

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2015/023679

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding claim 34, Cavallini et al. in view of Orcutt et al. teach the method of claim 30, Cavallini et al. teach wherein combined with said non-animal fat, and said one or more flavor precursors before heating said mixture ("blending in an oil to form an emulsion base; blending in a modified food starch and flavoring ingredients; to form a flavored emulsion base; and cooking the flavored emulsion base to produce a vegetable base meat analog", where the oil is vegetable oil and the flavors may include salt, Paras. [0023]-[0025]). Cavallini et al. do not explicitly disclose said iron salt. However, Orcutt et al. teach said iron salt (Orcutt et al., "A variety of ingredients that contain protein may be utilized in a thermal plastic extrusion process to produce structured protein products suitable for use in the ground meat simulated meat compositions.", Para. [0028], such as iron salt minerals, Para. [0064]; Claims 1, 11 and 12). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Cavallini et al. to add an iron containing salt, as taught by Orcutt et al. The motivation for doing so would be to mimic the texture and taste of meat with one or more "flavoring ingredients", (Cavallini et al., Abstract; Paras. [0024];[0045]). Cavallini et al. do not explicitly disclose a highly conjugated heterocyclic ring complexed to an iron ion. However, Sandhill teaches said second highly conjugated heterocyclic ring complexed to an iron ion and/or a second iron salt (Sandhill, "one or more isolated and purified proteins", Para. [0006], where "the replica contains between 0.01% and 5% by weight of a heme protein. In some embodiments, the replica contains between 0.01% and 5% by weight of leghemoglobin.", Para. [00354]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Cavallini et al. to add an iron containing protein, as taught by Sandhill. The motivation for doing so would be to mimic the texture and taste of meat with one or more "flavoring ingredients", (Cavallini et al., Abstract; Paras. [0024];[0045]).

Regarding claim 113 (as best interpreted), Cavallini et al. in view of Sandhill teach the replica of claim 110, Cavallini et al. do not explicitly disclose wherein a denaturation temperature of said binding agent is between about 40°C and about 80°C. However, Orcutt et al. teach wherein a denaturation temperature of said binding agent is between about 40°C and about 80°C (Orcutt et al., "ground meat simulated meat compositions", Para. [0028], with various plant extracts, such as those containing carotenoids, tocopherols, or flavonoids" are added to "the protein-containing materials that will be extruded", Para. [0062], and "Preferably the protein-containing mixture is heated to a temperature of from about 25.degree. C. to about 80.degree. C., more preferably from about 30.degree. C. to about 40.degree. C. in the preconditioner.", Para. [0079], "The dry pre-mix or the conditioned pre-mix is then fed into an extruder to heat, shear, and ultimately plasticize the mixture.", Para. [0083]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Cavallini et al. to denature the vegetable protein, as taught by Orcutt et al. The motivation for doing so would be to mimic the texture and taste of meat with one or more binding proteins, (Cavallini et al., Abstract; Paras. [0023]-[0024];[0040];[0045]-[0046]).

Claims 75, 76, 97 and 98 lack an inventive step under PCT Article 33(3) as being obvious over Lyrical Foods, Inc. [hereinafter Lyrical] in view of Krammer et al. and Sinha et al.

Regarding claim 75, Lyrical teaches a method of increasing the meat flavor or masking off flavors from plant material in a food product (a cheese replica, Abstract, where the replica is prepared from "proteins and fats from plants or other non-animal sources", and the replica incorporates flavor additives to mimic the flavor of cheese, Paras. [0012];[00125];[00127]). Lyrical does not explicitly disclose said method comprising adding, to said food product, one or more lactones, wherein said lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyl-dihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, 1-pentoyllactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, gamma-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1, 1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, delta-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. However, Krammer et al. teach said method comprising adding, to said food product, one or more lactones, wherein said lactones are selected from the group consisting of gamma-octalactone, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, delta-tetradecalactone (Krammer et al., "Cheese flavoring containing both volatile and non-volatile components which comprise constituents which contribute to the taste sensation "cheese".", Abstract, where the Group 2 flavoring additives comprise "milk-like and creamy flavors are saturated and unsaturated .delta.- and .gamma.-lactone having 6 to 14 carbon atoms. Preferred compounds of this group are delta.-tetradecalactone gamma.-octalactone ... compounds having a caramel-like flavor. Preferred compounds are ... 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone.", Para. [0031], and are added to food products, Paras. [0068]-[0069]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired flavoring, as taught by Krammer et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]). Lyrical does not explicitly disclose adding, to said food product, one or more lactones at a concentration of 10-3 to 10-11 of said food product. However, Sinha et al. teach adding, to said food product, one or more lactones at a concentration of 10-3 to 10-11 of said food product (Sinha et al., flavoring food products with lactones, where lactones are added "in desired and acceptable amount varying from ppm to mg based on the type of products", Paras. [0010];[0021];[0061];[0069]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired amount of flavoring, as taught by Sinha et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]).

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Regarding claim 76, Lyrical in view of Krammer et al. and Sinha et al. teach the method of claim 75, Lyrical does not explicitly disclose wherein said lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, gamma-octalactone, and delta-tetradecalactone. However, Krammer et al. teach wherein said lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, gamma-octalactone, and delta-tetradecalactone (Krammer et al., the Group 2 flavoring additives comprise "milk-like and creamy flavors are saturated and unsaturated .delta.- and .gamma.-lactone having 6 to 14 carbon atoms. Preferred compounds of this group are delta.-tetradecalactone gamma.-octalactone ... compounds having a caramel-like flavor. Preferred compounds are ... 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone.", Para. [0031]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired flavoring, as taught by Krammer et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]). Lyrical does not explicitly disclose butyrolactone. However, Sinha et al. teach butyrolactone (Sinha et al., flavoring food products with lactones, such as butyrolactone, Paras. [0010];[0021]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired flavoring, as taught by Sinha et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]).

Regarding claim 97, Lyrical teaches a food replica product (a cheese replica, Abstract, where the replica is prepared from "proteins and fats from plants or other non-animal sources", and the replica incorporates flavor additives to mimic the flavor of cheese, Paras. [0012]; [00125];[00127]). Lyrical does not explicitly disclose one or more lactones, wherein said one or more lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, deltaoctalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5 -penty-2(3H) furanone, dihydro-3-methylene-2,5-furandione, 1-pentoyllactone, tetrahydro-2H -pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, gamma-octalactone, 5 -hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5 -acetyldihydro-2(3 H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1, 1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, delta-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. However, Krammer et al. teach one or more lactones, wherein said one or more lactones are selected from the group consisting of gamma-octalactone, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, delta-tetradecalactone (Krammer et al., "Cheese flavoring containing both volatile and non-volatile components which comprise constituents which contribute to the taste sensation "cheese".", Abstract, where the Group 2 flavoring additives comprise "milk-like and creamy flavors are saturated and unsaturated .delta.- and .gamma.-lactone having 6 to 14 carbon atoms. Preferred compounds of this group are delta.-tetradecalactone gamma.-octalactone ... compounds having a caramel-like flavor. Preferred compounds are ... 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone.", Para. [0031], and are added to food products, Paras. [0068];[0069]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired flavoring, as taught by Krammer et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]). Lyrical does not explicitly disclose one or more lactones at a concentration of 10-3 to 10-11 of said food product. However, Sinha et al. teach one or more lactones at a concentration of 10-3 to 10-11 of said food product (Sinha et al., flavoring food products with lactones, where lactones are added "in desired and acceptable amount varying from ppm to mg based on the type of products", Paras. [0010];[0021];[0061];[0069]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired amount of flavoring, as taught by Sinha et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]).

Regarding claim 98, Lyrical in view of Krammer et al. and Sinha et al. teach the product of claim 97, Lyrical does not explicitly disclose wherein said one or more lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, gamma-octalactone, and delta-tetradecalactone. However, Krammer et al. teach wherein said lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, gamma-octalactone, and delta-tetradecalactone (Krammer et al., the Group 2 flavoring additives comprise "milk-like and creamy flavors are saturated and unsaturated .delta.- and .gamma.-lactone having 6 to 14 carbon atoms. Preferred compounds of this group are delta.-tetradecalactone gamma.-octalactone ... compounds having a caramel-like flavor. Preferred compounds are ... 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone.", Para. [0031]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired flavoring, as taught by Krammer et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]). Lyrical does not explicitly disclose butyrolactone. However, Sinha et al. teach butyrolactone (Sinha et al., flavoring food products with lactones, such as butyrolactone, Paras. [0010];[0021]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lyrical to add the desired flavoring, as taught by Sinha et al. The motivation for doing so would be to mimic the taste of cheese, (Lyrical, Abstract; Paras. [0012];[00125];[00127]).

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Claims 81 and 82 lack an inventive step under PCT Article 33(3) as being obvious over Cavallini et al. in view of Orcutt et al. and Ludwig.

Regarding claim 81, Cavallini et al. in view of Orcutt et al. teach the method of claim 80, Cavallini et al. do not explicitly disclose wherein said vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract is a Cucumis juice, puree, or extract. However, Ludwig teaches wherein said vegetable juice, vegetable puree, vegetable extract is a Cucumis juice, puree, or extract. (Ludwig, "A method of processing is disclosed that provides improved water retention and enhanced coloring and flavor, while preserving the meat and preventing bacterial contamination.", Abstract, where the process includes adding a material derived from a plant, i.e. an extract, juice or puree, such as "Cucumis sativus (cucumber)", and the concentration that contacts "meat product can be any suitable concentration", Paras. [0039];[0042];[0046]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Cavallini et al. to add the desired vegetable extract, as taught by Ludwig. The motivation for doing so would be to mimic the texture and taste of meat with one or more "flavoring ingredients", (Cavallini et al., Abstract; Paras. [0024];[0045]).

Regarding claim 82, Cavallini et al. in view of Orcutt et al. and Ludwig teach the method of claim 81 Cavallini et al. do not explicitly disclose wherein said Cucumis juice, puree, or extract is from a cucumber or a melon. However, Ludwig teaches wherein said Cucumis juice, puree, or extract is from a cucumber (Ludwig, "A method of processing is disclosed that provides improved water retention and enhanced coloring and flavor, while preserving the meat and preventing bacterial contamination.", Abstract, where the process includes adding a material derived from a plant, i.e. an extract, juice or puree, such as "Cucumis sativus (cucumber)", and the concentration that contacts "meat product can be any suitable concentration", Paras. [0039];[0042];[0046]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Cavallini et al. to add the desired vegetable extract, as taught by Ludwig. The motivation for doing so would be to mimic the texture and taste of meat with one or more "flavoring ingredients", (Cavallini et al., Abstract; Paras. [0024];[0045]).

Claims 85 and 86 lack an inventive step under PCT Article 33(3) as being obvious over Sandhill in view of Krammer et al. and Sinha et al.

Regarding claim 85, Sandhill teaches a food product or food replica product ("a meat substitute composition comprising a protein content, wherein one or more isolated and purified proteins accounts for 10% or more of said protein content by weight, wherein said meat substitute composition accurately mimics the taste, texture, or color of a meat product derived from animal sources", Paras. [0006];[00354]; Claims 163-166) comprising a heme-containing protein ("said one or more isolated and purified proteins are selected from the group consisting of leghemoglobin, non-symbiotic hemoglobin, hemoglobin, myoglobin", Para. [0018]). Sandhill does not explicitly disclose wherein said one or more lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, dihydro-5-pentyl-2(3 H)-furanone, dihydro-3-methylene-2,5-furandione, 1-pentoyllactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, gamma-octalactone, 5-hydroxymethyl dihydrofuran-2-one, 5-ethyl-2 (5H)-furanone, 5-acetyldihydro-2(3 H)furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1, 1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, delta-tetradecalactone, and dihydro-4-hydroxy-2 (3H)-furanone. However, Krammer et al. teach wherein said one or more lactones are selected from the group consisting of 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, gamma-octalactone, and delta-tetradecalactone (Krammer et al., the Group 2 flavoring additives comprise "milk-like and creamy flavors are saturated and unsaturated .delta.- and .gamma.-lactone having 6 to 14 carbon atoms. Preferred compounds of this group are delta.-tetradecalactone ... gamma.-octalactone ... compounds having a caramel-like flavor. Preferred compounds are ... 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone.", Para. [0031]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Sandhill to add the desired flavoring, as taught by Krammer et al. The motivation for doing so would be to mimic "the characteristic flavor and fragrance components" of meat, (Sandhill, Paras. [00317]; [00362]-[00376]). Sandhill does not explicitly disclose one or more lactones at a concentration of 10-3 to 10-11 of said food product. However, Sinha et al. teach one or more lactones at a concentration of 10-3 to 10-11 of said food product (Sinha et al., flavoring food products with lactones, where lactones are added "in desired and acceptable amount varying from ppm to mg based on the type of products", Paras. [0010];[0021];[0061];[0069]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Sandhill to add the desired amount of flavoring; as taught by Sinha et al. The motivation for doing so would be to mimic "the characteristic flavor and fragrance components" of meat, (Sandhill, Paras. [00317];[00362]-[00376]).

Regarding claim 86, Sandhill in view of Krammer et al. and Sinha et al. teach the method of claim 85, Sandhill does not explicitly disclose wherein said one or more lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, gamma-octalactone, and delta-tetradecalactone. However, Krammer et al. teach wherein said one or more lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, gamma-octalactone, and delta-tetradecalactone (Krammer et al., the Group 2 flavoring additives comprise "milk-like and creamy flavors are saturated and unsaturated .delta.- and .gamma.-lactone having 6 to 14 carbon atoms. Preferred compounds of this group are delta.-tetradecalactone ... gamma.-octalactone ... compounds having a caramel-like flavor. Preferred compounds are ... 5-ethyl-4-hydroxy-2-methyl-3 (2H)-furanone.", Para. [0031]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Sandhill to add the desired flavoring, as taught by Krammer et al. The motivation for doing so would be to mimic "the characteristic flavor and fragrance components" of meat, (Sandhill, Paras. [00317];[00362]-[00376]). Lyrical does not explicitly disclose butyrolactone. However, Sinha et al. teach butyrolactone (Sinha et al., flavoring food products with lactones, such as butyrolactone, Paras. [0010];[0021]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Sandhill to add the desired flavoring, as taught by Sinha et al. The motivation for doing so would be to mimic "the characteristic flavor and fragrance components" of meat, (Sandhill, Paras. [00317];[00362]-[00376]).

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Continuation of:

Claims 91 and 92 lack an inventive step under PCT Article 33(3) as being obvious over Sandhill in view of Orcutt et al. and Ludwig.

Regarding claim 91, Sandhill in view of Orcutt et al. teach the product of claim 90, Sandhill does not explicitly disclose wherein said vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract is a Cucumis juice, puree, or extract. However, Ludwig teaches wherein said vegetable juice, vegetable puree, vegetable extract is a Cucumis juice, puree, or extract (Ludwig, "A method of processing is disclosed that provides improved water retention and enhanced coloring and flavor, while preserving the meat and preventing bacterial contamination.", Abstract, where the process includes adding a material derived from a plant, i.e. an extract, juice or puree, such as "Cucumis sativus (cucumber)", and the concentration that contacts "meat product can be any suitable concentration", Paras. [0039];[0042];[0046]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Sandhill to add the desired vegetable extract, as taught by Ludwig. The motivation for doing so would be to mimic "the characteristic flavor and fragrance components" of meat, (Sandhill, Paras. [00317];[00362]-[00376]).

Regarding claim 92, Sandhill in view of Orcutt et al. and Ludwig teach the product of claim 91, Sandhill does not explicitly disclose wherein said Cucumis juice, puree, or extract is from a cucumber or a melon. However, Ludwig teaches wherein said Cucumis juice, puree, or extract is from a cucumber (Ludwig, "A method of processing is disclosed that provides improved water retention and enhanced coloring and flavor, while preserving the meat and preventing bacterial contamination.", Abstract, where the process includes adding a material derived from a plant, i.e. an extract, juice or puree, such as "Cucumis sativus (cucumber)", and the concentration that contacts "meat product can be any suitable concentration", Paras. [0039];[0042];[0046]). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Sandhill to add the desired vegetable extract, as taught by Ludwig. The motivation for doing so would be to mimic "the characteristic flavor and fragrance components" of meat, (Sandhill, Paras. [00317];[00362]-[00376]).

Claims 1-3, 18-36, 75-83, 85-94, 97-104, and 110-113 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

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Date of Search: 26 May 2015

Search History:

Limited Classification Search

The Patent Analyst performed a limited classification search within the following US, IPC, CPC, ECLA, or F-Term classification areas:

IPC (8) Class/Subclass(es): A23J 3/14, 3/22 (2015.01)

CPC Class/Subclass(es): A23J 3/14, 3/227 (2015.05)

U.S. Class/Subclass(es): 426/573, 574

See Global Search Results.

