10	10.70±0.56
同　上	100(経口)	10	11.41±0.52
同　上	20(経口)	10	12.28±0.63
チモサン	50(i.p.)	10	8.31±0.34*

＊ コントロールとの比較：P ＜0.05で有意差あり

第8表から明らかなように、本発明の凍結乾燥ヨーグルトは、1回投与では20mg/kg に弱い活性がみられただけであったが、3日間投与すると500 mg/kg と100mg/kgに有意な活性が見い出された。

以下、本発明の実施例を示す。

Motif Exhibit 1150, Page 484 of 1599

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

〔実施例〕

生乳17kgを 140℃で2秒殺菌した後、21℃に冷却し、これに活性化したケフイア粒0.6 kgを接種し、21℃で15時間培養した。

3～4分間攪拌し、21℃で更に培養し、再度攪拌した後に、ふるいで濾過し、ふるい下として得られた培養濾液17kgをケフイア・スターターとして用いた。

ふるい上には活性化ケフイア粒1.2 kgが残った。

一方、脱脂粉乳10kgと生乳 323kgを混合し、よく攪拌しながら70℃に加熱し、150kg/cm²に加圧して均質化処理した。次いで90℃で10分間殺菌した後に21℃に冷却し、攪拌しながら上記ケフイア・スターター17kgを接種し、21℃で時々攪拌しながら15時間、培養した。15℃に冷却し、この温度で5時間熟成させ、5℃に冷却して 350kgのケフイアを得た。

このケフイアをトレイに分割して積載し、－25～－35℃に冷却して凍結させた。

得られた凍結物を35～40℃で0.1～1.0 mmHgで乾燥し、本発明の凍結乾燥ヨーグルト49kgを得た。

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Motif Exhibit 1150, Page 485 of 1599

United States Patent [19]

Tokumaru et al.

[11] **Patent Number: 4,702,923**

[45] **Date of Patent: Oct. 27, 1987**

[54] **LYOPHILIZED KEFIR YOGHURT HEALTH FOOD**

[75] Inventors: **Sennosuke Tokumaru,** 10-3, Tsujido Taiheidai 1, Fujisawa-shi, Kanagawa-ken; **Michinori Kubo,** Sakai; **Mari Nogami,** Suita, all of Japan

[73] Assignee: **Sennosuke Tokumaru,** Kanagawa, Japan

[21] Appl. No.: **914,835**

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[30] **Foreign Application Priority Data**

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Feb. 12, 1986 [JP] Japan 61-26671

[51] **Int. Cl.**[4] **A23C 9/12;** A23L 1/29
[52] **U.S. Cl.** **426/61;** 426/648; 426/800; 426/34; 426/583
[58] **Field of Search** 426/43, 615, 34, 640, 426/583, 524, 444, 384, 385, 61, 648, 800-801; 424/95, 195.1

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[57] **ABSTRACT**

A lyophilized kefir yoghurt health food is disclosed.

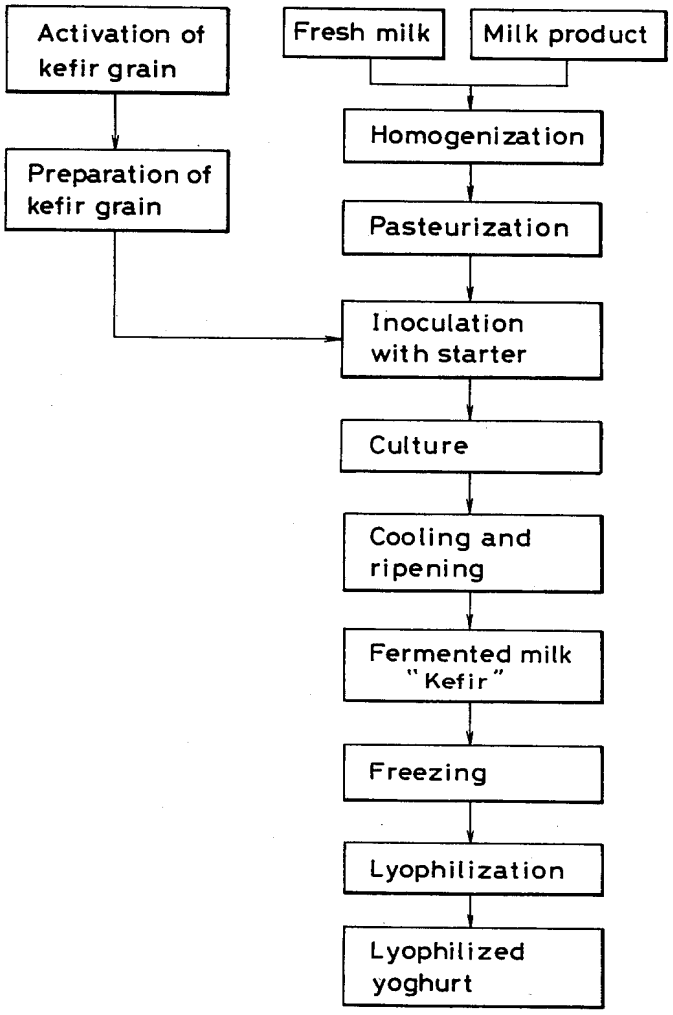
The lyophilized kefir yoghurt is a fermentation product obtained from milk with the use of kefir fungi and in the form of a lyophilized powder.

This lyophilized kefir yoghurt is produced by inoculating milk with a kefir starter, which is then cultured therein to thereby produce a yoghurt; and lyophilizing the obtained yoghurt.

The health food contains lyophilized kefir yoghurt as a main component.

5 Claims, 1 Drawing Figure

U.S. Patent Oct. 27, 1987 4,702,923



LYOPHILIZED KEFIR YOGHURT HEALTH FOOD

BACKGROUND

This invention relates to a lyophilized kefir yoghurt, a process for the production of the same and a health food containing the same as a main ingredient.

It has been known in the art that 37 kefir", which is a fermented milk originating in Caucasus, has been habitually taken in the Soviet Union and Eastern Europe. Recently the kefir has been rapidly attracting public attention since fermented milk is recognized as an excellent health food and Caucasus is world-famous for longevity.

However, examinations on the kefir, which is different from single-type lactic fermented milk such as yoghurt, are yet insufficient in spite of the recent increase in the attention thereto.

Kefir would form CO_2 as ripening is prolonged to thereby elevate the internal pressure of a container, which is an obstacle to the marketing of the same.

On the other hand, there are marketed various health foods riding the crest of the recent health food boom.

However many of these health foods contain vegetable or animal components as active ingredients and there are few solid health foods containing fermented milk as an active ingredient.

SUMMARY

It is a first object of the present invention to provide a powdery lyophilized kefir yoghurt, which will be sometimes called merely a lyophilized yoghurt hereinafter, obtained by lyophilizing kefir.

It is a second object of the present invention to provide a process for the production of the lyophilized kefir yoghurt.

Further it is a third object of the present invention to provide a health food containing the lyophilized kefir yoghurt as a main active ingredient.

The first object of the present invention as described above can be achieved by a powdery lyophilized kefir yoghurt which is prepared by lyophilizing a fermentation product obtained from milk with the use of kefir fungi.

The second object of the present invention can be achieved by inoculating milk with a kefir fungi starter, culturing the kefir fungi therein to thereby produce yoghurt and lyophilizing the obtained yoghurt.

Further the third object of the present invention can be achieved by a health food comprising the lyophilized yoghurt, assistants and excipients.

THE DRAWINGS

The sole appended FIGURE is a flow diagram of a process for the production of the lyophilized yoghurt of the present invention.

THE PREFERRED EMBODIMENTS

The lyophilized kefir yoghurt of the present invention is a fermentation product obtained from milk with the use of kefir fungi and in the form of a lyophilized powder. This lyophilized yoghurt may be produced by inoculating milk with a kefir fungi starter to thereby produce yoghurt which is then lyophilized.

The kefir fungi as described above, which have been obtained from Licensing Organization USSR by us and called YK-1, are a natural symbion which comprises various microorganisms and behaves as a single organism from a biological viewpoint. The kefir fungi are generally in the form of a white cauliflower as large as from an adzuki bean to the head of a little finger and supplied as lyophilized or water-immersed kefir grains.