Global Patent Literature Text Search

The Patent Analyst performed the following global text search, which was not limited by classification but may or may not have been limited by other criteria:

QUESTAL ORBIT:

Search Step	Result(s)	Query	Base
9	2	(PROTEIN AND MEAT AND REPLICA AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM	FAMPAT
8	739	(PROTEIN AND IRON AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM	FAMPAT
7	0	(PROTEIN AND IRON AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM AND (426574)/PCL	FAMPAT
6	0	(PROTEIN AND IRON AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM AND (426573)/PCL	FAMPAT
5	2	(PROTEIN AND IRON AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM AND (A23J-003/22)/IPC	FAMPAT
4	2	(PROTEIN AND IRON AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM AND (A23J-003/14)/IPC	FAMPAT
3	2	(PROTEIN AND IRON AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM AND (A23J-003/227)/CPC	FAMPAT
2	0	(PROTEIN AND HEME AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM AND (A23J-003/227)/CPC	FAMPAT
1	0	(PROTEIN AND HEME AND PLANT)/TI/AB/IW/OBJ/ADB/ICLM AND (A23J-003/14)/CPC	FAMPAT

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Google Patents: (<https://www.google.com/?tbn=pts>)

No. of Hits	Text String
1	2014/0127358
1	2014/0193547
24	"meat dough" isolated plant protein
7	"meat replica" isolated plant protein
27	cucumis juice cucumber food flavor meat vegetarian

Computer Accessed Text Databases Searched

The Patent Analyst searched the following computer accessed text databases:

Public AppFT and PatFT:

No. of Hits	Text String	Other Limiting Criteria
3	IN/"varadan ranjani"	Patent Apps
2	IN/"solomatin sergey"	Patent Apps
0	IN/"holtz schietinger celeste"	Patent Apps
0	IN/"cohn elysia"	Patent Apps
0	IN/"klapholz brown ariel"	Patent Apps
0	IN/"shiu jennifer"	Patent Apps
69	IN/"kale aniket"	Patent Apps
0	IN/"karr jessica"	Patent Apps
0	IN/"fraser rachel"	Patent Apps
0	AN/"impossible foods"	Patent Apps
1	AN/"impossible foods"	Patents
1	IN/"varadan ranjani"	Patents
56	IN/"kale aniket"	Patents

Google Scholar (<http://scholar.google.com/>)

No. of Hits	Text String
1	"meat replica" isolated plant protein
>1,000 First 50 Searched	cucumis juice cucumber food flavor meat vegetarian

Date search was completed: 26 May 2015

YLK/BT

Electronic Acknowledgement Receipt

EFS ID:	30679487
Application Number:	14796970
International Application Number:	
Confirmation Number:	8501
Title of Invention:	METHODS AND COMPOSITIONS FOR CONSUMABLES
First Named Inventor/Applicant Name:	Marija Vrljic
Customer Number:	26191
Filer:	M. Angela Parsons/Shannon Glaeser
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0010001
Receipt Date:	17-OCT-2017
Filing Date:	10-JUL-2015
Time Stamp:	15:28:59
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	69465	no	1
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Warnings:

Information:

This is not an USPTO supplied IDS fillable form

2	Information Disclosure Statement (IDS) Form (SB08)	1449.pdf	136449	no	3
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Warnings:**Information:**

This is not an USPTO supplied IDS fillable form

3	Foreign Reference	CN101156632.pdf	582162	no	8
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Warnings:**Information:**

4	Foreign Reference	CN101489422.pdf	3234714	no	61
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Warnings:**Information:**

5	Foreign Reference	CN101861895.pdf	838339	no	13
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Warnings:**Information:**

6	Foreign Reference	CN101897418.pdf	1157661	no	17
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Warnings:**Information:**

7	Foreign Reference	CN102440302.pdf	1244855	no	13
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Warnings:**Information:**

8	Foreign Reference	CN102578544.pdf	616731	no	8
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Warnings:

Case No. 1:20-cv-00727
U.S. Patent No. 10,039,300

Information:					
9	Foreign Reference	CN102835460.pdf	833806	no	16
			e910188f4c48d12b948fee2f60fe4b36fac4fe910188f4c48d12b948		

Case No.: IPR2023-00203
U.S. Patent No. 10,039,300

16	Non Patent Literature	AU_OA_20122281064_2016_01_25.pdf	2298422	no	5
			f5c940c08ccabd54eb7e4a076bd348e01b70000b		
Warnings:					
Information:					
17	Non Patent Literature	AU_OA_2012281069_2015_09_25.pdf	2474518	no	5
			745e3ac4e002a793e2ea08e9d393fd0a7ecf1342		
Warnings:					
Information:					
18	Non Patent Literature	Field_1980.pdf	437363	no	4
			38a044b22d825ad843e8c3936c7bc16a333fb0e1		
Warnings:					
Information:					
19	Non Patent Literature	Griffths_1934.pdf	252870	no	5
			076f872188691beaa794381aff2b7b3e799034d7		
Warnings:					
Information:					
20	Non Patent Literature	IPRP_PCTUS2012046560_2014_01_23_.pdf	402189	no	9
			f67483227baa2761bac16801bf5b27fa3928ceae		
Warnings:					
Information:					
21	Non Patent Literature	IPRP_PCTUS2012046552_2014_01_23.pdf	526057	no	9
			55c85b7cc66158bf86b269334a00761e602ea84f		
Warnings:					
Information:					
22	Non Patent Literature	IPRP_PCTUS201411347_2015_07_14.pdf	584870	no	9
			4dc17b21b6c093dfcb04536b144eaa41e3c40b09		
Warnings:					
Information:					

Case No.: IPR2023-0020

U.S. Patent No. 10,039,306

23	Non Patent Literature	IPRP_PCTUS201411362_2015_07_23.pdf	490028 a56c470c168b211577b6cf6cf2e500ce10d6507e	no	9
Warnings:					
Information:					
24	Non Patent Literature	ISR_PCTUS2012046560_2012_12_14.pdf	590243 a5b4450def1f4c9242d264d7a3ade91697209bfb	no	11
Warnings:					
Information:					
25	Non Patent Literature	ISR_PCTUS201246552_2012_11_19.pdf	702705 45e3f607586fe958a37e2be0b218b463b7a3c467	no	12
Warnings:					
Information:					
26	Non Patent Literature	ISRWO_PCTUS201411347_2014_07_03.pdf	900416 04b76e1ca2bb4da1e757ee853ae63aa8a760aeda	no	14
Warnings:					
Information:					
27	Non Patent Literature	ISRWO_PCTUS201411362_2014_06_13.pdf	708737 10c89227b10ab501909a5f16cca92801a59ff4e	no	12
Warnings:					
Information:					
28	Non Patent Literature	ISRWO_PCTUS2015017147_2015_05_01.pdf	1512940 5465827ac53f46f2f103335791b24f8911142157	no	15
Warnings:					
Information:					
29	Non Patent Literature	ISR_PCTUS2015023679_2015_08_28.pdf	1959616 bb383ccd0c0c4af7ea9fbfdd71363aea8d8dc2b6	no	26
Warnings:					
Information:					

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30	Non Patent Literature	Koutsidis_2008.pdf	698729	no	7
			b8563e9471043f89810c329cdad438b4c0e c7ad3		
Warnings:					
Information:					
31	Non Patent Literature	Naike_1996.pdf	3415330	no	17
			415ed32dd6b259ad6086db6457f79c03c79 a1c8d		
Warnings:					
Information:					
32	Non Patent Literature	CN_OA_2014800143499_2017_07_24.pdf	2825284	no	52
			a5c4197df8a8003ac8aa00256823a3d75d6 91236		
Warnings:					
Information:					
33	Non Patent Literature	CN_OA_2012800417131_2017_07_13.pdf	6746604	no	28
			f1fcc718b7503f56e6b768db5173a41855a0 80b3		
Warnings:					
Information:					
34	Non Patent Literature	CN_OA_2014800137784_2017_08_09.pdf	1251009	no	24
			0d9c22624f502a2c083faa815b03a9f37f0c0 897		
Warnings:					
Information:					
35	Non Patent Literature	Shimbayashi.pdf	364900	no	8
			bd10da91c70adeb7baaa295156afda49fa7 8c5c7		
Warnings:					
Information:					
36	Non Patent Literature	Tong.pdf	610795	no	10
			5d161e835d37686ee2805cea776ebc399a2 72395		
Warnings:					
Information:					

37	Non Patent Literature	Xiaoling.pdf	266416	no	10
			f3f7aabb1267df67a8cfbcae60c5f8e6c00e227		

Warnings:**Information:**

Total Files Size (in bytes):	60003323
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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.

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**Request
for
Continued Examination (RCE)
Transmittal**Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	14/796,970
Filing Date	July 10, 2015
First Named Inventor	Marija Vrljic
Art Unit	1791
Examiner Name	Elizabeth A. Gwartney
Attorney Docket Number	38767-0010001

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. **Submission required under 37 CFR 1.114** Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

- a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

ii. ☐ Other _____

- b. ☒ Enclosed

i. ☒ Amendment/Reply

iii. ☒ Information Disclosure Statement (IDS)

ii. ☒ Affidavit(s)/ Declaration(s)

iv. ☐ Other _____

2. **Miscellaneous**

Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a

- a. ☐ period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

- b. ☐ Other _____

3. **Fees**

The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

The Director is hereby authorized to charge the following fees any underpayment of fees or credit any overpayments to

- a. ☒ Deposit Account No. 06-1050

i. ☒ RCE fee required under 37 CFR 1.17(e)

ii. ☒ Extension of time fee (37 CFR 1.136 and 1.17)

iii. ☒ Other any deficiencies

- b. ☐ Check in the amount of \$ _____ enclosed

- c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.**SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED**

Signature	/M. Angela Parsons/	Date	/October 17, 2017/
Name (Print/Type)	M. Angela Parsons, Ph.D.	Registration No.	44,282

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature		Date	
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This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Marija Vrljic	Art Unit :	1791
Serial No. :	14/796,970	Examiner :	Elizabeth A. Gwartney
Filed :	July 10, 2015	Conf. No. :	8501

Title : METHODS AND COMPOSITIONS FOR CONSUMABLES

MAIL STOP AF

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

RESPONSE TO OFFICE ACTION MAILED APRIL 17, 2017

In response to the Office Action mailed April 17, 2017, please consider the following reply.

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Serial No. : 14/796,970
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Attorney's Docket No.: 38767-0010001 / M2CON-
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Amendments to the Claims:

Please amend claims 1, 5, 7, 12, 32, 37, 43, 45, 47 and 51 and add new claims 56-58 as follows. Please also cancel claims 53-55 without prejudice to continued prosecution. The claims and their status are shown below.

1. (Currently Amended) A method for imparting a beef-like aroma ~~flavor~~ to a meat replica matrix consumable product, wherein the ~~consumable product~~ comprises a meat replica matrix ~~[[that]]~~ contains no animal products and comprises one or more plant proteins, a sugar selected from glucose, ribose, sucrose, fructose, xylose, maltodextrin, and combinations thereof, and at least one sulfur compound selected from methionine, cysteine and thiamine, the method comprising

adding incorporating into the meat replica matrix of the consumable product 0.01%-5% (by weight of the meat replica matrix consumable) of ~~an isolated~~ a heme-containing protein to the meat replica matrix,

wherein, ~~after~~ upon cooking of the meat replica matrix, at least two volatile compounds are generated that are associated with a beef-like aroma, thereby imparting a beef-like aroma ~~flavor is imparted to the meat replica matrix consumable product.~~

2. (Original) The method of claim 1, wherein the heme-containing protein has an amino acid sequence with at least 70% homology to the amino acid sequence set forth in SEQ ID NO: 1.

3. (Original) The method of claim 1, wherein the heme-containing protein has a UV-VIS spectrum that is substantially similar to that of myoglobin from an animal.

4. (Original) The method of claim 1, wherein the heme-containing protein is selected from the group consisting of an androglobin, a cytoglobin, a globin E, a globin X, a globin Y, a

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hemoglobin, a leghemoglobin, a flavohemoglobin, Hell's gate globin I, a myoglobin, an erythrocrucorin, a beta hemoglobin, an alpha hemoglobin, a protoglobin, a cyanoglobin, a cytoglobin, a histoglobin, a neuroglobins, a chlorocruorin, a truncated hemoglobin, a truncated 2/2 globin, a hemoglobin 3, a cytochrome, and a peroxidase.

5. (Currently Amended) The method of claim 1, comprising incorporating into the meat replica matrix ~~of the consumable product~~ 0.4%-1% (by weight of the meat replica matrix consumable) of the ~~isolated~~ heme-containing protein.

6. (Canceled)

7. (Currently Amended) A method for making a meat replica matrix composition having a beef-like ~~flavor and~~ aroma, wherein the ~~meat replica composition comprises a~~ meat replica matrix ~~[[that]]~~ contains no animal products and comprises one or more plant proteins, a sugar selected from glucose, ribose, sucrose, fructose, xylose, maltodextrin, and combinations thereof, and at least one sulfur compound selected from methionine, cysteine and thiamine, the method comprising obtaining ~~a an isolated~~ heme-containing protein; and incorporating 0.01%-5% (by weight of the meat replica matrix consumable) of the ~~isolated~~ heme-containing protein into the meat replica matrix ~~of the meat replica composition~~, wherein, upon cooking of the meat replica matrix, at least two volatile compounds are generated that are associated with a beef-like aroma.

8. (Original) The method of claim 7, wherein the heme-containing protein has an amino acid sequence with at least 70% homology to the amino acid sequence set forth in SEQ ID NO: 1.

9. (Original) The method of claim 7, wherein the heme-containing protein has a UV-VIS spectrum that is substantially similar to that of myoglobin from an animal.

10. (Canceled)

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11. (Original) The method of claim 7, wherein the heme-containing protein is selected from the group consisting of an androglobin, a cytoglobin, a globin E, a globin X, a globin Y, a hemoglobin, a leghemoglobin, a flavohemoglobin, Hell's gate globin I, a myoglobin, an erythrocrucorin, a beta hemoglobin, an alpha hemoglobin, a protoglobin, a cyanoglobin, a cytoglobin, a histoglobin, a neuroglobins, a chlorocruorin, a truncated hemoglobin, a truncated 2/2 globin, a hemoglobin 3, a cytochrome, and a peroxidase.

12. (Currently Amended) The method of claim 7, comprising incorporating 0.4-1% (by weight of the meat replica matrix composition) of the ~~isolated~~ heme-containing protein into the meat replica matrix ~~of to the meat replica composition~~.

13 - 31. (Canceled)

32. (Currently Amended) The method of claim 1, wherein the ~~isolated~~ heme-containing protein is purified.

33. (Previously Presented) The method of claim 1, wherein at least one of the one or more plant proteins is isolated.

34. (Previously Presented) The method of claim 33, wherein at least one of the one or more isolated plant proteins is purified.

35. (Previously Presented) The method of claim 1, wherein the one or more plant proteins are selected from the group consisting of rubisco, pea proteins, lentil proteins, and other legume proteins.

36. (Previously Presented) The method of claim 35, wherein the pea proteins comprise pea albumin proteins.

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37. (Currently Amended) The method of claim 1, wherein the one or more plant proteins are present in the meat replica matrix consumable product in an amount of between 1% and 30% (by weight of the meat replica matrix consumable).

38. (Previously Presented) The method of claim 1, wherein the one or more plant proteins has a solubility in an aqueous solution of at least 25 g/L at a temperature of between 2°C and 32°C, wherein the aqueous solution has a pH between 3 and 8 and a sodium chloride content of 0 to 300 mM.

39. (Previously Presented) The method of claim 7, wherein at least one of the one or more plant proteins is isolated.

40. (Previously Presented) The method of claim 39, wherein at least one of the one or more isolated plant proteins is purified.

41. (Previously Presented) The method of claim 7, wherein the one or more plant proteins are selected from the group consisting of rubisco, pea proteins, lentil proteins, and other legume proteins.

42. (Previously Presented) The method of claim 41, wherein the pea proteins comprise pea albumin proteins.

43. (Currently Amended) The method of claim 7, wherein the one or more plant proteins are present in the meat replica matrix composition in an amount of between 1% and 30% (by weight of the meat replica matrix composition).

44. (Canceled)

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45. (Currently Amended) The method of claim 7, wherein the ~~isolated~~ heme-containing protein is purified.

46. (Previously Presented) The method of claim 7, wherein the one or more plant proteins has a solubility in an aqueous solution of at least 25 g/L at a temperature of between 2°C and 32°C, wherein the aqueous solution has a pH between 3 and 8 and a sodium chloride content of 0 to 300 mM.

47. (Currently Amended) A method for making a meat replica matrix composition having a beef-like ~~flavor and~~ aroma, wherein the ~~meat replica composition comprises a meat replica matrix~~ [[that]] contains no animal products and comprises one or more plant proteins, a sugar selected from glucose, ribose, sucrose, fructose, xylose, maltodextrin, and combinations thereof, and at least one sulfur compound selected from methionine, cysteine and thiamine, the method comprising incorporating 0.01%-5% (by weight of the meat replica matrix composition) of a ~~an isolated~~ heme-containing protein into the meat replica matrix of the meat replica composition, wherein, upon cooking of the meat replica matrix, at least two volatile compounds are generated that are associated with a beef-like aroma.