It is generally very difficult to directly examine the organized bacteria of these grains since the organized bacteria therein are in the dormant state and form a rigid structure together with polysaccharides and proteins.

Thus the organized bacteria of the grains are assumed by culturing the grains in an appropriate medium such as milk at 20° to 25° C. to thereby activate the same and examining the microbes liberated in the medium. The following Table 1 shows the organized bacteria.

TABLE 1

Yeast	
Candida pseudotropicalis	10^5–10^6/ml
Candida Krusei	$<10^5$/ml
Torulopsis holmii	$<10^5$/ml
Lactic acid bacteria	
Streptococcus lactis	10^8–10^9/ml
Lactobacillus brevis	
Lactobacillus fermentum	
Several species, which are highly symbiotical and can be hardly separated, are further observed.	
Acetic acid bacteria	
Highly symbiotical and hardly separated.	

As shown above, *S. lactis* is the most predominant bacterium and the lactic acid in the medium is mostly produced thereby. Among the yeasts, only *C. pseudotropicalis* ferments lactose to thereby form ethanol and CO_2.

Now a process for the production of a lyophilized yoghurt will be described by reference to the appended FIGURE.

In the present invention, the kefir grains as described above are first activated. Since the organized bacteria in the kefir grains are in the dormant state, it is required to activate the same to thereby make them sufficiently active in a medium to make a definite number of colonies with in a given period of time.

The activation may be usually achieved by subculturing the kefir grains several times, i.e., by culturing the kefir grains in milk at 20° to 25° C., separating the grains and culturing the same again.

An example of the activation process is as follows:

medium: UHT pasteurized milk (pasteurized at 130° C. for two seconds); and

culture condition: 21° C. and 24 hours.

The kefir grains are subjected to a pretreatment and added to a medium depending on the storage condition.

Lyophilized kefir grains are immersed in sterilized water or a sterilized physiological saline solution for approximately six hours and washed. Then they are added to a medium in an amount of approximately 7.5% by weight based on the medium.

Water-immersed kefir grains are washed with a physiological saline solution and added to a medium in an amount of approximately 7.5% by weight based on the medium.

Both kefir grains are treated in the same manner after the second subculture. Namely, approximately 7.5% by weight of the kefir grains are added to a medium. The

kefir grains capable of coagulating milk in about 20 hours are judged to be activated.

The activation of lyophilized kefir grains and water-immersed ones requires 10 days or longer and four to five days, respectively.

Then a kefir starter is prepared from the kefir grains thus activated.

Namely, one part by weight of the activated kefir grains are added to 30 to 50 parts by weight of fresh milk, i.e., 20 to 30 g of the grains per liter of the milk, and cultured therein. The culture may be usually performed at 15° to 25° C., preferably at 15 to 20° C. in summer and 18° to 22° C. in winter. After culturing the grains in the milk for 15 to 16 hours, the culturing broth is stirred for three or four minutes. The culture is performed for additional five to seven hours followed by stirring again. Then the culturing broth is filtered into a clean container.

The obtained filtrate, which has a pH value of 4.0 to 4.6, is the aimed kefir starter. The grains on the filter is introduced into fresh pasteurized cold milk again. The solution obtained by filtering the grains off, i.e., the kefir starter is used in the production of kefir.

Thus the whole process for the preparation of the starter usually required 24 hours.

Table 2 shows the organized bacteria of the kefir starter.

TABLE 2

Homofermentation mesophilic lactic acid streptococci (a)
Homofermentation thermophilic lactic acid bacilli
Perfume forming bacteria
Yeasts (b)
Acetic acid bacterium (c)

The streptococci (a) include perfume forming streptococci which rapidly elevate the acidity of the starter, e.g., *Streptococcus lactis, S. cremoris, Louconostoc citrovarum, L. dextranicum* and *S. diacetilactis.*

The yeasts (b) include those fermenting lactose as well as those not fermenting the same. The acetic acid bacterium (c) has fiagellums all over the surface and is capable of utilizing lactic acid as an energic material, different from *Acetobacter aceti.*

Then a fermented milk **37** kefir" is produced with the use of the starter. Fresh milk optionally containing milk product(s) is employed as the raw material. The milk products to be added to the fresh milk are powdered or condensed milk. Usually two to ten parts by weight of the milk products are added to 100 parts by weight of the fresh milk.

Then the mixture is heated to 60° to 70° C. under thoroughly stirring and subjected to further homogenization.

This homogenization is carried out in order to prevent the separation of fat during the fermentation or before the drying, as will be described hereinbelow. It is generally performed by applying a pressure of 100 to 150 kg/cm^2 and passing the mixture through, for example, a screen. After applying the pressure, the mixture is pasteurized by heating the same to 85° to 95° C. for five to 30 minutes.

After the pasteurization, the mixture is cooled to the same temperature (15° to 25° C.) as that of the starter to be used and inoculated with said starter under stirring.

100 parts by weight of the pasteurized material is inoculated with three to five parts by weight of the starter.

After inoculating with the starter, the material is cultured at the same temperature as that of the starter used under occasional stirring.

During this culture step, two processes, i.e., lactic fermentation and alcohol fermentation, proceed in parallel with each other, different from single-type lactic fermentation as typically observed in the production of yoghurt.

The culture usually completes within 12 to 24 hours.

After the completion of the culture, the mixture is cooled to 10° to 15° C. and allowed to ripen at this temperature for five to 20 hours.

After the completion of the ripening, it is cooled to 2° to 5° C. to thereby cease the fermentation.

Table 3 shows the properties of the fermented milk **37** kefir" thus obtained.

Table 4 shows the result of the counting of the lactic acid bacteria per gram of the kefir. Table 4 indicates that a sample obtained by culturing the material in an MRS agar medium at 25° C. for seven days shows the largest count, i.e., 1.6×10^7/g.

TABLE 3

Composition	
milk	92.4%
milk products	2.9%
kefir starter	4.7%
Nutritional components (per 100 g):	
energy	68 Kcal
water	86.1 g
protein	3.9 g
lipid	3.2 g
sugar	5.9 g
fiber	0.0 g
ash	0.9 g
calcium	129 mg
phosphorus	116 mg
iron	0.1 mg
sodium	65 mg
potassium	199 mg
retinol	29 ug
carotene	11 ug
A activity	111 IU
vitamin B1	0.05 mg
vitamin B2	0.19 mg
niacin	0.1 mg
vitamin C	0 mg

TABLE 4

Medium	Culture condition	Lactic acid bacteria count (/g)
BL agar	aerobic, 37° C., 72 hr	<1,000
	anaerobic, 37° C., 72 hr	<1,000
	aerobic, 30° C., 48 hr	<1,000
	aerobic, 25° C., 7 days	<1,000
MRS agar	aerobic, 37° C., 72 hr	<1,000
	anaerobic, 37° C., 72 hr	<1,000
	aerobic, 30° C., 48 hr	<1,000
	aerobic, 25° C., 7 days	1.6×10^7
BCP containing plate count agar	aerobic, 37° C., 72 hr	<1,000
tomato juice agar	aerobic, 37° C., 72 hr	<1,000
	anaerobic, 37° C., 72 hr	<1,000
skim milk agar	aerobic, 30° C., 48 hr	<1,000
Elliker agar	aerobic, 25° C., 7 days	1.6×10^6
glucose/yeast extract agar	aerobic, 25° C., 7 days	6.0×10^6
SI agar	aerobic, 25° C., 20 days	2.2×10^6

Bacteria are taken from one or two colonies on each of the MRS agar (separated bacteria a and b), Elliker agar (separated bacterium c), glucose/yeast extract agar (separated bacterium d) and SI agar (separated bacte-

Case No.: IPR2023-00208
U.S. Patent No. 11,224,241

rium e) media. Then the characteristics of the separated bacteria are examined. As a result, it is turned out that every separated bacterium is a hetero-type lactic acid bacillus.

The separated bacteria a, b, d and e are identified with *Lactobacillus brevis.* However the a, b and d show arabinose or maltose fermentabilities somewhat different from those typically observed. The properties of the separated bacterium c suggest that it might relate to *Lactobacillus brevis,* though it is negative in ribose fermentation. Table 5 shows the result of the characteristic test on these bacteria.

TABLE 5

	Separated bacterium Result				
Test item	a	b	c	d	e
morphology	bacillus	bacillus	bacillus	bacillus	bacillus
gram-stain	positive	positive	positive	positive	positive
catalase	−	−	−	−	−
formed lactic acid	DL	DL	DL	DL	DL
growth at 15° C.	+	+	+	+	+
growth at 45° C.	−	−	−	−	−
gas production from glucose	+	+	+	+	+
gas production from gluconate	+	+	+	+	+
sugar fermentability					
amygdaline	−	−	−	−	−
arabinose	−	−	−	−	+
cellobiose	−	−	−	−	−
fructose	+	+	+	+	+
glucose	+	+	+	+	+
gluconate	+	+	+	+	+
lactose	+	+	+	+	+
maltose	+	+	+	−	+
mannitol	−	−	−	+	+
mannose	−	−	−	−	−
melezitose	−	−	−	−	−
melibiose	+	+	+	+	+
raffinose	−	−	−	−	−
rhamnose	−	−	−	−	−
ribose	+	slight	−	+	+
sorbitol	−	−	−	−	−
sucrose	−	−	−	−	−
trehalose	−	−	−	−	−
xylose	−	−	−	−	−

The fermented milk kefir thus obtained is introduced into a container such as a tray and cooled to −25° to −35° C. to thereby freeze the same.

Thus a milk white frozen product is obtained.

The obtained frozen product is maintained at a temperature of 35° to 40° C. or below and dried under a reduced pressure of 0.1 to 1.0 mm Hg.

The lyophilized yoghurt thus obtained is in the form of a white or milk white powder having a milk-like odor.

Table 6 shows the composition of the lyophilized yoghurt thus obtained.

The obtained lyophilized yoghurt of the present invention is in the form of a white powder which can be readily handled and stably stored at room temperature for a prolonged period of time.

Further it may be readily formulated into granules. In addition, the milk-like odor thereof makes the intake thereof easy.

Accordingly the lyophilized yoghurt of the present invention per se can be marketed as a health food by appropriately dividing and packaging.

The lyophilized yoghurt of the present invention shows no increase in the inner pressure within a container caused by the formation of CO_2, which is observed in conventional unlyophilized kefir.

It is further noteworthy that the lyophilized yoghurt of the present invention has the following excellent physiological effects.

TABLE 6

Analysis item	Result	Lower limits of detection	Note	Analytical method
water	1.7%			heat-drying in vacuo
protein	26.3%		1	Kjeldahl method
lipid	27.4%			Rose-Gottlieb method
fiber	0%			modified Henneberg-Stohmann method
ash	6.0%			direct ashing
sugar	38.6%		2	
arsenic (as As_2O_3)	undetected	0.1 ppm		DDTC-Ag absorptiometry
heavy metal (as Pb)	undetected	1 ppm		sodium sulfide colorimetric method
coliform bacilli	negative		3	BGLB method
general viable bacterial count except lactic acid bacteria	<300/g		4	
yeast count	3.7×10^2/g			potato dextrose (10%) agar plate culture
lactic acid bacteria count	2.8×10^9/g		5	BCP-containing agar plate culture
lactic acid bacteria count	8.2×10^9/g		6	BL agar plate anaerobic culture

Notes:
1: nitrogen/protein coefficient = 6.38.
2: 100 − (water + protein + lipid + fiber + ash).
3: According to the Coliform Bacilli Test, Food Sanitation Guidance, Ministry of Health and Welfare.
4: According to the Bacterial Counting in Powdery Refreshing Beverage Containing Lactic Acid Bacteria, Provision Food-D: Standard for Food and Food additive (Notification No. 307 of the Ministry of Health and Welfare, 1959).
5: According to Test Method for Composition of Milk, Accompanying Table 2-(7) of Ministeral Ordinance on Specification for Milk and Milk Product (Notification No. 17 of the Ministry of Health and Welfare, 1979).
6: Cultured at 25° C. for seven days.

Now the health food of the present invention will be described in detail.

This health food comprises the lyophilized yoghurt as described above as well as assistants and excipients.

The assistants are added in order to impart to the product various physiological effects in addition to those brought about by the main ingredient, i.e., the lyophilized yoghurt to thereby enlarge the availability of the product as a health food. Animal or vegetable substances conventionally marketed as health foods or folk medicines are available therefor.

Preferable examples of these assistants include an extract of *Acanthopanax senticosus Harms.* (*Eleutherococus senticosus Maxim.*), that of *Cichorium Intylous L.* and antlers of a reindeer, though they are not restricted thereby.

One of these assistants or a mixture thereof may be added.

The extract of *A. senticosus Harms.* may be replaced by that of ginseng or calcium L(+)-lactate.

The extract of *A. senticosus Harms.*, which has been used to accelerate blood circulation for a long time, may be obtained in the following manner.

Roots and stems of *A. senticosus Harms.*, which belongs to *Araliaceae* and mainly occurs in Siberia and North East China, are cut into pieces. 500 l of water is

added to, for example, 100 kg of the finely divided product which is thus extracted for three hours at 90° to 100° C. After filtering, approximately 400 g of an extract is obtained.

To the extraction residue, 300 l of water is added again followed by extracting the same at 90° to 100° C. for one hour. After filtering, approximately 300 l of an extract is obtained.

These extracts are combined and concentrated to approximately 20 l at 60° C. or below in vacuo. The obtained concentrate is spray-dried and the dry product is passed through a screen to thereby give approximately 5 kg of a homogeneous powder.

A powder of reindeer antlers, which has been considered as a tonic, may be prepared by finely dividing reindeer antlers imported from Finland, further hammer-milling the same and filtering the same through a 100-mesh screen to give a homogeneous powder. A sterilization step may be optionally performed during the preparation.

The ginseng dried extract to be used in stead of the *A. senticosus Harms.* extract may be prepared by subjecting root of *Panax Ginseng C. A. Meyer* in a two-time repetition, concentrating the liquid extract under reduced pressure, and spray drying the concentrate. It may be added in the same amount as that of the *A. senticosus Harms.* as will be defined hereinbelow. Calcium L(+)-lactate may be prepared through a neutralizing reaction between fermented L(+) lactic acid and lime. It is added in an amount of 0.5 to 1% by weight of the total health food.

Further the *C. Intylous L.* extract, which has been used in immunoinvigoration in Europe for a long time, may be prepared in the following manner.

Namely, leaves and roots of *Cichorium Intylous L.*, which belongs to *Conpositqe* and mainly occurs in Europe, are cut into pieces. 800 l of water is added to, for example, 100 kg of the finely divided product which is thus extracted at 90° to 100,° C. for three hours. After filtering, approximately 700 l of an extract is obtained.

500 l of water is added to the extraction residue which is thus further extracted at 90° to 100° C. for one hour. After filtering, approximately 400 l of an extract is obtained.

These extracts are combined and concentrated to approximately 35 kg at 60° C. or below in vacuo to thereby give a soft syrup.

The excipients as used in the present invention include lactose and starch. One of these excipients or a mixture thereof may be employed.

In the health food of the present invention, the kefir, assistants and excipients are blended in the proportions as shown in the following Table 11.

TABLE 11

Lyophilized kefir	usually 30 to 50% by weight, preferably 35 to 45% by weight
Assistant	usually 7 to 15% by weight, preferably 9 to 14% by weight
Excipient	usually 40 to 55% by weight, preferably 45 to 55% by weight

Table 12 shows the composition of an example of the health food of the present invention which comprises an *A. senticosus Harms.* extract, a reindeer antler powder anc a *C. Intylous L.* extract as the assistants and lactose and starch as the excipients.