48. (Previously Presented) The method of claim 47, wherein the heme-containing protein has an amino acid sequence with at least 70% homology to the amino acid sequence set forth in SEQ ID NO: 1.

49. (Previously Presented) The method of claim 47, wherein the heme-containing protein has a UV-VIS spectrum that is substantially similar to that of myoglobin from an animal.

50. (Previously Presented) The method of claim 47, wherein the heme-containing protein is selected from the group consisting of an androglobin, a cytoglobin, a globin E, a globin X, a globin Y, a hemoglobin, a leghemoglobin, a flavohemoglobin, Hell's gate globin I, a myoglobin, an erythrocrucorin, a beta hemoglobin, an alpha hemoglobin, a protoglobin, a

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cyanoglobin, a cytoglobin, a histoglobin, a neuroglobins, a chlorocruorin, a truncated hemoglobin, a truncated 2/2 globin, a hemoglobin 3, a cytochrome, and a peroxidase.

51. (Currently Amended) The method of claim 47, comprising incorporating 0.4%-1% (by weight of the meat replica matrix composition) of the ~~isolated~~ heme-containing protein into the meat replica matrix ~~of the meat replica composition~~.

52. (Previously Presented) The method of claim 47, wherein the one or more plant proteins are selected from the group consisting of rubisco, pea proteins, lentil proteins, and other legume proteins.

53. (Canceled) ~~The method of claim 1, further comprising incorporating into the meat replica matrix of the consumable product, a sugar, sugar alcohol, sugar acid or sugar derivative; and at least one sulfur compound.~~

54. (Canceled) ~~The method of claim 53, wherein the sugar, sugar alcohol, sugar acid or sugar derivative is selected from the group consisting of glucose, fructose, galactose, mannose, arabinose, xylose, ribose, sucrose, lactose, melibiose, trehalose, cellobiose, maltose, arabitol, mannitol, dulcitol, sorbitol, galacturonate, glucuronate, gluconate and N-acetylglucosamine, and mixtures of two or more thereof.~~

55. (Canceled) ~~The method of claim 54, wherein the at least one sulfur compound is selected from the group consisting of cysteine, thiamine and methionine, and mixtures of two or more thereof.~~

56. (New) The method of claim 1, wherein the meat replica matrix is comprised within a consumable product, wherein the consumable product is selected from taco filling, a casserole, a sauce, a topping, soup, stew, and a loaf, wherein, upon cooking of the consumable product, the at least two volatile compounds are generated in substantially higher amounts than upon cooking of the consumable product lacking the meat replica matrix.

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57. (New) The method of claim 7, wherein the meat replica matrix is comprised within a meat replica composition, wherein the composition is selected from taco filling, a casserole, a sauce, a topping, soup, stew, and a loaf, wherein, upon cooking of the composition, the at least two volatile compounds are generated in substantially higher amounts than upon cooking of the composition lacking the meat replica matrix.

58. (New) The method of claim 47, wherein the meat replica matrix is comprised within a meat replica composition, wherein the composition is selected from taco filling, a casserole, a sauce, a topping, soup, stew, and a loaf, wherein, upon cooking of the composition, the at least two volatile compounds are generated in substantially higher amounts than upon cooking of the composition lacking the meat replica matrix.

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REMARKS

Applicants respectfully request entry of the amendments and remarks submitted herein. Claims 1, 5, 7, 12, 32, 37, 43, 45, 47 and 51 have been amended herein, and claims 53-55 have been canceled without prejudice to continued prosecution. In addition, new claims 56-58 have been added herein. Support for new claims 56-58 can be found, for example, at paragraph [0469] relative to the published application (US 2015/0305390).

Claims 1-5, 7-9, 11, 12, 32-43, 45-52 and 56-58 are currently pending. Reconsideration of the pending application is respectfully requested.

The Claim Objections

Claim 55 is objected to because it contains two punctuation marks at the end of the sentence. The Examiner is requiring appropriate correction.

For reasons unrelated to this rejection, claim 55 has been canceled herein without prejudice to continued prosecution. Accordingly, the objection to claim 55 is moot.

The Claim Rejections - 35 U.S.C. § 112

Claims 53-55 are rejected under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. According to the Examiner:

there is support for claiming that the meat replica may comprise sugars including monosaccharides, sugar alcohols, oligosaccharides, polysaccharides, sugar acids and sugar derivatives and the meat replica may comprises flavor precursor mix with specific combinations of sugars, amino acids and salts. There is no support for claiming incorporating, into the meat replica matrix of the consumable product, any combination of sugars and sulfur compounds. Moreover, while there is support for claiming sulfur compounds including methionine, cysteine and thiamine, when used in combination as part of a flavor precursor mix, there is no support for claiming any sulfur compound.

OA at page 3 (citations omitted; emphasis in original).

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Without acquiescing to the Examiner's rejection, Applicants have amended independent claims 1, 7 and 47 to require that the meat replica matrix contain a sugar selected from glucose, ribose, sucrose, fructose, xylose, maltodextrin, and combinations thereof and at least one sulfur compound selected from methionine, cysteine and thiamine. The sugars recited in independent claims 1, 7 and 47 find support in either or both Tables 5 and 8 of the present application, and each of the sulfur compounds recited in independent claims 1, 7 and 47 find support in both Tables 5 and 8. Therefore, the combinations of sugars and sulfur compounds now recited in independent claims 1, 7 and 47 are fully supported by the disclosure in at least Tables 5 and 8.

Based on the Examiner's comments, it is Applicants' understanding that independent claims 1, 7 and 47 as amended meet the written description requirement. In view of the amendments and remarks herein, Applicants respectfully request that the rejection of the pending claims under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AIA), first paragraph, be withdrawn.

The Claim Rejections - 35 U.S.C. § 103

Claims 1-5, 7-9, 11, 12, 32-43 and 45-54 are rejected under pre-AIA 35 U.S.C. § 103(a) as being unpatentable over Hsieh et al. (US 2012/0093994) in view of Proulx et al. (2006, "Iron Bioavailability of Hemoglobin from Soy Root Nodules Using a Caco-2 Cell Culture Model" - J. Agric. Food Chem., p. 1518-1522). According to the Examiner

Given Proulx et al. teach that iron deficiency is a major nutritional problem and that fortification of foods with iron has been a successful strategy from improving iron content, since Proulx et al. teach that bioavailability is higher from heme iron sources including, leghemoglobin from soy root nodules, it would have been obvious to one of ordinary skill in the art to have fortified the structured plant protein product of Hsieh et al. comprising no animal protein, with leghemoglobin from soy root nodules, to obtain a product with improved nutritional status, i.e. bioavailable source of iron.

OA at page 6. This rejection is respectfully traversed as follows with respect to the pending claims.

As discussed previously, a *prima facie* case of obviousness is established when three basic criteria have been met. First, there must be some suggestion or motivation, either in the prior art cited by the Examiner or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or combine the references being relied upon to reject the claimed

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invention. Second, there must be a reasonable expectation of success. Third, the prior art reference or combination of references must teach or suggest all of the requirements of the claimed invention. Thus, “one must have a motivation to combine accompanied by a reasonable expectation of achieving what is claimed.” (*Intelligent Biosystems, Inc. v. Illumina Ltd.*, page 12, Appeal No. 2015-1693 (Fed Cir 2016)).

The suggestion or motivation to modify the prior art reference or combine the prior art references being relied upon to reject the claimed invention must be found either in the prior art or in the knowledge generally available to one of ordinary skill in the art; it cannot be based on hindsight benefiting from the disclosure of the claimed invention. It is the Examiner's burden to demonstrate *both* that “a skilled artisan would have been motivated to combine the teachings of the prior art references to achieve the claimed invention, *and* that the skilled artisan would have had a reasonable expectation of success in doing so.” *Intelligent Biosystems, Inc. v. Illumina Ltd.*, page 13 (citations omitted; emphasis added).

Applicants respectfully submit that the present claims are not obvious. As set forth herein, a skilled artisan would not have been motivated, let alone have had a reasonable expectation of success, to modify the meat analog of Hsieh with the leghemoglobin of Proulx to provide a “product with improved nutritional status” (OA at page 6). Notably, Hsieh is silent with respect to the desirability of including an iron supplement in a meat analog or meat alternative and regarding the issue of iron bioavailability in a meat analog or meat alternative, despite the fact that Hsieh discusses various other nutritional aspects of meat and meat analogs. *See*, for example, paragraphs [0005], [0033], [0062], [0088], [0092] in Hsieh.

In the present case, the cited references do not provide the requisite motivation to combine, nor do they provide a reasonable expectation of success in making the proposed combination. Thus, a *prima facie* case of obviousness cannot be established by the instant combination of the references.

The Unpredictable, if Not Likely Negative, Effect of the Heme Protein on the Organoleptic Properties of a Meat Replica

A person having ordinary skill in the art would have not had a reasonable expectation of success that inclusion of leghemoglobin in the meat analogs and alternatives of the cited prior art

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would have resulted in meat replicas having acceptable organoleptic properties. The cited Proulx reference is silent regarding any predictable effect of leghemoglobin on the organoleptic properties of any given food product, let alone that of a meat replica matrix as claimed herein. The Proulx Thesis discloses that “[i]ron compounds should be added to the level *up to which they do not change the organoleptic qualities* of the food, usually between 20 ppm and 40 ppm” (Proulx, 2007, “Diversified strategies for improving iron bioavailability of maize,” Retrospective Theses and Dissertations, Iowa State University, Paper 15852 (“the Proulx thesis”); page 29; emphasis added). In addition, the Proulx Thesis states that “[f]ortification [with] iron has *a potential negative effect on organoleptic quality* of maize products, in that soluble forms of iron can cause *oxidative rancidity*” (see page 20; emphasis added). Accordingly, given such statements, a skilled artisan would not have been motivated to include leghemoglobin in a ground beef-like food product with any reasonable expectation of success that the organoleptic properties of the ground beef-like food product would not be adversely affected.

Indeed, a person having ordinary skill in the art would understand that heme iron has in fact been associated in the art with oxidation and off-flavors in meat. For example, Calkins et al. discloses that “[o]ff-flavor intensity [] was significantly related to pH and heme iron” ((2007, “A fresh look at meat flavor,” Meat Sci., 77:63-80, *see* the paragraph bridging pages 70-71). Yancey et al. discloses that their results “suggest that livery flavor may be related to total iron content in the *gluteus medius*, and to myoglobin concentration in the *infraspinatus*, *psoas major*, and *gluteus medius* muscles” (2006, “Effects of total iron, myoglobin, hemoglobin, and lipid oxidation of uncooked muscles on livery flavor development and volatiles of cooked beef steaks,” Meat Sci., 73:680-6) *see* page 686). And Brewer discloses that “[h]eme iron facilitates lipid oxidation producing peroxides which are enzymatically decomposed by catalase producing a variety of compounds, some of which contribute off-flavors” ((2006, “The Chemistry of Beef Flavor – Executive Summary,” National Cattlemen’s Beef Association, #12814), *see* page 10).

Contrary to the Examiner’s assertion, a skilled artisan likely would have avoided including heme in the claimed meat replica matrix. As evidence of this, Applicants respectfully refer the Examiner to the enclosed Declaration under 37 CFR § 1.132 by Dr. Keith R. Cadwallader (“the Cadwallader Declaration”; Exhibit 2), paragraph 11, which states in relevant part:

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This is a significant discovery since a person having ordinary skill in the art would not have anticipated that the inclusion of a heme protein would have any beneficial effect on flavor. In fact, iron, and specifically heme iron, has been most often associated with causing deleterious off-odor consequences in foods and especially meat products (Calkins, 2007).

Given the above understanding in the art related to the likely negative effects of heme-containing proteins on organoleptic properties, including oxidation, rancidity, and the production of off-flavors, a person of ordinary skill in the art would not have a reasonable expectation of success that including a heme-iron-containing protein in a ground beef-like food product would result in a ground beef-like food product having an acceptable organoleptic profile.

Secondary Considerations - The Actual Effect of a Heme Protein on the Organoleptic Properties of a Meat Replica Matrix is Unexpected and Surprising

The overwhelming evidence of objective indicia demonstrate that the instant claims were not obvious. Objective indicia “can be the most probative evidence of nonobviousness in the record, and enables the court to avert the trap of hindsight.” *Leo Pharm. Prods. v. Rea*, 726 F.3d 1346, 1358 (Fed. Cir. 2013). The Federal Circuit has “repeatedly held that . . . objective evidence of secondary considerations . . . must be considered before determining whether the claimed invention would have been obvious.” *Apple, Inc. v. ITC*, 725 F.3d 1356, 1365 (Fed. Cir. 2013). “[O]bjective evidence of nonobviousness includes factors such as commercial success, long-felt but unresolved need, failure of others, copying, and unexpected results.” *ABT Sys., LLC v. Emerson Elec. Co.*, 797 F.3d 1350, 1361 (Fed. Cir. 2015) (quotation marks and citations omitted). It also includes “acceptance by others[] and [] initial skepticism.” *Kinetic Concepts, Inc. v. Smith & Nephew, Inc.*, 688 F.3d 1342, 1367 (Fed. Cir. 2012).

As indicated in the present application:

The current state of the art for meat substitute compositions involves the extrusion of soy/grain mixture, resulting in products which largely fail to replicate the experience of cooking and eating meat. Furthermore, as the products must largely be sold pre-cooked, with artificial flavors and aromas built in, they fail to replicate aromas, flavors, and other key features associated with cooking meat.

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Page 2, lines 5-11, in the as-filed application. The present disclosure overcomes the organoleptic deficiencies of current meat analogs and meat alternatives and provides a consumable product (i.e., containing a meat replica matrix) that more accurately mimics the aroma of beef; see, e.g., Example 33 (page 131-134), which describes the production of beef flavors and aromas in replica burgers with the addition of heme and flavor precursors. See, also, page 112, lines 6-17 and the paragraph bridging pages 113-114.

The surprisingly and unexpectedly improved organoleptic properties, including, in particular, the beef-associated aroma profile of the claimed meat replica matrix, are supported by the attached Declaration under 37 CFR § 1.132 by Dr. Celeste Holz-Schietinger (the “Holz-Schietinger Declaration”; Exhibit 1). Specifically, Gas Chromatography-Mass Spectroscopy (GCMS) methods were used to evaluate the volatile compounds produced during cooking of three flavor reaction mixtures. As described in more detail in the Holz-Schietinger Declaration, the first flavor reaction mixture contained only Leghemoglobin (LegH-only); the second contained Ribose and Cysteine (Ribose + Cysteine); and the third contained Leghemoglobin with Ribose and Cysteine (LegH + Ribose + Cysteine). All flavor reaction mixtures were cooked and analyzed using GCMS as described in the Holz-Schietinger Declaration. Many different volatile compounds were produced, but the Holz-Schietinger Declaration focuses on those volatile compounds that have been associated in the art with a beef (or meat) aroma.

The Holz-Schietinger Declaration describes how the amount of each volatile compound was determined and explains the data presented in Exhibit B of the Holz-Schietinger Declaration. Significantly, as indicated in the Holz-Schietinger Declaration, there were **twenty-eight (28)** different volatile compounds that were produced in the Ribose + Cysteine sample *only when LegH was present*; these volatile compounds are indicated in Exhibit B as “Novel.”¹ As explained in more detail in the Holz-Schietinger Declaration, of the 28 novel volatile compounds whose generation can be attributed to the presence of the heme-containing protein, nine were sulfur (S)-containing heterocyclic compounds; six were oxygen (O)- and/or nitrogen (N)-

¹ “Novel” in the context of the present Response and Holz-Schietinger Declaration means “newly produced” only in the presence of LegH in the flavor reaction mixtures, and not that the compound itself is “novel,” i.e., never before disclosed or made in the patent sense.

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containing heterocyclic compounds; four were aldehydes; three were ketones; two were lactones; two were carboxylic acids; one was a sulfide and one was an alcohol.

Also as explained in the Holz-Schietinger Declaration, each of the 28 volatile compounds whose generation is attributable to the presence of the heme-containing protein have been associated in the art, directly or indirectly, with a beef (or meat) aroma. For example, 2-methyl-3-furanthiol (MFT) and its dimerized form bis(2-methyl-3-furyl)disulfide (MFT-MFT) are volatile compounds directly associated with beefy aroma in cooked meat (Tang et al., 2013). In addition, Applicants respectfully refer the Examiner to the Cadwallader Declaration, paragraph 15, which states:

Among the 28 compounds identified in [the Holz-Schietinger Declaration] experiments, two in particular are well-known to possess meaty sensory characteristics and to be important and potent odorants in cooked meat. Specifically, 2-methyl-3-furanthiol (MFT) and its dimerized form bis(2-methyl-3-furyl)disulfide (MFT-MFT) are important aroma compounds in cooked meat (Tang et al., 2013). Both compounds have extremely low odor detection thresholds of 0.7 parts-per-trillion (pptr) and 0.02 pptr, respectively (Belitz et al. 2006), and are usually only present in low abundance (pptr range) in cooked meat (Tang et al., 2013). Despite their low abundance in meat they are still among the most impactful aroma compounds because of their extremely low odor detection thresholds.

See also the Holz-Schietinger Declaration, paragraph 12, which states in relevant part:

[T]he presence of LegH in the flavor reaction mixture with Cysteine and Ribose resulted in the generation of two (2) novel volatile compounds upon heating: 2-methyl-3-furanthiol and bis(2-methyl-3-furyl)disulfide.

See also the Declaration under 37 CFR § 1.132 by Dr. Devin Grant Peterson (“the Peterson Declaration”; Exhibit 3), which states in relevant part:

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.

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(the Peterson Declaration, para. 21.)

As stated in the Cadwallader Declaration, both MFT and MFT-MFT have extremely low odor detection thresholds of 0.7 parts-per-trillion (pptr) and 0.02 pptr, respectively (Belitz et al. 2006), and are usually only present in low abundance (pptr range) in cooked meat (Tang et al., 2013). Despite their low abundance in meat they are still among the most impactful aroma compounds because of their extremely low odor detection thresholds.²

Also as indicated in the Holz-Schietinger Declaration, several volatile compounds were synergistically and dramatically increased in aqueous reaction mixtures of Ribose + Cysteine sample *only when LegH was present*. For example, the amount of 2-acetylthiazole increased **2,851-fold** in the presence of LegH and the amount of thiophene increased 89-fold in the presence of LegH. Again, as explained in the Holz-Schietinger Declaration, both 2-acetylthiazole and thiophene have been associated with beef (or meat) aroma.

Importantly, see also the Peterson Declaration, paragraphs 19-20, which states in relevant part:

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

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.

² See also Moon et al. (2006, Food Res. Int., 39:294-308): "2-Methyl-3-furanthiol, ... and bis(2-methyl-3-furyl) disulphide were identified as potent odourants with high aroma values from [cooked] beef."; "2-Methyl-3-furanthiol was identified in simulated beef flavour and confirmed to possess meaty flavour in the sniffing port of [gas chromatography-olfactometry] in this study"; and Song et al. (2012, J. Chromatog. A, 1254:115-24): "3-furanthiols are of special interest due to their key role in meat flavour. 2-Methyl-3-furanthiol (2-MF) and its disulphide (bis(2-methyl-3-furyl)disulphide) have been identified as the odour impact compounds of beef aromas in our previous research [citation omitted] and other model systems for preparing meat-like flavours, and two compounds gave various characteristic aromas described as "sweet meat", "beef broth" and "braised beef" [citation omitted]."

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In addition to the Holz-Schietinger Declaration, the present application discloses the production of beef aroma-associated volatile compounds in the presence of LegH and the consequent generation of beef flavors in the presence of LegH are contained within the application as filed. *See*, for instance, Example 16 (pages 111-114), Example 17 (pages 114-116), and Example 33 (pages 131-134).

Also enclosed herewith is a second Declaration under 37 CFR § 1.132 by Dr. Celeste Holz-Schietinger (the “Holz-Schietinger Heme Gradient Declaration”; Exhibit 4). The Holz-Schietinger Heme Gradient Declaration describes flavor reactions mixtures (1) containing only Leghemoglobin (LegH) at different concentrations (0.01%, 0.2%, 1 %, and 3%) (note 1 % =10 mg/mL); (2) containing a mixture representing the estimated concentrations of amino acids, sugars, and other small molecules in beef based on their values reported in literature as shown below (“the Precursor Mix”); and (3) and containing both Leghemoglobin at different concentrations (0.01 %, 0.05% 0.2%, 0.5%, 1 %, and 3%) and the Precursor Mix that have been cooked and then analyzed using GC-MS. Many different volatile compounds were produced, but the Holz-Schietinger Heme Gradient Declaration focuses on those volatile compounds that have been associated in the art with a beef (or meat) aroma. Significantly, described in the Holz-Schietinger Heme Gradient Declaration, there were at least two different volatile compounds that were produced in the Precursor Mixture sample only when LegH was present, i.e., volatile compounds whose generation is attributable to the presence of the heme-containing protein have been associated in the art, directly or indirectly, with a beef (or meat) aroma.

Also enclosed herewith is a third Declaration under 37 CFR § 1.132 by Dr. Celeste Holz-Schietinger (the “Holz-Schietinger Low Sulfur Declaration”; Exhibit 5). The Holz-Schietinger Low Sulfur Declaration describes flavor reactions mixtures (1) containing only Leghemoglobin (LegH); (2) containing ribose and cysteine (ribose + 1.5 mM cysteine); and (3) containing Leghemoglobin with ribose and cysteine (LegH + ribose + 1.5 mM cysteine) that have been cooked and then analyzed using GC-MS. The Holz-Schietinger Low Sulfur Declaration also describes flavor reactions mixtures (1) containing only Leghemoglobin (LegH); (2) containing ribose and thiamine (ribose + 1.5 mM thiamine); and (3) containing Leghemoglobin with ribose and thiamine (LegH + ribose + 1.5 mM thiamine) that have been cooked and then analyzed using

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GC-MS. Many different volatile compounds were produced, but the Holz-Schietinger Low Sulfur Declaration focuses on those volatile compounds that have been associated in the art with a beef (or meat) aroma. Significantly, described in the Holz-Schietinger Low Sulfur Declaration, there were at least two different volatile compounds that were produced in the ribose and cysteine sample, or the ribose and thiamine sample, only when LegH was present, i.e., volatile compounds whose generation is attributable to the presence of the heme-containing protein have been associated in the art, directly or indirectly, with a beef (or meat) aroma.

Additional Evidence of the Patentability of the Pending Claims

Free Amino Acids Containing Sulfur

Enclosed herewith is a Declaration under 37 CFR § 1.132 by Dr. S. Christopher Davis (“the Davis Declaration”; Exhibit 6). As indicated in the Davis Declaration, the amount of most of the sulfur-containing free amino acids in masa flour as employed in Proulx were below the limit of quantitation of the particular assay used (e.g., 0.00050% in the assay used by Danforth; see Paragraphs 18 - 26 in the Davis Declaration). In fact, cysteine is present in masa in an amount less than 0.04 mM (Paragraph 22 in the Davis Declaration), cysteic acid is present in masa in an amount less than 0.03 mM (Paragraph 23 in the Davis Declaration), disulfide bonded cysteine, also known as cystine, is present in masa in an amount less than 0.02 mM (Paragraph 24 in the Davis Declaration), methionine is present in masa in an amount less than 0.03 mM (Paragraph 25 in the Davis Declaration), and methionine sulfoxide is present in masa in an amount less than 0.03 mM (Paragraph 26 in the Davis Declaration). Accordingly, the masa flour used in the tortillas of Proulx contains, at best, no more than 0.15 mM of sulfur-containing free amino acids.

Heme-Iron-Containing Proteins

Enclosed herewith is a Declaration under 37 CFR § 1.132 by Dr. David J. Lipman (“the Lipman Declaration”; Exhibit 7). As explained in the Lipman Declaration, an alignment of 27 heme-containing proteins from a diverse range of organisms was performed. Despite adopting a highly conservative approach to align such sequences, heme-containing proteins are highly diverse and share very little sequence similarity. Paragraph 10 of the Lipman Declaration.

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The Lipman Declaration also refers to data presented in U.S. Application No. 15/398,479, in which the production of volatile compounds was evaluated, including those associated with a meaty aroma, during cooking of aqueous flavor reaction mixtures containing four distinct globin heme-containing proteins as well as reaction mixtures containing myoglobin free in solution or hemin (heme unbound to protein) coupled to a solid support (paragraphs 11 and 15 of the Lipman Declaration). According to the Lipman Declaration, the ability of four very distinct members of heme-containing proteins to generate meat-associated volatile aroma compounds upon heating, coupled with the experimental data demonstrating that even heme unbound to protein (i.e., hemin) shared such meat aroma and flavor production properties when heated in a reaction mixture, make it likely that these functional properties are not based on protein sequence similarity, but are instead based on the commonality among the proteins and hemin, namely, the inclusion of the heme co-factor (paragraph 18 of the Lipman Declaration).

Conclusion--Applicants' Claims are Non-Obviousness.

Applicants were the first to discover a method for imparting a beef-like flavor to a consumable product. As recited in the claims, the consumable product includes a meat replica matrix that contains no animal products and contains one or more plant proteins, a sugar, sugar alcohol, sugar acid or sugar derivative, and at least one sulfur compound selected from the group of methionine, cysteine and thiamine. A beef-like flavor is imparted to the consumable product by incorporating 0.01%-5% (by weight of the consumable) of a heme-containing protein into the meat replica matrix and cooking the consumable product.

As shown above, prior to the present application, a skilled artisan would not have had a reasonable motivation to add a heme-containing protein to a meat replica matrix that contained no animal products. To support a *prima facie* case, there must be a clear and explicit articulation of reasons why the claimed invention would have been obvious. *KSR International Co. v. Teleflex Inc.*, 550 U.S. 398, 418 (2007). Yet the Office can point to no reasonable guideposts or rationales based on the combination of Hsieh and Proulx for one of ordinary skill in the art to add a heme-containing protein to a meat analog. Indeed, the cited art cautions those skilled in the art against doing so. A “rejection on obviousness cannot be sustained with mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to

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support the legal conclusion of obviousness. *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006). *See KSR International Co. v. Teleflex Inc.*, 550 U.S. 398, 418 (2007) (quoting Federal Circuit statement with approval).

Moreover, as shown above, neither Hsieh nor Proulx provide any evidence that one of ordinary skill in the art would have a reasonable expectation of success that doing so would result in the production of at least two volatile compounds that have a beef-associated aroma. A showing that one could have combined known elements to arrive at something within the claims does not render a claimed invention obvious unless the results would have been predictable to one of ordinary skill in the art. “Although predictability is a touchstone of obviousness, the ‘predictable result’ discussed in *KSR* refers not only to the expectation that prior art elements are capable of being physically combined, but also that the combination **would have worked for its intended purpose.**” *Depuy Spine, Inc. v. Medtronic Sofamor Danek, Inc.*, 567 F.3d 1314, 1326 (Fed. Cir. 2009); see also *Senju Pharm. Co. v. Lupin Ltd.*, 780 F.3d 1337, 1356 (Fed. Cir. 2015) (same). Further, “[t]o the extent an art is unpredictable, as the chemical arts often are, *KSR*’s focus on these ‘identified, predictable solutions’ may present a difficult hurdle because potential solutions are less likely to be genuinely predictable.” *Eisai Co. v. Dr. Reddy’s Labs., Ltd.*, 533 F.3d 1353, 1359 (Fed. Cir. 2008); see also *Otsuka Pharm. Co., Ltd. v. Sandoz, Inc.*, 678 F.3d 1280, 1298 (Fed. Cir. 2012) (“As *KSR* makes clear, predictability is a vital consideration in the obviousness analysis.”).

The record now provides overwhelming evidence of objective indicia that demonstrate the instant claims were not obvious. In particular, the Declarations now of record set forth objective evidence that establishes the surprisingly and unexpectedly improved organoleptic properties, including, in particular, the aroma profile of the claimed ground beef-like food product.

As discussed above, a person of ordinary skill, based on the prior art, would not only have had no motivation to add such a heme-iron containing protein to a meat replica matrix, but also would have had no reasonable expectation of success in so doing, because he/she would have understood that such a heme-iron containing protein would likely adversely impact the organoleptic properties of the meat replica matrix. Further, the addition of a heme-iron-containing protein to a non-animal-based meat replica matrix provides several unexpected

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advantages including, for example, mimicking the taste, aroma, flavor, and color of animal meat in a raw state and a cooked state. Prior to the present application, a skilled artisan would have had no expectation that the addition of a heme-iron-containing protein to a non-animal-based meat replica matrix would result in these beneficial characteristics.

In view of the amendments and remarks herein, Applicants respectfully request that the rejection of the pending claims under 35 U.S.C. § 103 be withdrawn.

Request for Extension of Time (RCE) and Petition for Extension of Time

Applicants submit herewith a Request for Continued Examination (RCE), and a Petition for Extension of Time. Please extend the period of time for responding to the Office Action mailed April 17, 2017, for three months to and including October 17, 2017, as provided for under 37 CFR § 1.136. Please apply all required fees to Deposit Account No. 06-1050.

CONCLUSION

Applicants respectfully request that claims 1-5, 7-9, 11, 12, 32-43, 45-52 and 56-58 be allowed. If a telephone call to the undersigned would expedite prosecution, the Examiner is encouraged to do so.

Please apply any other charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: /October 17, 2017/

/M. Angela Parsons/

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Serial No. :	14/797,006	Examiner :	Elizabeth A.
Filed :	July 10, 2015		Gwartney
		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, Celeste Holz-Schietinger, Ph.D., hereby declare as follows:

1. I am currently a Principal Scientist at Impossible Foods in Redwood City, California. I have held this position since 2015, and I am responsible for leading the Flavor Department. In this role, I direct flavor technology research as well as flavor product integration. I began working for Impossible Foods in 2012 as a Scientist, and I was elevated to a Senior Scientist in 2013. While at Impossible Foods, I have been the lead chemist on the identification, characterization and manipulation of biological flavor catalysis and potentiation, including through the use of heme-containing proteins.
2. I received my Bachelors of Science (B.S.) degree in Molecular Biology and Evolution, with a minor in Chemistry, in 2005 from The Evergreen State College in Olympia, Washington, and I received my Doctor of Philosophy (Ph.D.) degree in Biochemistry from the University of California at Santa Barbara in 2012. I was an Enzymology Research Associate at the University of California at Santa Barbara from 2007 to 2012, and I also was a Biochemistry Visiting Associate at Stanford University in 2009.
3. A copy of my curriculum vitae is attached as **Exhibit A**.
4. I am a named inventor on the above-referenced patent application.
5. I have read U.S. Application No. 14/797,006 ("the '006 application") as well as the Office Action mailed June 6, 2016, in which the claims were rejected as being anticipated by Proulx et al. (2006, *J. Agric. Food Chem.*, 54:1518-22) and obvious over Hseih et al. (US 2012/0093994) in view of Proulx et al.

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6. Gas Chromatography-Mass Spectroscopy (GCMS) and Gas Chromatography-Olfactory (GCO) methods were used to evaluate the production of volatile compounds during cooking of three flavor reaction mixtures, and in particular, to evaluate the production of volatile compounds that are associated with meaty aroma. The first flavor reaction mixture contained 1% (10 mg/ml) Leghemoglobin (LegH-only); the second contained 50 mM Ribose with 50 mM Cysteine (Ribose + Cysteine); and the third contained 1% Leghemoglobin with 50 mM Ribose and 50 mM Cysteine (LegH + Ribose + Cysteine). All flavor reaction mixtures were adjusted to pH 6.5 using 50 mM of potassium phosphate buffer and 100 mM of sodium chloride buffer. Two mls of each flavor reaction mixture were prepared in 10 ml GCMS glass vials (referred to as "flavor reaction mixture samples" or "samples" hereinafter). Each sample was analyzed in duplicate.

7. An Agilent 7890A GC system coupled with a Leco TruTOF HT system was used along with a Gerstel MultiPurpose Sampler for auto-sampling. The Gerstel auto-sample was used to cook, cool, and perform HS-SPME on each of the samples. Each sample was heated to 150°C for 3 minutes with 750 rpm of agitation to "cook" the material. This was followed by a two-phase active cooling step that decreased the agitator temperature from 150°C to 50°C. The sample was extracted for 20 minutes at 50°C with 500 rpm of agitation using a 50/30 µm Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS), Stable flex, 23 Ga, Autosampler (Supleco, Cat# 57298-U) fiber.

8. Extracted samples were run on the GCMS by desorbing the SPME fiber in the Gerstel controlled CIS inlet for 6 minutes at 240°C. The desorbed sample was separated on a 60-meter wax column (Agilent, VF-Waxms 60 m x 0.25 mm x 0.25 mm, Part# CP9207) using a 35 minutes GCMS method (35°C for 2 minutes, ramp of 5C°/min until 172°C, ramp of 60C°/min until 255°C, 255°C for 4 minutes) and 1.75 mL/min Helium flow rate and splitless mode. Separated compounds were analyzed by the mass spectrometer, and all data was collected over a 14-500 µ mass range, with an acquisition rate of 10 spectra/sec, and a detector voltage off-set of 100.

9. Samples were then analyzed using Leco ChromaTOF optimized for TruTOF HT (Version 4.51.6.0) coupled with the NIST MS Search 2.2. The identity of each peak in each sample was identified in a two-step process. First, the mass spectrum of each peak with a signal

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to noise greater than 50 was matched to a mass spectrum in the NIST library using a match similarity score of 650, which in my experience is a conservative match similarity score. Additionally, an internally developed calibration method was applied to the data set to confirm the identity of the compounds of interest. In the second step, the Statistical Compare function of ChromaTOF was used to align the named analytes across all samples in a set. The criteria for aligning a single peak across all samples were a similarity score (similarity between the peak spectra across all samples) of 700, a maximum retention time difference of 3 secs, and the peak must have been present in at least two samples.