TABLE 12

Lyophilized kefir	usually 30 to 50% by weight, preferably 35 to 45% by weight
A. senticosus Harms. extract	usually 1.5 to 4.0% by weight, preferably 2.0 to 3.5% by weight
Reindeer antler powder	usually 0.3 to 1.0% by weight, preferably 0.4 to 0.8% by weight
C. Intylous L. extract	usually 5.5 to 10% by weight, preferably 6.5 to 9.5% by weight
Lactose	usually 30 to 38% by weight, preferably 32 to 37% by weight
Starch	usually 10 to 18% by weight, preferably 12 to 17% by weight

When the reindeer antler powder is contained in an amount less than 0.3% by weight, it shows no tonic effect. On the other hand, when it is contained in an amount exceeding 1.0% by weight, no significant increase in said effect is observed.

When the lactose is contained in an amount less than 30% by weight, it shows a poor excipient property for formulating granules or tablets, as will be shown hereinbelow. On the other hand, an amount of the same exceeding 38% by weight is excessive for the lyophilized yoghurt or the assistants.

The starch would accelerate the dissolution of other ingredients taken in the body while at the same time it acts as an assistant and as an excipients. When it is contained in an amount less than 10% by weight, the bulking and excipient effect of the same is insufficient. On the other hand, when it is contained in an amount exceeding 18% by weight, no significant difference in said effects is observed.

The health food of the present invention may further contain sweeteners such as sugar, colorants and preservatives if required.

Now a process for the production of the health food of the present invention in granular form will be described.

The assistants and excipients as described above are weighed and mixed together. Then water and ethanol are added thereto and the obtained mixture is kneaded.

The kneaded mixture is passed through a screen of 1 mm in diameter and the particles thus obtained are dried at 60° to 80° C. for one hour. The dried particles are arranged by passing through a 12-mesh screen and further passed through a 12-mesh screen.

Separately a lyophilized kefir is prepared in the abovementioned manner and formulated into granules by, for example, the same procedure as the one used in the granulation of the assistants and excipients.

Finally the lyophilized kefir and other ingredients, each formulated in granules as described above, are weighed and mixed together to thereby give the health food of the present invention.

Further the presence of coliform bacilli and general viable bacteria therein is optionally examined.

In addition, the obtained health food may be weighed out in a given amount and singly packaged with, for example, a plastics film, if required. Thus a product suitable for marketing and intake may be obtained.

Thus a process for the production of the health food of the present invention in granular form has been described. However it is to be understood that the present invention is not restricted thereby. Therefore it may be formulated into tablets or provided as a powder by omitting the granulation step.

The health food of the present invention may be generally taken in a dose of 2.5 to 3.0 g a day. The singly

packaged granules as mentioned above is preferable since it would not scatter at the intake.

Alternately the granules may be packed in a bottle and taken in a given amount with the use of, for example, a measuring spoon.

The health food of the present invention is in a grayish white or grayish brown color depending on the types and amounts of the added assistants and has a milk-like odor and taste. Thus it can be easily taken. Further it shows no change even when stored for a prolonged period of time.

As described above, the health food of the present invention contains the lyophilized kefir.

Furthermore, the health food of the present invent contains assistants in addition to the lyophilized yoghurt.

In addition, the health food of the present invention may be formulated into, for example, granules by adding excipients. Thus the milk-like odor and taste of the lyophilized kefir are maintained as such, which makes the intake of a given amount of the product easy.

To further illustrate the present invention, the following Examples will be given.

EXAMPLE 1

17 kg of fresh milk was pasteurized at 140° C. for two seconds and cooled to 21° C. Then the milk was inoculated with 0.6 kg of activated kefir grains which were thus cultured therein at 21° C. for 15 hours.

After stirring for three to four minutes, the culture was further performed at 21° C. After stirring again, the culture broth was filtered through a screen and 17 kg of the obtained culture filtrate was used as a kefir starter.

Thus 1.2 kg of the activated kefir grains remained on the screen.

Separately 10 kg of skim milk was mixed with 323 kg of fresh milk and the mixture was heated to 70° C. under thorough stirring. Then it was homogenized under an elevated pressure of 150 kg/cm^2. After pasteurizing at 90° C. for ten minutes, it was cooled to 21° C. and inoculated with 17 kg of the above kefir starter under stirring followed by culturing at 21° C. under occasional stirring. Then the culture was cooled to 15° C. and allowed to ripen at this temperature for five hours. After cooling to 5° C., 350 kg of kefir was obtained.

The obtained kefir was divided, placed on a tray and frozen at −25° to −35° C.

The frozen product was dried at 35° to 40° C. under a pressure of 0.1 to 1.0 mmHg to thereby give 49 kg of the lyophilized yoghurt of the present invention.

EXAMPLE 2

A health food of the composition as shown in the following Table 13 was produced.

TABLE 13

lyophilized kefir	1000 mg
A. senticosus Harms. extract	81.90 mg
reindeer antler powder	17.55 mg
C. Intylous L. extract	234.00 mg
lactose	833.275 mg
starch	333.275 mg

Thus these ingredients in amounts as shown in Table 13 were thorougly mixed together and the obtained mixture was granulated.

The granules thus obtained, which were in a pale brown color and showed a milk-like odor and flavor, were tasty and could be easily taken.

We claim:

1. A health food which comprises 30 to 50% by weight lyophilized kefir yoghurt, 7 to 15% by weight at least one ingredient selected from the group consisting of an extract of *Acanthopanax senticosus Harms.*, an extract of *Cichorium Intylous L.* and a powder of reindeer antlers, and 40 to 55% by weight of at least one excipient.

2. A health food as set forth in claim **1**, which comprises 35 to 45% by weight of the lyophilized kefir yoghurt, 9 to 14% by weight of the assistant(s) and 45 to 55% by weight of the excipient(s).

3. A health food as set forth in claim **1**, wherein said lyophilized kefir yoghurt is a fermentation product obtained from milk and kefir fungi and wherein the kefir yogurt fermentation product is in the form of a lyophilized powder.

4. A health food as set forth in claim **1**, wherein said excipients are selected from the group consisting of lactose and starch.

5. A health food as set forth in claim **2**, which comprises 1.5 to 4.0% by weight of an extract of *A. senticosus Harms.*, 0.3 to 1.0% by weight of a powder of reindeer antlers and 5.5 to 10% by weight of an extract of *C. Intylous L.*

* * * * *

SU291395

SUBJECT MATTER OF THE INVENTION

1. A method for preparing a product capable of imparting a taste of meat, by mixing organic carboxylic acids with a liquid medium, characterized in that in order to impart the taste and aroma of fried meat, a sulfur-containing compound is introduced to the mixture, the sulfur-containing compound being selected from the group comprising 3-acetyl-3-mercaptopropanol-1 and thiamine, wherein the whole mixture is heated to initiate reaction.

2. The method according to claim 1, characterized in that the heating of the mixture is carried out at a temperature of from 180ºF (82.2ºC) to 350ºF (176.7ºC).

3. The method according to claims 1 and 2, characterized in that the pH of the medium during the reaction is maintained within a pH range of 3.5 to 7.0.

4. The method according to claims 1-3, characterized in that a nonsubstituted aliphatic acid, an amino-substituted and an oxy-substituted acid, predominantly acetic, lactic, glutamic and tartaric acid, are used as the organic carboxylic acids.

5. The method according to claims 1-3, characterized in that the acids are a product of protein hydrolysis.

6. The method according to claims 1-5, characterized in that in order to accelerate the reaction, additional sulfur-containing compounds are introduced into the mixture, wherein the additional sulfur-containing compounds are selected from the group comprising amino acids that release hydrogen sulfide during the reaction, lower alkyl mercaptans, lower alkyl sulfides, lower alkyl disulfides, hydrogen sulfide, and inorganic compounds having the formula MS_x, wherein M is selected from the group comprising an alkali metal, an alkaline earth metal and ammonium, and S_x is selected from the group comprising sulfide and hydrosulfide.