10. The amount of each volatile compound was determined by averaging the signal intensities from the duplicate experiments. The amount of each volatile compound that can be attributed to Leghemoglobin (LegH)¹ was determined by using the average signal intensity for each volatile compound produced in the LegH-only sample as a baseline to standardize the average signal intensity for the same volatile compound produced in the LegH + Ribose + Cysteine sample. The fold-change in each volatile compound was determined by dividing the signal intensity produced in the presence of LegH (i.e., in the LegH + Ribose + Cysteine sample) by the signal intensity produced in the absence of LegH (i.e., the Ribose + Cysteine sample). If the signal intensity in the absence of LegH (i.e., the Ribose + Cysteine sample) was not detectable, the volatile compound produced in the presence of LegH (i.e., in the LegH + Ribose + Cysteine sample) was indicated as "novel," meaning that the volatile compound was newly produced (within the limits of detection of the technique) in the LegH + Ribose + Cysteine sample.

11. The results of the above-described experiments are shown in **Exhibit B**. The first column shows a list of volatile compounds, grouped into eight (8) different classes of chemicals. The next three columns show the amount of each volatile compound, calculated as described above, for each of the samples (LegH only, Ribose + Cysteine, and LegH + Ribose + Cysteine) followed by a column indicating the fold-change, calculated as described above, or indicating that the volatile compound is novel as described above. The next column provides, where

¹ Volatile compounds that can be attributed to LegH refer to compounds that are, independently, either not detectably produced or produced in a detectably smaller amount upon cooking compared with the control samples (e.g., LegH-alone or Ribose + Cysteine in the absence of LegH).

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available, one or more peer-reviewed publications in which that particular volatile compound is associated with meaty aroma.

12. The presence of LegH resulted in the production of twenty-eight (28) novel volatile compounds that were not produced in the absence of LegH. The 28 novel volatile compounds can be divided into eight (8) different classes of compounds as follows. Representative volatile compounds in each class are shown below; the full list can be found in **Exhibit B**.

- *Oxygen (O)- and/or Nitrogen (N)-containing heterocyclic:* six (6) novel O- and/or N-containing heterocyclic compounds were produced (i.e., 2-methyl furan; 2-pentyl furan; bis(2-methyl-3-furyl) disulfide; 2,5-dimethyl-pyrazine; methyl-pyrazine; and 2-ethyl-5-methyl-pyrazine);
- *Sulfur (S)-containing heterocyclic:* nine (9) novel S-containing heterocyclic compounds were produced (e.g., 2-methyl-3-furanthiol; 3-thiophenemethanol; and 2-methyl-thiazole);
- *sulfides:* one (1) novel sulfide compound was produced (i.e., dimethyl sulfide);
- *aldehydes:* four (4) novel aldehydes were produced (e.g., (E,E)-2,4-hexadienal; and decanal);
- *lactones:* two (2) novel lactones were produced (i.e., 5-ethyldihydro-2(3H)-furanone; and dihydro-5-pentyl-2(3H)-furanone);
- *ketones:* three (3) novel ketones were produced (e.g., 2-octanone; and 3-nonanone);
- *alcohols:* one (1) novel alcohol was produced (i.e., p-cresol); and
- *carboxylic acids:* two (2) novel carboxylic acids were produced (i.e., hexanoic acid; and hexadecanoic acid).

Significantly, each of the 28 different novel volatile compounds have been associated, directly or indirectly, with a meaty aroma; see, for example, the references provided in **Exhibit B**. Of further significance, the presence of LegH in the flavor reaction mixture with Cysteine and Ribose resulted in the generation of two (2) novel volatile compounds upon heating: 2-methyl-3-furanthiol and bis(2-methyl-3-furyl)disulfide. As noted in the Cadwallader

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Declaration, which I have reviewed, both compounds are important aroma compounds in cooked meat and have extremely low odor detection thresholds of 0.7 parts-per-trillion (pptr) and 0.02 pptr, respectively (Belitz et al., 2006, Food Chemistry, Springer-Verlag, Berlin, p. 368). Both compounds usually are only present in low abundance (pptr range) in cooked meat (Tang et al., 2013, J. Sulfur Chem., 34:38-47). Despite their low abundance in meat, they are still among the most impactful aroma compounds because of their extremely low odor detection thresholds.

13. The presence of LegH in the flavor reaction mixture with Cysteine and Ribose also resulted in a synergistic and dramatic increase in the amount of two (2) volatile compounds produced upon heating compared to the amount of the same volatile compounds produced upon heating in the absence of LegH (i.e., in the Ribose + Cysteine sample) or in the LegH-only sample. In the presence of LegH, the amount of:

- 2-acetylthiazole was increased 2,851-fold; and
- thiophene was increased 89-fold.

Significantly, 2-acetylthiazole and thiophene have been associated with a meaty aroma. See, for example, the references cited in **Exhibit B**.

14. In sum, the presence of LegH with Cysteine and Ribose dramatically increased the number and amount of aroma compounds associated with meat upon cooking.

15. 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 2nd 2016


Celeste Holz-Schietinger, Ph.D.

EXHIBIT A

Celeste Holz-Schietinger, PhD

Principal Scientist, Impossible Foods

Redwood City, CA
celeste.holz@impossiblefoods.com

Innovative R&D Scientist and Product Developer responsible for leading the Flavor Department at Impossible Foods. Technical skills include enzymology, flavor chemistry, sensory science, lipid and protein biochemistry, protein purification, analytical chemistry, metabolic pathways and small molecule identification.

Education

Ph.D. Biochemistry, **University of California** at Santa Barbara 2007-2012
from the Biomolecular Science and Engineering Program

B.S. Molecular Biology & Evolution, minor in Chemistry, 2001- 2005
The Evergreen State College, Olympia, WA

Research

Principal Scientist, *Impossible Foods, Dr. Pat Brown* 2015- Current

Senior Scientist, *Impossible Foods, Dr. Peg Scott, Dr. Chris Davis* 2013- 2015

Scientist, *Impossible Foods, Dr. Chris Davis* 2012-2013

- R&D scientist and flavor department leader at Impossible Foods, directing flavor technology research and flavor product integration.
- Manage a team of scientists to understand the fundamental biology and chemistry of flavor perception and flavor creation. We identify the catalysis and precursors that create aroma compounds, determine how aroma compounds create particular flavors profiles, and then how to recreate these flavors in products.
- Lead chemist on the identification, characterization and manipulation of biological flavor catalysis, including heme-proteins, microbes, and enzymes.
- Support off flavor identification and removal in products and feedstock with product developers, protein biochemist and process engineers.
- Flavor product integration, leading efforts for creating meat replicas from plants that taste and smell like animal based meats, working closely with manufacturing to scale the process, and marketing to meet consumers desires.
- Previous roles also included but not limited to sensory panel leader, lipid group leader, and product developer creating nut-base cheese (Kite Hill Cheese).

Enzymology Research Associate, *UC Santa Barbara, Dr. Norbert Reich* 2007 –2012

- Researched the underlying chemical mechanisms that regulate how the genetic code is translated to creates phenotypic variation in human cells; particularly, understanding the developmental enzymes (DNMT3B and DNMT3A) and their modulators.
- Identified the mechanisms and structural changes that occurred with mutated forms of DNMT3A, highly associated with diseased states that change gene expression.

- Particular specialty was the development and execution of kinetic and biophysical assays to characterize the enzymology and regulation of the *de novo* DNA methyltransferase (DNMT3A).
- Mentored and lead 6 undergraduate researchers.

Biochemistry Visiting Associate, Stanford University, Dr. Pat Brown (collaboration 2008-2010) 06-11/2009

- Global identification of RNA associated with developmental enzymes (DNMT3B and DNMT3A) through cellular differentiation and correlated to gene expression and DNA methylation patterns. Developed RNA library construction protocols for next generation sequencing, and became skilled in microarray, hES cell culture, and fluorescence microscopy.

Scientist in Endocrinology, U.S. Geological Survey 05-09/2006

- Conducted research on the stress response of translocation on the desert tortoise, though ELISA-based assays, leading team of 5 interns.

Molecular Biology NSF Fellow, University of Georgia, Dr. Travis Glenn 05-09/2005

- Conducted independent research on pair bonding in alligators and determining mutation rates by carrying out ABI Prism sequencing for SNP and microsatellites, multiplexed PCR, and sequence analyzes for genotyping.

Genetics Research Assistant, Evergreen State College, Dr. Erik Thusen 2004-2005

- Independent research on the population genetics of moon jellies, gaining experience in genomic DNA purification, PCR for species identification, and phylogenetic analyzes.

Developmental Biology Research Assistant, Evergreen State College, Dr. Donald Morisato 2003-2004

- Investigated the molecular mechanisms of generating the anterior-posterior axis in *Drosophila*, by carrying out *lac z* staining, *in situ* hybridization, and genetic crosses.

Environmental Science Research Assistant, Pacific Crest Community School, Dr. Tigerin Peare 2000-2001

- Independent research on the *E. coli* levels in the Willamette River, Portland, OR. Data collected results in new city testing and regulations on the city water treatment facilities.

Teaching/Mentoring Experience

Research Mentor for Undergraduate Students, UC Santa Barbara 2008-2011

- Lead undergraduate students in research projects; taught techniques and guided experiments.

Teaching Assistant for epigenetics, developmental & biology laboratory, UCSB, intermittently 2007-2010

- Prepared lectures, gave lectures, and instructed laboratory sections.

Teaching Assistant, Santa Barbara City College, 2006 - 2007

- Molecular biology, botany and general biology laboratories.

Scientific Computing Specialist, Evergreen State College 2003-2006

- Taught and helped students with science software - including biological modeling, statistics, phylogenetic, mathematical, and graphical programs.

EXHIBIT B

Compound	Signal intensity (ND = Not detected (signal to noise of 50, ~ signal of > 20,000))			Fold Increase or Novel (((LegH + Ribose + Cysteine) - (LegH)))/(Ribose + Cysteine)	References
	LegH	Ribose + Cysteine	LegH + Ribose + Cysteine		
O- and N-Heterocyclic compounds					
Furan, 2-methyl-	ND	ND	162,329	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); Kerth et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences); Moon et al. (2006, Food Res. Int., 39:294-308); www.thegoodscentscompany.com
Furan, 2-pentyl-	ND	ND	3,574,602	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); Moon et al. (2006, Food Res. Int., 39:294-308); Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8); Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8); www.thegoodscentscompany.com
bis(2-methyl-3-furyl) disulfide*	ND	ND	1,215,514	Novel	Mottram (1998, Food Chem., 62:415-24); Macleod (1994, The flavour of beef in "The flavour of Meat and Meat Products," ed Shahidi); Moon et al. (2006, Food Res. Int., 39:294-308); Song et al. (2012, J. Chromatog. A, 1254:115-24); www.thegoodscentscompany.com
Pyrazine, 2,5-dimethyl-	ND	ND	224,117	Novel	Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Morita et al. (2003, J. Sci. Food Agric., 83:289-97); www.thegoodscentscompany.com
Pyrazine, methyl-	1049328	ND	12,559,346	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Morita et al. (2003, J. Sci. Food Agric., 83:289-97)
Pyrazine, 2-ethyl-5-methyl-	40894	ND	87,684	Novel	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
S-Heterocyclic compounds					
2-Methyl-3-furanthiol	ND	ND	793,456	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); Mottram (1998, Food Chem., 62:415-24); Moon et al. (2006, Food Res. Int., 39:294-308); www.thegoodscentscompany.com ;
2-Acetylthiazole	ND	153,183	436,716,305	2851	Song et al. (2012, J. Chromatog. A, 1254:115-24); McGorin et al. (2001, Special Publication - Royal Soc. Chem., 274:67-84); Kerth et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8); www.thegoodscentscompany.com ;
Dihydro-3-(2H)-thiophenone	ND	ND	1,168,594	Novel	www.thegoodscentscompany.com ;
Thiophene, 3-methyl-	ND	1,127,036	2,650,365	2	Shibamoto (1980, J. Agric. Food Chem., 28:237-43)

3-Thiophenemethanol	ND	ND	430,476	Novel	Chen et al. (2000, J. Agric. and Food Chem., 48:3512-6)
Thiophene, 2,3-dihydro-5-methyl-	ND	ND	11,799,570	Novel	Elmore et al. (1999, J. Agric. Food Chem., 47:1619-25)
Thiophene	ND	2,422,423	216,296,040	89	Shibamoto (1980, J. Agric. Food Chem., 28:237-43); Macleod (1994, The flavour of beef in "The flavour of Meat and Meat products" ed Shahidi); www.thegoodscentscompany.com
3(2H)-Thiophenone, dihydro-2-methyl-	ND	ND	204,409	Novel	www.thegoodscentscompany.com
5-Methyl-2-thiophenecarboxaldehyde	ND	ND	3,046,036	Novel	Shibamoto (1980, J. Agric. Food Chem., 28:237-43); www.thegoodscentscompany.com;
3-Methyl-2-thiophenecarboxaldehyde	ND	ND	657,464	Novel	Shibamoto (1980, J. Agric. Food Chem., 28:237-43); www.thegoodscentscompany.com
Ethanone, 1-(4,5-dihydro-2-thiazolyl)-	ND	ND	1,304,816	Novel	www.thegoodscentscompany.com;
Thiazole, 2-methyl-	ND	ND	18,835,504	Novel	Shibamoto (1980, J. Agric. Food Chem., 28:237-43)
Sulfides					
Dimethyl sulfide	ND	ND	1,601,935	Novel	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Kerth et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences); Grigorakis et al. (2003, Aquaculture, 225:109-19); Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8); www.thegoodscentscompany.com
Aldehydes					
2,4-Hexadienal, (E,E)-	ND	ND	9,495,304	Novel	Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8); Grigorakis et al. (2003, Aquaculture, 225:109-19); www.thegoodscentscompany.com;
Benzaldehyde, 4-ethyl-	ND	ND	24,320,418	Novel	Morita et al. (2003, J. Sci. Food Agric., 83:289-97); www.thegoodscentscompany.com
Decanal	ND	ND	26,163	Novel	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Morita et al. (2003, J. Sci. Food Agric., 83:289-97); Moon et al. (2006, Food Res. Int., 39:294-308); Song et al. (2012, J. Chromatog. A, 1254:115-24); Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8); www.thegoodscentscompany.com
Undecanal	ND	ND	813,152	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Morita et al. (2003, J. Sci. Food Agric., 83:289-97)
Lactones					
2(3H)-Furanone, 5-ethyldihydro-	ND	ND	72,377,175	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24)
2(3H)-Furanone, dihydro-5-pentyl-	ND	ND	31,360,192	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24)
Ketones					

2-Octanone	ND	ND	1,242,778	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Song et al. (2012, J. Chromatog. A, 1254:115-24); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8); Grigorakis et al. (2003, Aquaculture, 225:109-19)
3-Nonanone	ND	ND	650,546	Novel	Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8); www.thegoodscentcompany.com
3,5-Octadien-2-one, (E,E)-	ND	ND	537,770	Novel	Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8); Grigorakis et al. (2003, Aquaculture, 225:109-19); Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8); www.thegoodscentcompany.com;
Alcohols					
p-Cresol	ND	ND	536,008	Novel	Kerth et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences)
Carboxylic Acids					
Hexanoic acid	ND	ND	14,935	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Song et al. (2012, J. Chromatog. A, 1254:115-24); Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8); Selli et al. (2009, Food Chem., 114:317-22)
Hexadecanoic acid	ND	ND	1,959,921	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); www.thegoodscentcompany.com;
* in addition to being categorized as an O- and N-containing heterocyclic, bis(2-methyl-3-furyl) disulfide also can be categorized as a sulfide and as a S-containing heterocyclic compound.					

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 35 U.S.C. § 1.132

I, Keith R. Cadwallader, Ph.D., hereby declare as follows:

1. I am currently a Professor of Food Chemistry in the Department of Food Science and Human Nutrition at the University of Illinois in Urbana, Illinois. I have held this position since 2007. I have worked and taught in the fields of Food Science, Food Chemistry and Flavor Science since 1985. Prior to my current position, I held Assistant/Associate Professor positions in Food Chemistry at Louisiana State University and Mississippi State University and was an Associate Professor at the University of Illinois.

2. I received my B.S. degree in Food Science from the University of Georgia in 1985, my M.S. degree in Food Science from the University of Florida in 1987, and my Ph.D. degree in Food Science (minor in Chemistry) from the University of Florida in 1990.

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

4. My research includes: chemistry and analysis of food and beverage flavors, flavor (aroma) characterization and flavor quality determination, instrumental and sensory identification and quantification of character-impact flavor compounds in foods/beverages and agricultural/ industrial materials, process flavor development, recovery of flavor constituents

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from food processing by-products, development of methods to stabilize labile flavors, occurrence and mechanisms of flavor formulation in foods and beverages, technology for production of food and beverage flavors, and physical chemistry of food/beverage matrix-flavor binding, flavor release and flavor-packaging interactions. I have taught courses relating to food analysis, flavor chemistry, food products analysis, applied food chemistry, flavor and food acceptance, food composition, and flavor/color of foods.

5. I am a nationally and internationally recognized expert and scholar in the field of flavor science. I have co-edited five books and published 57 refereed book chapters and 96 refereed journal articles in the area of food flavor chemistry and analysis. My research has been published in well-respected journals in the field of flavor chemistry, including the *Journal of Agricultural and Food Chemistry*, *Food Chemistry*, *Journal of Food Science*, *Analytical Chemistry*, *Journal of Sensory Studies*, *Journal of the American Oil Chemists Society*, *Cereal Chemistry*, *Journal of Dairy Science*, *Flavour and Fragrance Journal* and others. I have presented more than 50 invited symposium talks and over 115 scientific papers at national and international scientific meetings. I am co-inventor of an invention described in a U.S. patent application related to stabilized compositions and methods of manufacture (Publication No. US 2014/0275543 A1).

6. Through my research and teaching, I have made both fundamental and applied contributions to the current understanding of flavor chemistry and analysis. In 2016, I received the University of Illinois, College of Agriculture, Consumer and Environmental Sciences, *Senior Faculty Award for Excellence in Research*. I was awarded the *2007 Stephen S. Chang Award for Excellence in Lipid or Flavor Science* from the Institute of Food Technologists. In 2010, I was

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elected a *Fellow in the Agricultural and Food Chemistry Division of the American Chemical Society* in recognition of my scientific contributions to the field of agricultural and food chemistry, specifically in the area of flavor science.

7. I am a professional member of the Institute of Food Technologists and the American Chemical Society (Agricultural and Food Chemistry Division). From 2003-2005, I was the Chair and Program Chair of the Agricultural and Food Chemistry Division of the American Chemical Society. I currently serve on the editorial board of the *Flavour and Fragrance Journal* and as an associate editor of the *Journal of Food Science*.

8. In the preparation of my declaration, I have reviewed the U.S. Application Serial 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 have also considered additional materials listed in **Exhibit B**.

9. Flavor is the integrated response to the simultaneous perception (total or combined sensation) of the taste, aroma and somatosensory (nerve response) characteristics of substances naturally present in foods. Although the peripheral sensory organs for detection of taste and smell stimuli are quite distinct, their signals are integrated in the orbitofrontal and other areas of the cerebral cortex of the brain to generate the perception of "flavor" and to mediate food recognition and overall acceptance (Chaudhari and Roper, 2010). It is generally accepted by most researchers that olfaction (aroma) plays the dominant role in the flavor of food (Spence, 2015), and aroma has been regarded as the single most important factor contributing to the

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characteristic flavor of meat (Shahidi et al., 1986).

10. Gas chromatography-mass spectrometry (GC-MS) has been the method of choice for the analysis of aroma components of meat for over five decades (Cadwallader and MacLeod, 1998). Before GC-MS can be conducted, the volatile components of the product must first be isolated from the sample and then transferred to the instrument for subsequent analysis.

Headspace solid phase microextraction (HS-SPME) combined with GC-MS has been widely and successfully used for analysis of aroma components of meat (Rochat et al., 2007) and “meat aroma” reaction mixtures (Jublot et al., 2010). During analysis, the GC-MS system separates the individual volatile compounds in the mixture and then the mass-spectrometer detects and characterizes each component individually, enabling both qualitative (compound identification) and quantitative (compound concentration) analyses to be performed.

11. In my opinion, Applicants discovered a “ground beef-like food product” containing no animal products and including:

0.01%-5% by weight of a heme-containing protein comprising an amino acid sequence having at least 80% sequence identity to the polypeptide set forth in SEQ ID NO:4;
a sugar, sugar alcohol, sugar acid, or sugar derivative selected from glucose, ribose, fructose, lactose, xylose, arabinose, glucose-6-phosphate, maltose, and galactose, and mixtures of two or more thereof; and
at least 10 mM of a compound selected from cysteine, cystine, selenocysteine, thiamine, methionine, and mixtures of two or more thereof; and
at least 10% or more by weight one or more plant proteins,
which has the surprising capability of promoting, upon heating, the efficient conversion of

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nonvolatile flavor precursors into desirable meaty aroma compounds. This is a significant discovery since a person having ordinary skill in the art would not have anticipated that the inclusion of a heme protein would have any beneficial effect on flavor. In fact, iron, and specifically heme iron, has been most often associated with causing deleterious off-odor consequences in foods and especially meat products (Calkins, 2007). As a flavor scientist who has worked in the field for over 30 years, I consider Applicants' discovery to be completely unexpected.

12. 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) is required or essential for the development of meaty aroma in a “ground beef-like food product” comprising no animal products. These experiments are described in the ‘006 application. In addition, I reviewed the experiments in the Declaration Under 37 CFR §1.132 of Dr. Celeste Holz-Schietinger, Ph.D. (“Holz-Schietinger Declaration”).

13. The Holz-Schietinger Declaration describes the application of state-of-the-art instrumental aroma analysis methods, specifically the aforementioned 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 the Maillard reaction and other 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

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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 (150C for 3 min) and then analyzed by HS-SPME-GC-MS.

14. It is my opinion that the above experimental approach and subsequent data analyses techniques used by Dr Celeste Holz-Schietinger are appropriate and acceptable for the purpose of evaluating and comparing aroma compound formation in the three reaction systems under heating conditions. It is also my opinion that these conditions which 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.

15. In my opinion, Applicants' experiments in the '006 application clearly demonstrate that inclusion of LegH in the reaction mixture has the surprising result of greatly enhancing the reaction of ribose and cysteine to form a variety of volatile compounds possessing meaty aromas. Moreover, it is my opinion that Dr. Celeste Holz-Schietinger's experiments confirm these surprising results. Among the 28 compounds identified in her experiments, two in particular are well-known to possess meaty sensory characteristics and to be important and potent odorants in cooked meat. Specifically, 2-methyl-3-furanthiol (MFT) and its dimerized form bis(2-methyl-3-furyl)disulfide (MFT-MFT) are important aroma compounds in cooked meat (Tang et al., 2013). Both compounds have extremely low odor detection thresholds of 0.7

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parts-per-trillion (pptr) and 0.02 pptr, respectively (Belitz et al. 2006), and are usually only present in low abundance (pptr range) in cooked meat (Tang et al., 2013). Despite their low abundance in meat they are still among the most impactful aroma compounds because of their extremely low odor detection thresholds.

16. On the basis of my considerable knowledge and experience in the field of flavor science, it is my opinion that Applicants have indeed discovered and demonstrated the unique ability of heme-protein, including LegH, to serve as an efficient catalyst or promoter of the Maillard reaction and/or other relevant thermal reactions (e.g. thiamine degradation) which can form meaty aroma compounds in a “ground beef-like food product” containing no animal products.

17. It is also my opinion that a person having ordinary skill in the art would not have anticipated or expected this discovery based on the prior art cited. My opinion takes into consideration the Proulx et al. (2006) study, which did not disclose, mention or otherwise anticipate the potential for a heme-protein to influence or promote the formation of desirable meaty aroma compounds. Furthermore, Applicants' unique flavor precursor system, in which a heme-protein is intentionally added to act as an essential promotor of meat flavor generation during cooking, was not disclosed, mentioned or otherwise anticipated in the '994 application. My opinion also takes into consideration the common understanding of those in the field that the inclusion of iron, including heme iron, has been most often associated with causing deleterious off-odor consequences in foods and especially meat products.

18. 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

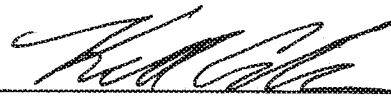
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statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize 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 McGorin, 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/Plenum 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-acetyl-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., Briske-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 Warburg 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.

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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.

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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).

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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 American 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.

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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., McGorin, R.J., organizers. 234th ACS National Meeting, August 19-23. AGFD Paper 206.

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Cadwallader, K.R. 2007. Protein-based functional beverages and flavoring considerations. In IFT Symposium: *Functional Beverages and Human Health*. Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 28 - August 1, Session-Paper 174-02.

Cadwallader, K.R. 2006. Chemical and sensory characterization of food aroma: An academic approach. Meeting of the Society of Flavor Chemists, Chicago, IL, October 19.

Cadwallader, K.R. 2006. Flavor chemistry of fish and shellfish. ACS Symposium: Molecular Cuisine. (Tunick, M.H., Risch, S.J. and Johnson, S.C., organizers). 232nd ACS National Meeting, San Francisco, CA, September 10-14, AGFD paper 61.

Cadwallader, K.R. and Zhou, Q. 2006. Characteristic aroma components of tree nuts. ACS Symposium: Nutrition and Sensory Quality of Nuts. (Pattee, H.E., Shahidi, F., Yu, L., organizers). 331st ACS National Meeting, Atlanta, GA, March 26 – March 30, AGFD paper 40.

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.

Cadwallader, K.R., Bordignon, J.R., Wang, P. and Sugiharto, R. 2005. Identification of odorants responsible for off-flavor of corn gluten meal. ACS Symposium: *Grain-based Products: Health and Flavor, and Safety Aspects* (Cadwallader, K.R., Schieberle, P. and Morello, M.J., organizers). 330th ACS National Meeting, Washington, DC, August 28 – September 1, AGFD paper 167.

Cadwallader, K.R., Drake, M.A., Carunchia-Whetstine, M.E. and Singh, T.J. 2005. Characterisation of Cheddar cheese flavour by sensory directed instrumental analysis and model studies. 11th Weurman Flavour Research Symposium, Roskilde, Denmark, June 21-24.

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.

Lozano, P.R., Nicolalde, C.L. and Cadwallader, K.R. 2005. Identification of characteristic aroma components of mate (*Ilex paraguariensis*) tea. 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.

Lecona-Urbe, S., Loarca-Piña, G., Arcila-Lozano, C.C. and Cadwallader, K.R. 2005. Chemical composition of essential oil from Mexican oregano. 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.

Cadwallader, K.R. Importance of sample preparation technique on the composition of aroma extracts from fresh herbs. ACS Symposium: *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 McGorin, 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.

Back, 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 Symposium 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

Tinchai, 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-Elk, P., Herrera Corredor, J., Prinyawiwatukul, 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.

Back, 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.

Cadwallader, K.R. 1996. Potent odorants in hickory and mesquite smokes and liquid smoke extracts. 57th Annual Meeting of the Institute of Food Technologists, New Orleans, LA, #34-6, p. 74.

Cadwallader, K.R., Baek, H.H., Chang-Lee, G.-H. 1996. Aroma extract dilution analysis of cooked freshwater prawn. 57th Annual Meeting of the Institute of Food Technologists, New Orleans, LA, #80D-20, p.197.

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 (*Pamulirus 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 (*Callinectes 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 (*Callinectes 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 β -pinene by *Aspergillus cellulosa*. 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 β -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 extractability 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 Wageningen, 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 University 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
Sompoeche 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 Tinchai (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 Briske (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 Pischevar (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 Fragrance 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 Flavor 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 McGorin, 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 McGorin, 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 (KMUTL), 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 Nutraceuticals 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)

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.

First Named Inventor : Rachel Fraser
Serial No. : 14/797,006
Filed : July 10, 2015
Page : 2 of 9

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

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

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Track 1

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 Holz-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)

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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.

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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

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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

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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

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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, Kerschler 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.

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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

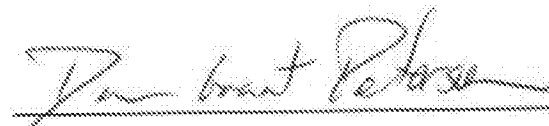

Devin Grant Peterson, Ph.D.

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, Brownd 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.
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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>
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<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.2002.tb11360.x/abstract>

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”)
- Office Action Mailed June 6, 2016 and draft Response to same
- Declaration Under 37 CFR §1.132 of Celeste Holz-Schietinger, Ph.D.
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DECLARATION UNDER 37 CFR §1.132

I, Celeste Holz-Schietinger, Ph.D., hereby declare as follows:

1. I am currently a Principal Scientist at Impossible Foods in Redwood City, California. I have held this position since 2015, and I am responsible for leading the Flavor Department at Impossible Foods. In this role, I direct flavor technology research as well as flavor product integration. I began working for Impossible Foods in 2012 as a Scientist, and I was elevated to a Senior Scientist in 2013. While at Impossible Foods, I have been the lead chemist on the identification, characterization and manipulation of biological flavor catalysis and potentiation, including through the use of heme-containing proteins.
2. I received my Bachelors of Science (B.S.) degree in Molecular Biology and Evolution, with a minor in Chemistry, in 2005 from The Evergreen State College in Olympia, Washington, and I received my Doctor of Philosophy (Ph.D.) degree in Biochemistry from the University of California at Santa Barbara in 2012. I was an Enzymology Research Associate at the University of California at Santa Barbara from 2007 to 2012, and I also was a Biochemistry Visiting Associate at Stanford University in 2009.
3. A copy of my curriculum vitae is attached as **Exhibit A**.
4. Gas Chromatography-Mass Spectroscopy (GCMS) and Gas Chromatography-Olfactory (GCO) methods were used to evaluate the production of volatile compounds during cooking of flavor reaction mixtures, and in particular, to evaluate the production of volatile compounds that are associated with meaty aroma. The first flavor reaction mixture set contained only Leghemoglobin (LegH-only) at different concentrations (0.01%, 0.2%, 1%, and 3%) (note 1% = 10 mg/mL); the second set contained a mixture representing the estimated concentrations of amino acids, sugars, and other small molecules in beef based on their values reported in literature as shown below (Table 1, "the Precursor Mix"):

Table 1

Precursor Mix	
Chemical entity	mM
Alanine	5.6
Arginine	0.6
Asparagine	0.8
Aspartate	0.8
Cysteine	0.8

Glutamic acid	3.4
Glutamine	0.7
Glycine	1.3
Histidine	0.6
Isoleucine	0.8
Leucine	0.8
Lysine	0.7
Methionine	0.7
Phenylalanine	0.6
Proline	0.9
Threonine	0.8
Tryptophan	0.5
Tyrosine	0.6
Valine	0.9
Glucose	5.6
Ribose	6.7
Maltodextrin	5
Thiamine	0.5
GMP	0.24
IMP	0.6
Lactic acid	1
Creatine	1
NaCl	10
KCl	10
Potassium phosphate pH 6.5	10

and the third contained both Leghemoglobin at different concentrations (0.01%, 0.05%, 0.2%, 0.5%, 1%, and 3%) and the Precursor Mix. All flavor reaction mixtures were adjusted to pH 6.5 using 50 mM of potassium phosphate buffer and 100 mM of sodium chloride buffer. Two mL of each flavor reaction mixture were prepared in 10 mL GCMS glass vials (referred to as “flavor reaction mixture samples” or “samples” hereinafter). Each sample was analyzed in duplicate.

5. An Agilent 7890A GC system coupled with a Leco TruTOF HT system was used along with a Gerstel MultiPurpose Sampler for auto-sampling. The Gerstel auto-sample was used to cook, cool, and perform HS-SPME on each of the samples. Each sample was heated to 150°C for 3 minutes with 750 rpm of agitation to “cook” the material. This was followed by a two-phase active cooling step that decreased the agitator temperature from 150°C to 50°C. The sample was extracted for 20 minutes at 50°C with 500 rpm of agitation using a 50/30 µm

Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS), Stable flex, 23 Ga, Autosampler (Supleco, Cat# 57298-U) fiber.

6. Extracted samples were run on the GCMS by desorbing the SPME fiber in the Gerstel controlled CIS inlet for 6 minutes at 240°C. The desorbed sample was separated on a 60-meter wax column (Agilent, VF-WAXms 60 m x 0.25 mm x 0.25 mm, Part# CP9207) using a 35 minutes GCMS method (35°C for 2 minutes, ramp of 5C°/min until 172°C, ramp of 60C°/min until 255°C, 255°C for 4 minutes) and 1.75 mL/min Helium flow rate and splitless mode. Separated compounds were analyzed by the mass spectrometer, and all data was collected over a 14-500 μ mass range, with an acquisition rate of 10 spectra/sec, and a detector voltage off-set of 100.

7. Samples were then analyzed using Leco ChromaTOF optimized for TruTOF HT (Version 4.51.6.0) coupled with the NIST MS Search 2.2. The identity of each peak in each sample was identified in a two-step process. First, the mass spectrum of each peak with a signal to noise greater than 50 was matched to a mass spectrum in the NIST library using a match threshold of 650. Additionally, an internally developed calibration method was applied to the data set to confirm the identity of the compounds of interest. In the second step, the Statistical Compare function of ChromaTOF was used to align the named analytes across all samples in a set. The criteria for aligning a single peak across all samples were a similarity score (similarity between the peak spectra across all samples) of 700, a maximum retention time difference of 3 seconds, and the peak must have been present in at least two samples.