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(54) **Title:** BODY TASTE IMPROVER COMPRISING LONG-CHAIN HIGHLY UNSATURATED FATTY ACID AND/OR ESTER THEREOF

(54) 発明の名称: 長鎖高度不飽和脂肪酸及び／又はそのエステル体を含むコク味向上剤

(57) **Abstract:** Means for improving the body taste of a food and improving the taste or flavor thereof. Provided are: a body taste improver which comprises as major components a long-chain highly unsaturated fatty acid and/or ester thereof and a body taste enhancer; a method of enhancing the effect of a body taste improver comprising a long-chain highly unsaturated fatty acid and/or ester thereof, which comprises adding a body taste enhancer to the body taste improver; a body taste improver which comprises a long-chain highly unsaturated fatty acid and/or ester thereof and is derived from a specific vegetable oil containing a large amount of γ-linolenic acid; a vegetable fat composition containing a long-chain highly unsaturated fatty acid and/or ester thereof, characterized by having an α-linolenic acid content of 5% or lower; various foods each containing any of the body taste improvers and vegetable fat composition; and a method of improving the body taste of a food, which comprises incorporating any of the body taste improvers and vegetable fat composition into the food.

(57) 要約: 本発明の目的は、食品のコク味を向上させ、更に、その味又は風味を向上させる手段を提供することである。本発明は、主成分としての長鎖高度不飽和脂肪酸及び／又はそのエステル体及びコク味増強成分を含むコク味向上剤、長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤にコク味増強成分を添加することによりコク味向上剤の効果を増強する方法、γリノレン酸を多量に含む特定の植物油由来の長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤、長鎖高度不飽和脂肪酸及び／又はそのエステル体を含む植物油脂組成物であって、αリノレン酸の含量が５％以下であることを特徴とする植物油脂組成物、これらのコク味向上剤又は植物油脂組成物を含む各種食品、並びに、コク味向上剤又は植物油脂組成物を食品に含有させることから成る食品のコク味を向上させる方法に係る。

WO 2005/046354 A1

明 細 書

長鎖高度不飽和脂肪酸及び／又はそのエステル体を含むコク味向上剤

技術分野

[0001] 本発明は、長鎖高度不飽和脂肪酸及び／又はそのエステル体を主成分として含み、更に、αトコフェロール及び鉄分等から成るコク味増強成分を含むコク味向上剤、長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤にコク味増強成分を添加することによりコク味向上剤の効果を増強する方法、長鎖高度不飽和脂肪酸及び／又はそのエステル体を含むコク味向上剤、及びαリノレン酸含量が低い植物油脂から成る植物油脂組成物、更にこれらを含むコク味が向上した食品、並びに、コク味向上剤又は植物油脂組成物を食品に含有させることから成る食品のコク味を向上させる方法等に関する。

背景技術

[0002] γリノレン酸及びアラキドン酸（シス－5，8，11，14－エイコサテトラエン酸）は長鎖高度（多価）不飽和脂肪酸の一種であり動物の臓器及び組織から得られるリン脂質中に存在する。これは必須脂肪酸でありプロスタグランジン、トロンボキサンチン及びロイコトリエン等の合成の前駆体となる重要な化合物である。

[0003] 従来、γリノレン酸を比較的多量に含む月見草油やボラージ油、又はアラキドン酸等の長鎖高度不飽和脂肪酸及びそのエステルを、栄養強化及び各種生理的作用を付与する目的で油脂及び食品等の各種組成物へ添加することが試みられてきた。

[0004] 特開平10－99048に記載の栄養強化組成物には、母乳に近い成分を実現する為に添加される成分の一種としてアラキドン酸が0．1ー10重量％含有されている。

[0005] 一方、エステル体の例としては、特開平4－197134に蒸発潜熱による生地内部の温度降下が防止されたフライ用油脂組成物が記載されているが、そこにはアラキドン酸等の不飽和脂肪酸が構成脂肪酸として20ー60重量％含有されている。

[0006] 特開平9－13075では、アラキドン酸等の長鎖多価不飽和脂肪酸を含むグリセリドからなる血中脂質濃度を低減する作用のある油脂が記載されているが、このグリセリドはエステル交換法により得られ、該長鎖多価不飽和脂肪酸の総量の40モル％未

満がグリセリドの2位に結合するという天然油とは異なる構造を有するものである。特開平9−13076では、同じ構成から成る血小板凝集能を抑制する作用のある油脂が記載されている。

[0007] 又、特開平11−89513に記載のヒト乳脂肪に近似する合成脂質組成物には、アラキドン酸を含む長鎖n−6高度不飽和脂肪酸がトリグリセリドを構成する脂肪酸の一種として使用されている。

[0008] 更に、特開平10−70992及び特開平10−191886にはアラキドン酸をトリグリセリドの形で豊富に含有する微生物由来の食用油脂が記載されており、この好適用途として、未熟児用調製乳、乳児用調製乳、幼児用食品及び妊婦用食品等が挙げられている。

[0009] しかしながら、上記の従来技術のいずれにおいても、アラキドン酸等の長鎖多価不飽和脂肪酸を食品や植物油脂のコク等の風味を向上させる目的で使用することに関する開示はなく、このような目的に使用できる可能性を示唆する記載も見当たらない。

[0010] 一方で、このような長鎖高度不飽和脂肪酸を添加した場合には、その酸化分解物の臭い(所謂「戻り臭」)が食品等の風味を損ねるという問題点があり、その解決策として様々な手段が講じられてきた。

[0011] かかる手段の例として、例えば、特開昭63−44843に記載の油中水中油型乳化油脂組成物においては高度不飽和脂肪酸を内相油に含有することを特徴とする技術が開示されている。特開平6−172782では高度不飽和脂肪酸を含有する油脂を粉末化する技術が記載されている。特開平9−176679では不飽和脂肪酸粉状体に粉末状の抗酸化剤を混合させる技術が開示されている。特開平9−263784では多価不飽和脂肪酸を含有する油脂にδ−トコフェロールを含有させる技術が開示されている。又、特開平11−12592では高度不飽和脂肪酸を含有する魚脂類にしょうゆ油を添加することを特徴とする技術が開示されている。

[0012] また、食品分野における長鎖高度不飽和脂肪酸の利用例として、特開2001−78702には、油脂とエキスを水中油型に乳化させることで、まろやかさ、後味、うま味が増強された調味料が開示されており、油脂の例として魚油又は魚油を含有する油脂が

挙げれられており、魚油を構成する脂肪酸のうちω3系高度不飽和脂肪酸を10%以上含む例が記載されている。

[0013] この調味料においては、含まれる油脂の酸化を抑制する為に乳化剤としてポリグリセリン脂肪酸エステルを用いたり、油脂の酸化を防ぐためにエキスに天然物から抽出されたカルノシン、及びアンセリン等の抗酸化物質が含まれていることがこの好ましいとされている。又、調味料を製造する段階で、加熱処理等の酸化処理は一切行われていない。更に、この調味料の効果を有効に発揮できる食品の例としては、水産練り製品、魚類、魚類加工品が挙げられているのみである。

[0014] 又、特許第3220155号公報には、乳脂肪以外の脂肪酸等の酸化によって得ることができる香味料組成物が開示され、この脂肪酸がω−3非共役二重結合系をもつ少なくとも1種類のポリ不飽和脂肪酸を0．01重量%を超える量含むことを特徴としている。この香味料組成物はバター様フレーバーに強く認められている甘く、クリーミィなノートが含まれるものであるが、かかるノートを得る為には油脂を必ず酸化処理する必要がある。そしてこの酸化処理は、酸化を若干遅らせるような量の酸化防止剤が存在するプロセスによる制御された酸化処理である必要がある。又、かかる酸化処理によって発生するフレーバーは揮発性成分であるので、酸化処理は密閉系で行うことが好ましいとされ、実施例においては還流凝縮器を使用して酸化処理を行っている。香味料組成物の用途としてはバターフレーバーをもつと有益な食品の香味付与に特に適していると記載されている。

[0015] 米国特許第3689289号明細書（US3689289）には還元糖とアミノ酸及びアラキドン酸またはそのアラキドン酸メチルエステルを特定の条件下で加熱反応させることで人工的なチキンフレーバーを製造する方法が記載されている。また国際公開第WO03/051139号パンフレットには還元糖とアミノ酸及びアラキドン酸を特定の条件下で加熱反応させることによる人工的なチキンフレーバーを製造する方法においてアラキドン酸をグリセリンエステルの形で使用することにより、生成したチキンフレーバーの香りに耐熱性や持続性が生じることが記載されている。