8. The amount of each aroma compound was determined by averaging the signal intensities from the duplicate experiments. The amount of each aroma compound that can be attributed to leghemoglobin (LegH)¹ was determined by using the average signal intensity for each aroma compound produced in the LegH-only sample as a baseline to standardize the average signal intensity for the same aroma compound produced in the LegH + Precursor Mix sample. The fold-change in each aroma compound was determined by dividing the signal intensity produced in the presence of LegH (i.e., in the LegH + Precursor Mix sample) by the signal intensity produced in the absence of LegH (i.e., Precursor Mix sample). If the signal intensity in the absence of LegH (i.e., Precursor Mix) was not detectable, the aroma compound

¹ Aroma compounds that can be attributed to LegH refer to compounds that are, independently, either not detectably produced or produced in a detectably smaller amount upon cooking compared with the control samples (e.g., LegH-alone or Precursor Mix in the absence of LegH).

produced in the presence of LegH (i.e., in the LegH + Precursor Mix sample) was indicated as “novel,” meaning that the aroma compound was newly produced (to the limits of detection of the technique) in the LegH + Precursor Mix sample.

9. The results of the above-described experiments are shown in **Exhibit B**. The first column shows the list of aroma compounds, grouped into eight (8) different classes of chemicals. The next columns show the amount of each aroma compound, calculated as described above, for each of the samples (LegH only, Precursor Mix only, and LegH + Precursor Mix) followed by a column indicating the fold-change, calculated as described above, or indicating that the aroma compound is novel as described above. The next column provides, if available, one or more peer-reviewed publications in which that particular aroma compound is associated with meaty aroma.

10. The presence of 0.01% LegH with precursor mix resulted in the production of twenty-two (22) novel meat associated aroma compounds upon cooking that were not produced in the absence of the LegH. The 22 different novel aroma compounds can be divided into eight (8) different classes of compounds as follows. Representative aroma compounds in each class are shown below; the full list can be found in Exhibit B.

- *Lactones*: one (1) novel lactone was produced (i.e., dihydro-5-pentyl-2(3H)-furanone);
- *Oxygen (O)- and/or Nitrogen (N)-containing heterocyclic*: no (0) novel O- and/or N-containing heterocyclic compounds were produced;
- *Sulfur (S)-containing heterocyclic*: five (5) novel S-containing heterocyclic compounds were produced (i.e., 3-thiophenemethanol, 3-methyl-thiophene, 2-ethyl-thiophene, 2-methyl-5-propyl-thiophene, 4-(methylthio)thiophenol);
- *Carboxylic acids*: three (3) novel carboxylic acids were produced (i.e., hexanoic acid, octanoic acid, propanoic acid);
- *Aldehydes*: eight (8) novel aldehydes were produced (i.e., decanal, undecanal, butanal, heptanal, 2-methyl-propanal, propanal, 3-methyl-butanal, 3-methyl-2-butenal);
- *Sulfides*: two (2) novel sulfide compounds were produced (i.e., dimethyl sulfide, methanethiol);

- *Ketones*: three (3) novel ketones were produced (i.e., 2-nonanone, 2-heptanone, 2,3-pentanedione); and
- *Alcohols*: no (0) novel alcohols were produced.

11. The presence of 0.2% LegH with precursor mix resulted in the production of twenty-seven (27) novel meat associated aroma compounds upon cooking that were not produced in the absence of the LegH. The 27 different novel aroma compounds can be divided into eight (8) different classes of compounds as follows. Representative aroma compounds in each class are shown below; the full list can be found in Exhibit B.

- *Lactones*: no (0) novel lactones were produced;
- *Oxygen (O)- and/or Nitrogen (N)-containing heterocyclic*: two (2) novel O- and/or N-containing heterocyclic compound was produced (i.e., 2,5-dimethyl-pyrazine, indole);
- *Sulfur (S)-containing heterocyclic*: five (5) novel S-containing heterocyclic compounds were produced (i.e., 3-thiophenemethanol, 3-methyl-thiophene, 2-ethyl-thiophene, 2-methyl-5-propyl-thiophene, 4-(methylthio)thiophenol);
- *Carboxylic acids*: four (4) novel carboxylic acids were produced (i.e., hexanoic acid, hexadecanoic acid, octanoic acid, propanoic acid);
- *Aldehydes*: nine (9) novel aldehydes were produced (i.e., decanal, undecanal, butanal, heptanal, 2-methyl-propanal, propanal, hexanal, 3-methyl-butanal, 3-methyl-2-butanal);
- *Sulfides*: four (4) novel sulfide compounds were produced (i.e., dimethyl sulfide, dimethyl trisulfide, dimethyl disulfide, methanethiol);
- *Ketones*: three (3) novel ketones were produced (i.e., 2-nonanone, 2-heptanone, 2,3-pentanedione); and
- *Alcohols*: no (0) novel alcohols were produced.

12. The presence of 1% LegH with precursor mix resulted in the production of thirty two (32) novel meat associated aroma compounds upon cooking that were not produced in the absence of the LegH. The 32 different novel aroma compounds can be divided into eight (8) different classes of compounds as follows. Representative aroma compounds in each class are shown below; the full list can be found in Exhibit B.

- *Lactones*: no (0) novel lactones were produced;

- *Oxygen (O)- and/or Nitrogen (N)-containing heterocyclic*: four (4) novel O- and/or N-containing heterocyclic compounds were produced (i.e., 2-pentyl-pyridine, 2-ethyl-5-methyl- pyrazine, 2,5-dimethyl-pyrazine, indole);
- *Sulfur (S)-containing heterocyclic*: seven (7) novel S-containing heterocyclic compounds were produced (i.e., 3-thiophenemethanol, 1-(4,5-dihydro-2-thiazolyl)-ethanone, 3-methyl-thiophene, 2-ethyl-thiophene, 2-methyl-5-propyl-thiophene, 4-(methylthio)thiophenol, 2,4-dimethyl-thiazole);
- *Carboxylic acids*: four (4) novel carboxylic acids were produced (i.e., hexanoic acid, hexadecanoic acid, octanoic acid, propanoic acid);
- *Aldehydes*: ten (10) novel aldehydes were produced (i.e., decanal, undecanal, butanal, heptanal, 2-methyl-propanal, propanal, hexanal, 3-methyl-butanal, pentanal, 3-methyl-2-butanal);
- *Sulfides*: three (3) novel sulfide compounds were produced (i.e., dimethyl sulfide, dimethyl trisulfide, dimethyl disulfide);
- *Ketones*: three (3) novel ketones were produced (i.e., 2-nonanone, 2-heptanone, 2,3-pentanedione); and
- *Alcohols*: one (1) novel alcohol was produced (i.e., furaneol).

13. The presence of 3% LegH with precursor mix resulted in the production of thirty-three (33) novel meat associated aroma compounds upon cooking that were not produced in the absence of the LegH. The 33 different novel aroma compounds can be divided into eight (8) different classes of compounds as follows. Representative aroma compounds in each class are shown below; the full list can be found in Exhibit B.

- *Lactones*: one (1) novel lactone was produced (i.e., 5-ethyl-dihydro-2(3H)-furanone);
- *Oxygen (O)- and/or Nitrogen (N)-containing heterocyclic*: four (4) novel O- and/or N-containing heterocyclic compounds were produced (i.e., 2-pentyl-pyridine, 2-ethyl-5-methyl- pyrazine, 2,5-dimethyl-pyrazine, indole);
- *Sulfur (S)-containing heterocyclic*: seven (7) novel S-containing heterocyclic compounds were produced (i.e., 3-thiophenemethanol, 1-(4,5-dihydro-2-thiazolyl)-ethanone, 3-methyl-thiophene, 2-ethyl-thiophene, 2-methyl-5-propyl-thiophene, 4-(methylthio)thiophenol, 2,4-dimethyl-thiazole);

- *Carboxylic acids*: four (4) novel carboxylic acids were produced (i.e., hexanoic acid, hexadecanoic acid, octanoic acid, propanoic acid);
- *Aldehydes*: ten (10) novel aldehydes were produced (i.e., decanal, undecanal, butanal, heptanal, 2-methyl-propanal, propanal, hexanal, 3-methyl-butanal, pentanal, 3-methyl-2-butanal);
- *Sulfides*: three (4) novel sulfide compound was produced (i.e., dimethyl sulfide, dimethyl trisulfide, dimethyl disulfide);
- *Ketones*: three (3) novel ketones were produced (i.e., 2-nonanone, 2-heptanone, 2,3-pentanedione); and
- *Alcohols*: one (1) novel alcohol was produced (i.e., furaneol).

14. Across the four concentration ranges of LegH, 35 different novel aroma compounds were identified, each of which have been associated, directly or indirectly, with meaty aroma; see, for example, the references provided in **Exhibit B**. Specifically,

- twenty (20) of those novel compounds were produced in all four LegH reactions (0.01%, 0.2%, 1% and 3%) (i.e., 3-thiophenemethanol, 3-methyl-thiophene, 2-ethyl-thiophene, 2-methyl-5-propyl-thiophene, 4-(methylthio)thiophenol, hexanoic acid, octanoic acid, propanoic acid, decanal, undecanal, butanal, heptanal, 2-methyl-propanal, propanal, 3-methyl-butanal, 3-methyl-2-butanal, dimethyl sulfide, 2-nonanone, 2-heptanone, and 2,3-pentanedione);
- six (6) of those novel compounds were produced in all LegH reactions except at the lowest concentration (i.e., 0.2%, 1% and 3% LegH, but not 0.01% LegH) (i.e., 2,5-dimethyl-pyrazine, hexadecanoic acid, hexanal, dimethyl trisulfide, dimethyl disulfide, and indole); and
- four (4) of those novel compounds were produced in the presence of the higher concentrations of LegH (i.e., 1% and 3% LegH, but not 0.01% or 0.2% LegH) (i.e., 1-(4,5-dihydro-2-thiazolyl)-ethanone, 2,4-dimethyl-thiazole, pentanal, and furaneol).

15. The presence of LegH in the flavor reaction mixture with the Precursor Mix also resulted in a synergistic and dramatic increase in the amount of numerous meat associated aroma compounds produced upon heating compared to the amount of the same aroma compounds

produced upon heating in the absence of LegH (i.e., in the Precursor Mix sample) or in the LegH sample. For example, in the presence of LegH, the amount of:

- 3-thiophenecarboxaldehyde was increased almost 10-fold in the presence of 0.01% LegH, more than 250-fold in the presence of 0.2% LegH, about 3600-fold in the presence of 1% LegH, and over 4300-fold in the presence of 3% LegH;
- methyl-2-thiophenecarboxaldehyde was increased over 350-fold in the presence of 1% LegH and over 1460-fold in the presence of 3% LegH.;
- methyl pyrazine was increased over 10-fold in the presence of 0.2% LegH, over 120-fold in the presence of 1% LegH, and almost 150-fold in the presence of 3% LegH;
- 2-methyl-3-furanthiol was increased over 120-fold in the presence of 0.01% LegH, and about 320-fold in the presence of 0.2% LegH;
- dihydro-3-(2H)-thiophenone was increased almost 5-fold in the presence of 0.2% LegH, over 15-fold in the presence of 1% LegH, and over 30-fold in the presence of 3% LegH; and
- 2,3-butanedione was increased over 8-fold in the presence of 0.2% LegH, almost 650-fold in the presence of 1% LegH, and almost 620-fold in the presence of 3% LegH.
- 2-butanone was increased over 15-fold in the presence of 0.2% LegH, over 125-fold in the presence of 1% LegH, and over 165-fold in the presence of 3% LegH.
- 2-acetylthiazole was increased over 8-fold in the presence of 0.2% LegH, almost 215-fold in the presence of 1% LegH, and almost 275-fold in the presence of 3% LegH.
- furfural was increased almost 40-fold in the presence of 1% LegH and over 30-fold in the presence of 3% LegH.

Significantly, each of these compounds have been associated, either directly or indirectly, with meaty or beefy aroma. See, for example, the references cited in **Exhibit B**. Specifically, 2-

methyl-3-furanthiol^{2,3} and 2-acetylthiazole⁴, for example, are considered important components in the aroma of meat or beef.

² See paragraph 15 in the Declaration under 37 CFR § 1.132 by Dr. Keith R. Cadwallader ("the Cadwallader Declaration"), submitted in U.S. Application No. 14/797,006, now U.S. Patent No. 9,700,067, on December 5, 2016, which states:

Among the 28 compounds identified in [the accompanying Holz-Schietinger Declaration] experiments, two in particular are well-known to possess meaty sensory characteristics and to be important and potent odorants in cooked meat. Specifically, 2-methyl-3-furanthiol (MFT) and its dimerized form bis(2-methyl-3-furyl)disulfide (MFT-MFT) are important aroma compounds in cooked meat (Tang et al., 2013). Both compounds have extremely low odor detection thresholds of 0.7 parts-per-trillion (pptr) and 0.02 pptr, respectively (Belitz et al. 2006), and are usually only present in low abundance (pptr range) in cooked meat (Tang et al., 2013). Despite their low abundance in meat they are still among the most impactful aroma compounds because of their extremely low odor detection thresholds.

³ See paragraph 21 in the Declaration under 37 CFR § 1.132 by Dr. Devin Grant Peterson ("the Peterson Declaration"), submitted in U.S. Application No. 14/797,006, now U.S. Patent No. 9,700,067, on December 5, 2016, which states:

Additionally, the [accompanying 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.

⁴ See paragraph 19 of the Peterson Declaration, submitted in U.S. Application No. 14/797,006, now U.S. Patent No. 9,700,067, on December 5, 2016, which states:

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).

16. 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: 8/17/17


Celeste Holz-Schietinger

EXHIBIT A

Celeste Holz-Schietinger, PhD

Principal Scientist, Impossible Foods

Redwood City, CA
celeste.holz@impossiblefoods.com

Innovative R&D Scientist and Product Developer responsible for leading the Flavor Department at Impossible Foods. Technical skills include enzymology, flavor chemistry, sensory science, lipid and protein biochemistry, protein purification, analytical chemistry, metabolic pathways and small molecule identification.

Education

Ph.D. Biochemistry, **University of California** at Santa Barbara 2007-2012
from the Biomolecular Science and Engineering Program

B.S. Molecular Biology & Evolution, minor in Chemistry, 2001- 2005
The Evergreen State College, Olympia, WA

Research

Principal Scientist, *Impossible Foods, Dr. Pat Brown* 2015- Current

Senior Scientist, *Impossible Foods, Dr. Peg Scott, Dr. Chris Davis* 2013- 2015

Scientist, *Impossible Foods, Dr. Chris Davis* 2012-2013

- R&D scientist and flavor department leader at Impossible Foods, directing flavor technology research and flavor product integration.
- Manage a team of scientists to understand the fundamental biology and chemistry of flavor perception and flavor creation. We identify the catalysis and precursors that create aroma compounds, determine how aroma compounds create particular flavors profiles, and then how to recreate these flavors in products.
- Lead chemist on the identification, characterization and manipulation of biological flavor catalysis, including heme-proteins, microbes, and enzymes.
- Support off flavor identification and removal in products and feedstock with product developers, protein biochemist and process engineers.
- Flavor product integration, leading efforts for creating meat replicas from plants that taste and smell like animal based meats, working closely with manufacturing to scale the process, and marketing to meet consumers desires.
- Previous roles also included but not limited to sensory panel leader, lipid group leader, and product developer creating nut-base cheese (Kite Hill Cheese).

Enzymology Research Associate, *UC Santa Barbara, Dr. Norbert Reich* 2007 –2012

- Researched the underlying chemical mechanisms that regulate how the genetic code is translated to creates phenotypic variation in human cells; particularly, understanding the developmental enzymes (DNMT3B and DNMT3A) and their modulators.
- Identified the mechanisms and structural changes that occurred with mutated forms of DNMT3A, highly associated with diseased states that change gene expression.

- Particular specialty was the development and execution of kinetic and biophysical assays to characterize the enzymology and regulation of the *de novo* DNA methyltransferase (DNMT3A).
- Mentored and lead 6 undergraduate researchers.

Biochemistry Visiting Associate, Stanford University, Dr. Pat Brown (collaboration 2008-2010) 06-11/2009

- Global identification of RNA associated with developmental enzymes (DNMT3B and DNMT3A) through cellular differentiation and correlated to gene expression and DNA methylation patterns. Developed RNA library construction protocols for next generation sequencing, and became skilled in microarray, hES cell culture, and fluorescence microscopy.

Scientist in Endocrinology, U.S. Geological Survey 05-09/2006

- Conducted research on the stress response of translocation on the desert tortoise, though ELISA-based assays, leading team of 5 interns.

Molecular Biology NSF Fellow, University of Georgia, Dr. Travis Glenn 05-09/2005

- Conducted independent research on pair bonding in alligators and determining mutation rates by carrying out ABI Prism sequencing for SNP and microsatellites, multiplexed PCR, and sequence analyzes for genotyping.