[0016] これらはいずれも糖とアミノ酸とアラキドン酸の3種の加熱反応物が人工的なチキン様の臭いを発現するもので、アラキドン酸等の長鎖高度不飽和脂肪酸が単独で、又

は長鎖高度不飽和脂肪酸と糖若しくはアミノ酸とで食品の呈味を改善するという記載は見られない。

[0017] 更に、特開2002−95439号公報には、高度不飽和脂肪酸グリセリドを含有することを特徴とする調味料が開示されている。この発明の目的は、酸化劣化されやすい高度不飽和脂肪酸グリセリドの酸化安定性を高めることであり、その為に、大豆の発酵加工食品、魚介類の発酵加工食品もしくはトマト成分を主体とした調味料に高度不飽和脂肪酸グリセリドを含ませることを特徴とするものである。従って、この発明は、高度不飽和脂肪酸グリセリドを含有させることにより食品のコク味を向上することに関しては何等開示又は示唆していない。

[0018] 特許文献1:特許第3220155号公報

特許文献2:米国特許第3689289号明細書

特許文献3:国際公開第WO03/051139号パンフレット

特許文献4:特開2002−95439号公報

発明の開示

発明が解決しようとする課題

[0019] ところで、食品の分野において、とんかつ等の油調食品やカレー、餃子等の油脂含有食品等、食品によっては「コク」や「濃厚感」が必要なものがある。そのような食品へ「コク」や「濃厚感」を賦与するため、従来は、香料を添加したり、動物性油脂を単独または植物油脂に配合して使用していた。

[0020] しかしながら、香料を添加したものにおいては、加熱調理中に香料が揮発し、賦与した「コク味」が持続しないという課題があった。また動物油脂の使用は同油脂に含まれるコレステロールや飽和脂肪酸の健康への影響が懸念されていた。植物油脂はノンコレステロールで飽和脂肪酸が少ないが、植物油脂で調理した食品は「あっさり」しており、「コク味」を必要とする食品には「物足りなさ」が感じられるという課題があった。よって、ノンコレステロールで飽和脂肪酸が少なく、しかも「コク味」のある油脂の実現が望まれていた。

[0021] 本発明者等は上記の課題を解決すべく研究した結果、従来は肉類等の腐臭等、オフフレーバーの原因物質とされてきたアラキドン酸等の長鎖高度不飽和脂肪酸及び

／又はそのエステル体を単独で食品に含有させるか、又はそれを特定の範囲で含有する植物油脂で加熱調理などの酸化処理をした場合に、食品のコク味が増し、味を引き立たす効果が得られることを既に見出した（PCT/JP03/00182）。

課題を解決するための手段

[0022] 本発明者は上記の課題を解決すべく更に研究を重ねた結果、長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤に、更に、αトコフェロール及び鉄分等から成るコク味増強成分を含ませることによって、コク味向上効果が一層向上することを見出した。更に、αリノレン酸及び／又はリノール酸の含量が低い植物油脂に長鎖高度不飽和脂肪酸及び／又はそのエステル体を添加することによって、コク味が一層向上した植物油脂組成物が得られることを見出し、本発明を完成した。

[0023] 即ち、本発明は、主成分としての長鎖高度不飽和脂肪酸及び／又はそのエステル体及びコク味増強成分を含むコク味向上剤、γリノレン酸を多量に含む特定の植物油由来の長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤、長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤にコク味増強成分を添加することによりコク味向上剤の効果を増強する方法、長鎖高度不飽和脂肪酸及び／又はそのエステル体を含む植物油脂組成物であって、αリノレン酸の含量が5％以下であることを特徴とする植物油脂組成物、これらのコク味向上剤又は植物油脂組成物を含むコク味が向上した各種食品、コク味向上剤又は植物油脂組成物を食品に含有させることから成る、食品のコク味を向上させる方法、並びに、長鎖高度不飽和脂肪酸及び／又はそのエステル体及びコク味増強成分を個別に食品に添加することから成る、食品のコク味を向上させる方法に係る。

[0024] 本明細書中において、「コク味向上剤」とは、これを添加した食品に有意な「コク味」や「濃厚感」を新たに付与したり、それらを向上させるという本発明の効果が得られるものをいう。このような本発明の効果に関する評価は、本明細書中の実施例に示した官能評価法によって行うことが出来る。

[0025] 更に本明細書中において、「コク味増強成分」とは、長鎖高度不飽和脂肪酸及び／又はそのエステル体によるこのような効果を相加的又は相乗的に高める作用を有する成分をいう。

発明の効果

[0026] 長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤に、更に、αトコフェロール及び鉄分等から成るコク味増強成分を含ませることによって、コク味向上効果が一層向上することを見出した。更に、αリノレン酸及び／又はリノール酸の含量が低い植物油脂に長鎖高度不飽和脂肪酸及び／又はそのエステル体を添加することによって、コク味が一層向上した植物油脂組成物が得られることを見出した。

発明を実施するための最良の形態

[0027] 本発明におけるコク味増強成分の種類・構造等に特に制限はないが、例えば、その代表的な例として、αトコフェロール及び鉄分を挙げることが出来る。理論上、本発明を何等拘束するものではないが、αトコフェロール及び鉄分は、油脂の酸化を促進する効果があることが知られている。

[0028] 鉄分の供給原料として、硫酸第1鉄、クエン酸第1鉄、クエン酸鉄、グルコン酸第1鉄、塩化第2鉄、乳酸鉄等の鉄塩類、ヘム鉄、血漿粉末、血球粉末等の、市販されている当業者に公知の任意の鉄含有素材等を使用することができる。Feの形態として、鉄、第1鉄塩及び第2鉄塩があるが、好ましくは第1鉄塩である。尚、鉄分は、溶解・分散・乳化等の任意の方法で長鎖高度不飽和脂肪酸及び／又はそのエステル体と混合することが出来る。

[0029] αトコフェロールの供給原料としても、当業者に公知の任意のものを使用することが出来る。例えば、全トコフェロール組成中でαトコフェロールが最も多く含まれているようなトコフェロール製剤、具体的には、全量中の50％以上がαトコフェロールから成るトコフェロール製剤が市販されているので、それをそのままαトコフェロールの供給原料として使用することが出来る。又、高濃度のαトコフェロールを含有する植物油脂をαトコフェロールの供給原料として使用することも出来る。或いは、適当なトコフェロール製剤から、当業者に公知の方法でβ、γ、δトコフェロールを低減ないし除去することによってαトコフェロール濃度を高めたものを使用することも出来る。

[0030] これらコク味増強成分のコク味向上剤中の含量に特に制限はないが、効果及び経済性などの点から、αトコフェロールの場合には、好ましくは50ー15000ppm、より

好ましくは50ー5000ppm、更に好ましくは100ー2000ppmである。又、鉄分の場合には、Fe換算で0．1ー200ppm、好ましくは0．5ppmー100ppmを含有する。

[0031] 本発明で「長鎖高度不飽和脂肪酸」とは、n−3系の長鎖高度不飽和脂肪酸の場合には炭素数が20以上かつ二重結合を3つ以上有する脂肪酸を意味し、n−6系の長鎖高度不飽和脂肪酸の場合には炭素数が18以上かつ二重結合を3つ以上有する脂肪酸を意味する。更に、n−3系及びn−6系の双方ともに炭素数が20ー24かつ二重結合を4ー6有する長鎖高度不飽和脂肪酸が好ましい。n−6系の長鎖高度不飽和脂肪酸の例としてγリノレン酸、アラキドン酸（AA）及びドコサテトラエン酸（DTA）を挙げることが出来、特に、アラキドン酸が好適である。又、n−3系の長鎖高度不飽和脂肪酸の例として、ドコサヘキサエン酸（DHA）及びエイコサペンタエン酸（EPA）を挙げることが出来る。

[0032] アラキドン酸等の本発明で使用する長鎖高度不飽和脂肪酸及びそのエステル体の由来に特に制限はなく、各種動植物、微生物、藻類等から得られたものが市販されており、これら当業者には公知のものを適宜使用することが出来る。

[0033] 例えば、上記の特開平10−70992及び特開平10−191886に記載されたアラキドン酸をトリグリセリドの形で豊富に含有する微生物由来の食用油脂を使用することが出来る。このようにアラキドン酸等の本発明の活性成分である脂肪酸及びそのエステル体として微生物由来のものを使用した場合には、動物由来の油脂を実質的に含有しないノンコレステロールで飽和脂肪酸含量が少ないコク味向上剤又は食品を得ることが出来る。

[0034] 2種以上の長鎖高度不飽和脂肪酸とは異なる原料から得られた同種の脂肪酸を適宜混合して使用することも可能である。

[0035] エステル体の構造及びその製造方法に特に制限はなく、これを構成するアルコール類としては、一価及び多価アルコールを使用することが出来る。多価アルコールの中でも、安全性やコストの点から好ましいものの例としてグリセロールをあげることができ、この場合にエステル体としてトリグリセリド、ジグリセリド及びモノグリセリドを構成する。これらのエステル体を構成する脂肪酸中に本発明の長鎖高度不飽和脂肪酸以外の脂肪酸が含まれていても良い。

[0036] 大豆油、トウモロコシ油、オリーブ油、HOS、及びカノーラ油等の植物油にはγリノレン酸は含まれていない。一方、これに対して、ボラージ(ボリジ)油、月見草油、ローズヒップ油、及びブラックカラント油には、約3%ー22%程度のγリノレン酸が含まれている。特に、ボラージ油には約22%もγリノレン酸が含まれており、本発明の長鎖高度不飽和脂肪酸の原料として好適に使用できる。

[0037] 従って、本発明は、ボラージ油、月見草油、ローズヒップ油、又はブラックカラント油から選ばれた植物油由来の長鎖高度不飽和脂肪酸及び/又はそのエステル体から成るコク味向上剤、特に、ボラージ油由来の長鎖高度不飽和脂肪酸及び/又はそのエステル体から成るコク味向上剤に係る。

[0038] 本発明のコク味向上剤中の長鎖高度不飽和脂肪酸及び/又はそのエステル体の含有量に特に制限はないが、含有量があまり少ないと所望の効果を得るために多量のコク味向上剤が必要とされ、その他の成分に由来する雑味の悪影響が生じる可能性もある。従って、本発明のコク味向上剤は、長鎖高度不飽和脂肪酸及び/又はそのエステル体を、好ましくは10重量%以上、より好ましくは30重量%以上含有するものである。

[0039] コク味向上剤中には、上記活性成分の他に、コク味向上剤の効果に悪影響を与えない限り、当業者に公知の他の任意成分、例えば、乳化剤、ステロール類、リン脂質、その他の脂肪酸、その他の脂肪酸より構成されるトリグリセリド、ジグリセリド及びモノグリセリド等が含まれていても良い。

[0040] 尚、n—3系の長鎖高度不飽和脂肪酸及び/又はそのエステル体を使用する場合に効果を充分得るためにはそれらを酸化処理することが望ましい。酸化処理の方法に特に制限はないが、その一例として加熱処理があげられる。加熱処理の方法に特に制限はないが、酸化防止剤を存在させる必要はなく、又、酸化処理を密閉系で行う必要もない。開放系において、通常、40℃ー200℃において0.1時間ー240時間加熱する方法があり、好ましくは、80℃ー180℃において0.5時間ー72時間加熱する。

[0041] 又、n—6系の長鎖高度不飽和脂肪酸及び/又はそのエステル体の効果を発揮するために酸化処理は必要ないが、酸化処理することによって、その効果が一層増す。

[0042] 尚、本発明の長鎖高度不飽和脂肪酸及び／又はそのエステル体から成るコク味向上剤、又はそれを含有する食品を同様に加熱等の酸化処理することによっても、同様の効果を実施することが出来る。

[0043] 尚、エステル体の中でも特にグリセリンエステルの場合には、長鎖高度不飽和脂肪酸はそれらのエステル体に比べて揮発性が高いので、より少量で本発明の効果を得ることが出来る。対して、エステル体は揮発性が比較的低い一方で持続性に優れているので、エステル体を含む場合には相対的に含有量が多くなる。

[0044] 更に、本発明は、以上のコク味向上剤を含む植物油脂組成物も含むものである。ここで、ベースとなる植物油脂としては、当業者に公知に任意のものを使用することが出来る。このような植物油脂の例として、例えば、大豆油、菜種油、コーン油、ひまわり油、HOS、米油、ごま油、オリーブ油、及びパーム油等を挙げることが出来る。この中でも、特に、フライや炒め物などの加熱調理に主に利用される大豆油、菜種油、コーン油、及びパーム油等に本発明のコク味向上剤を添加することによって、本発明の効果を有利に達成することが出来る。任意の2種類以上の植物油脂を適宜混合して使用することも出来る。

[0045] かかる植物油脂組成物中の長鎖高度不飽和脂肪酸はそれらのエステル体の含有量は、長鎖高度不飽和脂肪酸の種類、植物油脂組成物の用途等に応じて当業者が適宜選択することが出来るが、コク味が向上した植物油脂組成物であるためには、長鎖高度不飽和脂肪酸として通常、10ー100，000ppm、好ましくは、10ー20，000ppm、より好ましくは10ー5000ppm、更に好ましくは20ー1000ppmである。特に、γリノレン酸等の植物油由来の長鎖高度不飽和脂肪酸を含む植物油脂組成物を、炒めやフライ用といった油調用油脂、風味油又は香味油のベース油等として使用する場合には、植物油脂組成物中のγリノレン酸等の長鎖高度不飽和脂肪酸の含量は、通常、0．05ー10％、好ましくは0．1ー5％含有する。

[0046] 尚、既に記載したように、ボラージ（ボリジ）油、月見草油、ローズヒップ油、及びブラックカラント油には約3％ー22％程度のγリノレン酸が既に含まれている。従って、これらの植物油は、特に長鎖高度不飽和脂肪酸はそれらのエステル体を外部から添加しなくとも、それ自体本発明の植物油脂組成物として有利に使用することも出来る

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[0047] 又、本発明者は、本発明で使用する長鎖高度不飽和脂肪酸及び／又はそのエステル体と混合される植物油脂の脂肪酸組成がある条件を満たしているような場合に、より効果的にコク味の向上効果が得られることを見出した。

[0048] 即ち、本発明の植物油脂組成物において、αリノレン酸の含量が5%以下、例えば、αリノレン酸の含量が0．01%ー5%、好ましくは0．05%ー3%である植物油脂組成物が好ましい。更に、オレイン酸含量が20ー86%及び／又はリノール酸含量が3ー25%であるものがより好ましい。

[0049] 既に記載したように、植物油脂組成物における長鎖高度不飽和脂肪酸及び／又はそのエステル体の含量は、長鎖高度不飽和脂肪酸として通常、10ー100，000ppm、好ましくは、10ー20，000ppm、より好ましくは10ー5000ppm、更に好ましくは20ー1000ppmである。尚、このような植物油脂組成物において、コク味増強成分は必ずしも含まれている必要はない。

[0050] 尚、ボラージ（ボリジ）油及びブラックカラント油にはαリノレン酸が実質的に含有されておらず、オレイン酸含量も上記の範囲である。従って、これらの油はこの点からもそれ自体植物油脂組成物として好適なものである。

[0051] こうして得られる本発明の植物油脂組成物は、様々な加熱調理法、例えば、80ー300℃、より好ましくは110ー300℃に加熱する調理法に使用することができる。このような温度範囲で加熱すると、本発明の植物油脂組成物を用いて調理した食品のコク味が増し、食品の味を一層引き立たせることが出来る。

[0052] 最後に、本発明は、本発明のコク味向上剤、又は植物油脂組成物を含むコク味が向上した食品にも係る。ここで、対象となる食品の種類に特に制限はなく、例えば、各種フライ食品及び炒め物等の油調食品、並びに、カレー、餃子、及び焼売等の油脂添加食品を挙げることが出来る。本発明の食品は、それに含まれる本発明に係るコク味向上剤又はこれを含む植物油脂組成物によって、有意な「コク味」や「濃厚感」が付与されている。これらの食品は、既に記載したような加熱処理されることによって、更にそのコク味が向上する。

[0053] 従って、本発明は更に、本発明のコク味向上剤又は植物油脂組成物を食品に含有

させることから成る、食品のコク味を向上させる方法に係る。ここで、本発明のコク味向上剤に含まれる長鎖高度不飽和脂肪酸及び／又はそのエステル体並びにコク味増強剤は、夫々個別に食品に添加することによっても同様な効果を得ることが出来る。従って、本発明は、更に、長鎖高度不飽和脂肪酸及び／又はそのエステル体、及びコク味増強成分を個別に食品に添加することから成る、食品のコク味を向上させる方法にも係る。尚、このように個別に添加する際には、食品中の長鎖高度不飽和脂肪酸及び／又はそのエステル体並びにコク味増強剤の最終的な含量は、本発明のコク味向上剤を添加した場合と同様なものとなるようにすることが好ましい。尚、これら各成分の添加時期・順序等は食品の種類等に応じて、当業者が適宜選択することが出来る。

[0054] 食品中に含有される本発明のコク味向上剤又はこれを含む植物油脂組成物の量に特に制限はないが、通常、喫食時に長鎖高度不飽和脂肪酸及び／又はそのエステル体の量に換算して、0．1ー100，000ppm、より好ましくは0．1ー10，000ppmを含有させることによって、食品のコク味向上効果が得られる。例えば、醤油、スープ等の液状食品に対しては、喫食時に長鎖高度不飽和脂肪酸及び／又はそのエステル体の量に換算して、好ましくは1ー1，200ppm、より好ましくは1ー500ppmを含有させることによって、食品のコク味向上効果が得られる。又、ハンバーグのような固形食品においては、長鎖高度不飽和脂肪酸及び／又はそのエステル体の量に換算して、好ましくは20ー2，500ppm、より好ましくは20ー1，000ppmを含有させることによって、食品のコク味向上効果が得られる。

[0055] 以下に、実施例により本発明をさらに具体的に説明するが、本発明の技術的範囲はそれに限定されるものではない。尚、本明細書中、特に断わりがない限り、「％」は「重量％」を意味する。

[0056] 本発明において、トコフェロール組成は基準油脂分析法（日本油化学会編）2.4.10−1996に従って分析することが出来る。又、脂肪酸含量は基準油脂分析法（日本油化学会編）2.4.2.2−1996に基づき分析することが出来る。

[0057] ＜官能評価法＞

以下の官能評価において「コク味が向上する」とは各表中の評価項目にある「香り

の強さ」、「風味の強さ」、「味の強さ」及び「後味の強さ」がそれぞれの「香りの好ましさ」、「風味の好ましさ」、「味の好ましさ」及び「後味の好ましさ」を向上させる若しくは損なうことなく増大することである。

[0058] パネル n＝7

以下の実施例の結果を示した表における記号の意味は以下のとおりである。

×:コントロールより弱いまたは好ましくない;△:コントロールと同等;○:コントロールより強いまたは好ましい;◎コントロールより明らかに強いまたは好ましい

実施例

[0059] ＜使用した長鎖不飽和脂肪酸＞

アラキドン酸含有トリグリセリド(AATG:純度40ー45%:ナカライテスク(株)販売、サントリー(株)製造)、ボラージ油(γリノレン酸20%:Statfold社製)、低αリノレン酸菜種油(Low Linolenic Canola Oil、以下、「LLC」、味の素製油(株))、菜種油(以下、「CO」、味の素製油(株))、高オレイン酸種紅花油(High Oleic Safflower Oil、以下、「HOS」、味の素製油(株))を使用した。

[0060] [表1]

脂肪酸組成(%)

	C16	C18:0	C18:1	C18:2	C18:3 (n-3)
CO	3.9	1.9	62.5	19.5	8.8
HOS	4.6	1.9	76.3	14.2	0.2
LLC	3.3	1.7	73.5	15.4	2.4

[0061] [表2]

トコフェロール製剤及び含量
「ビタミンE5－67」ADM社

	α	β	γ	δ
含量(mg/g)	707	16	4	3

[0062] ＜αトコフェロールのコク味増強成分としての効果＞

以下の組成で長鎖高度不飽和脂肪酸及び/またはそのエステル体及びαトコフェロール製剤と植物油脂を混合しコク味向上剤を調製した。

(1)LLC

(2)1%AATG／LLC(AA0．4%)

(3)0．5%AATG/LLC(AA0．2%)

(4)1%AATG＋0．002%αトコフェロール製剤/LLC

(AA0．4%、αトコフェロール14.1ppm)

(5)1%AATG＋0．01%αトコフェロール製剤/LLC

(AA0．4%、αトコフェロール70.7ppm)

(6)1%AATG＋0．02%αトコフェロール製剤/LLC

(AA0．4%、αトコフェロール141ppm)

(7)1%AATG＋0．2%αトコフェロール製剤/LLC

(AA0．4%、αトコフェロール1410ppm)

(8)1%AATG＋2%αトコフェロール製剤/LLC

(AA0．4%、αトコフェロール14100ppm)

(9)0．5%AATG＋0．02%αトコフェロール製剤/LLC

(AA0．2%、αトコフェロール141ppm)

[0063] 調製油15gに薄力粉15gを混合し120℃で30分炒めた後にカレー粉(「特製エスビーカレー」、エスビー食品(株))3gを添加しさらに10分間加熱しカレールーを調製した。コンソメスープ(「コンソメ」味の素(株)1．7%溶液)500mlを添加し加熱しコンソメスープを調製した。カレールーをコンソメスープで希釈しカレースープを調製した。

[0064] [表3]

(1)(コントロール)と(2)の比較

調製油(コク味向上剤)	(1)	(2)
AA喫食時濃度(ppm)	0	112.5
香りの強さ	−	◎
香りの好ましさ	−	◎
風味の強さ	−	◎
風味の好ましさ	−	◎
味の強さ	−	◎
味の好ましさ	−	◎
後味の強さ	−	◎
後味の好ましさ	−	◎

[0065] [表4]

（2）（コントロール）と（3）、（4）、（5）、（6）、（7）、（8）、（9）の比較

調製油	（2）	（3）	（4）	（5）	（6）	（7）	（8）	（9）
油中αトコフェロール濃度（ppm）	0	0	14	71	141	1410	14100	141
AA喫食時濃度(ppm)	112.5	56.3	112.5	112.5	112.5	112.5	112.5	56.3
香りの強さ	−	×	△	○	○	◎	◎	○
香りの好ましさ	−	×	△	◎	◎	◎	◎	○
風味の強さ	−	×	△	○	○	◎	◎	○
風味の好ましさ	−	×	△	○	◎	◎	◎	○
味の強さ	−	×	△	◎	◎	◎	◎	○
味の好ましさ	−	×	△	◎	◎	◎	○	○
後味の強さ	−	×	△	◎	◎	◎	◎	○
後味の好ましさ	−	×	△	○	○	○	○	○

[0066] 上記表4に示した結果において、(2)(コントロール：αトコフェロールを含有しない)と(4)、(5)、(6)、(7)、(8)の比較において、AATGがカレー中に同濃度でも、(5)、(6)、(7)、(8)の濃度のαトコフェロールを含有することによりコク味向上効果が増強されたことが判明した。更に、(2)と(3)の比較において、コク味向上効果はAATG濃度の低い(3)は(2)に比べ劣るが、αトコフェロールを添加した(9)は(3)とAATG濃度が同じでも(2)以上のコク味向上効果を発現したことが判明した。

[0067] ＜鉄成分のコク味増強成分としての効果＞

2.5%AATG/LLCに対し、硫酸第1鉄・7水塩を0.00005ー0.1%(Fe: 0.1ー200ppm)添加して均一に分散させた後、0.8%食塩水に0.1%添加した。

(1)2.5%AATG/LLC

(2)硫酸第1鉄・7水塩　0.00005%添加(Fe:0.1ppm)/2.5%AATG/LLC

(3)　〃　0.00025%(Fe:0.5ppm)/2.5%AATG/LLC

(4)　〃　0.005%(Fe:10ppm)/2.5%AATG/LLC

(5)　〃　0.05%(Fe:100ppm)/2.5%AATG/LLC

(6)　〃　0.1%(Fe:200ppm)/2.5%AATG/LLC

[0068] [表5]