Genetics Research Assistant, Evergreen State College, Dr. Erik Thusen 2004–2005

- Independent research on the population genetics of moon jellies, gaining experience in genomic DNA purification, PCR for species identification, and phylogenetic analyzes.

Developmental Biology Research Assistant, Evergreen State College, Dr. Donald Morisato 2003–2004

- Investigated the molecular mechanisms of generating the anterior-posterior axis in *Drosophila*, by carrying out *lac z* staining, *in situ* hybridization, and genetic crosses.

Environmental Science Research Assistant, Pacific Crest Community School, Dr. Tigerin Peare 2000–2001

- Independent research on the *E. coli* levels in the Willamette River, Portland, OR. Data collected results in new city testing and regulations on the city water treatment facilities.

Teaching/Mentoring Experience

Research Mentor for Undergraduate Students, UC Santa Barbara 2008-2011

- Lead undergraduate students in research projects; taught techniques and guided experiments.

Teaching Assistant for epigenetics, developmental & biology laboratory, UCSB, intermittently 2007-2010

- Prepared lectures, gave lectures, and instructed laboratory sections.

Teaching Assistant, Santa Barbara City College, 2006 - 2007

- Molecular biology, botany and general biology laboratories.

Scientific Computing Specialist, Evergreen State College 2003–2006

- Taught and helped students with science software - including biological modeling, statistics, phylogenetic, mathematical, and graphical programs.

Publications

- Holz-Schietinger, C.,** Matje, D.M., Reich, N.O. Mutations in DNA methyltransferase (DNMT3A) observed in Acute Myeloid Leukemia patients disrupt methylation of clustered CpG sites. *J. Biol. Chem.* 287: 30941-30951 (2012).
- Holz-Schietinger, C.,** and Reich, N.O. RNA modulation of the human *de novo* methyltransferase 3A (DNMT3A). *Nucleic Acids Res.* 40: 8550-8557 (2012).
- Holz-Schietinger, C.,** Matje, D.M., Flexer Harrison, M. and Reich, N.O. DNA oligomerization controls the mechanism of *de novo* DNA methylation. *J. Biol. Chem.* 286, 41479-41488 (2011).
- Holz-Schietinger, C. &** Reich, N.O. The inherent processivity of the human *de novo* methyltransferase 3A (DNMT3A) is enhanced by DNMT3L. *J. Biol. Chem.* 285, 29091–29100 (2010).
- Holz-Schietinger, C.,** Matje, D.M. and Reich, N.O. *De novo* DNA methyltransferase DNMT3A: regulation of oligomeric state and mechanism of action in response to physiological pH changes. *Biochimica et Biophysica Acta (BBA)*. 1850:1131-1139 (2015).
- Purdy, M.M., **Holz-Schietinger, C. &** Reich, N.O. Identification of a second DNA binding site in human DNA methyltransferase 3A by substrate inhibition and domain deletion. *Arch. Biochem. Biophys.* 498, 13-22 (2010).
- Lance, S., Tuberville, T., Dueck, L., **Holz-Schietinger, C.,** Trosclair, P., Elsey, R., Glenn, T. Multi-year multiple paternity and mate fidelity in the American alligator, *Alligator mississippiensis*. *Molecular Ecology*. 2009. 18, 4508-4520 (2009).

Awards/Fellowships

- 2010-2011 Philip & Aida Siff Fellowship for excellent undergraduate student mentoring
- 2010, 2011 Best graduate presentation for USCB BMSE and MCDB departments
- 2009 Honorable Mention NSF Graduate Research Fellowship
- 2007-2008 Amgen Fellowship
- 2005 NFS Undergraduate Research Fellowship
- 2005 Evergreen foundation grant (research funding)
- 2002-2004 Evergreen State College foundation scholarship for academic excellence
- 2001-2002 USAA All-American Scholar, Philip S. Wilder Award for Academic Distinction, National Deans List

Training Courses /Research Conferences

- 14th Weurman Flavour Research Symposium, (2014) Cambridge, UK
- FKS Flavor Reaction Course, (2014), University of Missouri St Louis
- American Oil Chemistry Society (AOCS) conference, Lecithin Functions in Technology and Nutrition Course, (2013) AOCS Montreal QC, Canada
- Enzymes, Coenzymes & Metabolic Pathways Gordon conference, (2011, 2010) Waterville Valley, NH

Motif Exhibit 1148, Page 2816 of 3341

Propenal	NA	ND	49,776	448,102	ND	270,076	211,574	291,138	2,371,841	3,348,197	3,435,023	Novel	Novel	Novel	Novel	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
Hexanal	75,075	95,183	112,957	325,332	ND	ND	20,228	32,489	227,058	191,953	302,262	ND	Novel	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Rochat et al. (2007, J. Chromatogr., 1147:85-94)
Butanal, 3-methyl-	557,072	18,263,998	31,231,931	110,254,766	ND	2,362,618	2,533,443	12,904,160	202,809,346	308,133,887	327,395,687	Novel	Novel	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Rochat & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85)
Pentanal	ND	112,415	ND	719,891	ND	ND	ND	ND	1,199,001	1,334,055	ND	ND	Novel	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308)
3-methyl-2-Butenal	ND	92,647	147,360	220,819	ND	153,253	60,188	1,457,862	27,806,744	26,383,307	18,675,745	Novel	Novel	Novel	Novel	www.thegoodscentscompany.com
Aldehydes																
Dimethyl sulfide	NA	ND	16,805	138,735	ND	608,212	4,277	46,842	175,231	115,614	363,092	Novel	Novel	Novel	Novel	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Kerthi et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences); Grigorakis et al. (2003, Aquaculture, 225:109-119); Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8); www.thegoodscentscompany.com
Dimethyl trisulfide	9,651	625,067	696,993	1,561,353	ND	ND	ND	8,894	49,681	145,651	427,713	ND	Novel	Novel	Novel	Rochat et al. (2007, J. Chromatogr., 1147:85-94); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
Disulfide, dimethyl	166,715	2,216,160	3,343,993	6,841,436	ND	ND	ND	17,319	125,598	169,793	1,107,514	ND	Novel	Novel	Novel	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
Methanethiol	ND	ND	ND	ND	ND	13,850	68,075	1,032,552	15,252	ND	ND	Novel	Novel	ND	ND	Moon et al. (2006, Food Res. Int., 39:294-308); Guth & Grosch (1995, Z. Lebensm. Unters. Forsch., 201:25-6); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
Ketones																
2-Butanone	422,992	10,234,816	15,836,368	58,951,787	1,801,674	4,793,578	5,923,348	39,080,140	174,676,895	246,577,952	360,484,573	2.4	16.0	128.1	167.4	Moon et al. (2006, Food Res. Int., 39:294-308); Rochat & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85)
2-Norone	62,751	403,694	810,729	3,070,051	ND	63,605	76,414	94,606	204,317	599,275	1,663,501	Novel	Novel	Novel	Novel	Rochat & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2-Undecanone	43,609	963,841	1,575,957	3,492,050	3,719	82,402	87,638	204,052	703,973	1,491,956	3,177,997	10.4	Decrease	Decrease	Decrease	Rochat & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2,3-Butanedione	566,203	523,951	45,103,463	46,719,319	950,004	2,282,634	2,136,841	8,438,865	246,427,606	658,789,553	755,251,721	1.8	8.3	646.0	619.0	Rochat & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2-Heptanone	69,010	129,443	183,580	549,173	ND	59,309	160,170	154,193	129,189	115,276	262,944	Novel	Novel	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Rochat & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
Acetophenone	41,840	111,440	169,138	564,399	28,223	105,993	88,379	189,534	570,870	396,314	708,004	2.3	2.8	8.2	5.1	www.thegoodscentscompany.com
2,3-Pentanedione	ND	333,214	627,685	2,199,141	ND	39,380	14,589	98,573	700,537	993,562	2,173,388	Novel	Novel	Novel	Novel	www.thegoodscentscompany.com
Alcohols																
Furanol	ND	235,105	385,068	1,925,164	ND	ND	ND	ND	47,673	153,459	774,672	ND	ND	Novel	Novel	Guth & Grosch (1995, Z. Lebensm. Unters. Forsch., 201:25-6); Moon et al. (2006, Food Res. Int., 39:294-308); Rochat et al. (2007, J. Chromatogr., 1147:85-94); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Farmer
2-Ethyl-1-hexanol	ND	ND	ND	ND	311,874	1,016,073	920,983	ND	755,739	ND	424,100	3.3	ND	ND	1.4	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)

DECLARATION UNDER 37 CFR §1.132

I, Celeste Holz-Schietinger, Ph.D., hereby declare as follows:

1. I am currently a Principal Scientist at Impossible Foods in Redwood City, California. I have held this position since 2015, and I am responsible for leading the Flavor Department at Impossible Foods. In this role, I direct flavor technology research as well as flavor product integration. I began working for Impossible Foods in 2012 as a Scientist, and I was elevated to a Senior Scientist in 2013. While at Impossible Foods, I have been the lead chemist on the identification, characterization and manipulation of biological flavor catalysis and potentiation, including through the use of heme-containing proteins.
2. I received my Bachelors of Science (B.S.) degree in Molecular Biology and Evolution, with a minor in Chemistry, in 2005 from The Evergreen State College in Olympia, Washington, and I received my Doctor of Philosophy (Ph.D.) degree in Biochemistry from the University of California at Santa Barbara in 2012. I was an Enzymology Research Associate at the University of California at Santa Barbara from 2007 to 2012, and I also was a Biochemistry Visiting Associate at Stanford University in 2009.
3. A copy of my curriculum vitae is attached as **Exhibit A**.
4. Gas Chromatography-Mass Spectroscopy (GCMS) and Gas Chromatography-Olfactory (GCO) methods were used to evaluate the production of volatile compounds during cooking of flavor reaction mixtures, and in particular, to evaluate the production of volatile compounds that are associated with meaty aroma. The first flavor reaction mixture contained 0.5% (note 0.5% = 5 mg/mL) leghemoglobin (LegH); the second contained 10 mM ribose with 1.5 mM thiamine (ribose + thiamine 1.5 mM); the third contained 0.5% leghemoglobin with 10 mM ribose with 1.5 mM thiamine (LegH + ribose + thiamine 1.5 mM); the fourth contained 10 mM ribose with 1.5 mM cysteine (ribose + cysteine 1.5 mM); the fifth contained 0.5% leghemoglobin with 10 mM ribose with 1.5 mM cysteine (LegH + ribose + cysteine 1.5 mM). All flavor reaction mixtures were adjusted to pH 6.5 using 50 mM of potassium phosphate buffer and 100 mM of sodium chloride buffer. Two mL of each flavor reaction mixture were prepared in 10 mL GCMS glass vials (referred to as "flavor reaction mixture samples" or "samples" hereinafter). Each sample was analyzed in duplicate.

5. An Agilent 7890A GC system coupled with a Leco TruTOF HT system was used along with a Gerstel MultiPurpose Sampler for auto-sampling. The Gerstel auto-sampler was used to cook, cool, and perform HS-SPME on each of the samples. Each sample was heated to 150°C for 5 minutes with 750 rpm of agitation to “cook” the material. This was followed by a two-phase active cooling step that decreased the agitator temperature from 150°C to 50°C. The sample was extracted for 20 minutes at 50°C with 500 rpm of agitation using a 50/30 µm Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS), Stable flex, 23 Ga, Autosampler (Supleco, Cat# 57298-U) fiber.

6. Extracted samples were run on the GCMS by desorbing the SPME fiber in the Gerstel controlled CIS inlet for 6 minutes at 240°C. The desorbed sample was separated on a 60-meter wax column (Agilent, VF-WAXms 60 m x 0.25 mm x 0.25 mm, Part# CP9207) using a 35 minutes GCMS method (35°C for 2 minutes, ramp of 5C°/min until 172°C, ramp of 60C°/min until 255°C, 255°C for 4 minutes) and 1.75 mL/min Helium flow rate and splitless mode. Separated compounds were analyzed by the mass spectrometer, and all data was collected over a 14-500 µ mass range, with an acquisition rate of 10 spectra/sec, and a detector voltage off-set of 100.

7. Samples were then analyzed using Leco ChromaTOF optimized for TruTOF HT (Version 4.51.6.0) coupled with the NIST MS Search 2.2. The identity of each peak in each sample was identified in a two-step process. First, the mass spectrum of each peak with a signal to noise greater than 50 was matched to a mass spectrum in the NIST library using a match threshold of 650. Additionally, an internally developed calibration method was applied to the data set to confirm the identity of the compounds of interest. In the second step, the Statistical Compare function of ChromaTOF was used to align the named analytes across all samples in a set. The criteria for aligning a single peak across all samples were a similarity score (similarity between the peak spectra across all samples) of 700, a maximum retention time difference of 3 seconds, and the peak must have been present in at least two samples.

8. The amount of each aroma compound was determined by averaging the signal intensities from the duplicate experiments. The amount of each aroma compound that can be

attributed to leghemoglobin (LegH)¹ was determined by using the average signal intensity for each aroma compound produced in the LegH sample as a baseline to standardize the average signal intensity for the same aroma compound produced in the LegH + ribose + thiamine 1.5 mM sample and/or the LegH + ribose + cysteine 1.5 mM sample. The fold-change in each aroma compound was determined by dividing the signal intensity produced in the presence of LegH (i.e., in the LegH + ribose + thiamine 1.5 mM sample) by the signal intensity produced in the absence of LegH (i.e., in the ribose + thiamine 1.5 mM sample); or the fold-change in each aroma compound was determined by dividing the signal intensity produced in the presence of LegH (i.e., in the LegH + ribose + cysteine 1.5 mM sample) by the signal intensity produced in the absence of LegH (i.e., in the ribose + cysteine 1.5 mM sample). If the signal intensity in the absence of LegH (i.e., in the ribose + thiamine 1.5 mM sample *or* in the ribose + cysteine 1.5 mM sample) was not detectable, the aroma compound produced in the presence of LegH (i.e., in the LegH + ribose + thiamine 1.5 mM sample *or* in the LegH + ribose + cysteine 1.5 mM sample) was indicated as “novel,” meaning that the aroma compound was newly produced (to the limits of detection of the technique) in the LegH + ribose + thiamine (or cysteine) sample.

9. The results of the above-described experiments are shown in **Exhibit B**. The first column shows the list of aroma compounds, grouped into six (6) different classes of chemicals. The next columns show the amount of each aroma compound, calculated as described above, for each of the samples (LegH; ribose + thiamine 1.5 mM; LegH + ribose + thiamine 1.5 mM; ribose + cysteine 1.5 mM; and LegH + ribose + cysteine 1.5 mM) followed by columns indicating the fold-change, calculated as described above, or indicating that the aroma compound is novel as described above. The next column provides, if available, one or more peer-reviewed publications in which that particular aroma compound is associated with meaty aroma.

10. The presence of LegH in combination with thiamine resulted in the production of **twenty five (25)** novel meat associated aroma compounds that were not produced in the absence of LegH. The 25 different novel aroma compounds can be divided into six (6) different classes of compounds as follows.

- *Ketones*: three (3) novel ketones were produced (i.e., 2-butanone, 2-heptanone, 3-octanone);

¹ Aroma compounds that can be attributed to LegH refer to compounds that are, independently, either not detectably produced or produced in a detectably smaller amount upon cooking compared with the control samples (e.g., LegH-alone or Ribose + Cysteine in the absence of LegH).

- *Sulfur (S)-containing heterocyclic*: four (4) novel S-containing heterocyclic compounds were produced (i.e., 2-methyl-3-furanthiol, 2-methyl-thiazole, thiazole, 4-methyl-5thiazoleethanol);
- *Sulfides*: no (0) novel sulfide compounds were produced;
- *Aldehydes*: nine (9) novel aldehydes were produced (i.e., 2-methyl-propanal, pentanal, nonanal, undecanal, 3-methyl-butanal, furfural, decanal, 3-methyl-2-butanal, octanal);
- *Alcohols*: two (2) novel alcohol was produced (i.e., 1-hexanol, 2-furanmethanol); and
- *Oxygen (O)- and/or Nitrogen (N)-containing heterocyclic*: seven (7) novel O- and/or N-containing heterocyclic compounds were produced (i.e., 2-acetyl-1-pyrroline, methyl-pyrazine, 2-methyl-furan, pyrazine, 2-pentyl-furan, 2,5-dimethyl-pyrazine, 2,3-dimethyl-pyrazine).

Significantly, each of the twenty five (25) different novel aroma compounds have been associated, directly or indirectly, with meaty aroma; see, for example, the references provided in **Exhibit B**. For example, 2-methyl-3-furanthiol^{2,3}, 2-methyl-thiazole, thiazole, 4-methyl-5-

² See also the enclosed Declaration under 37 CFR § 1.132 by Dr. Keith R. Cadwallader ("the Cadwallader Declaration"), attached as **Exhibit C**, paragraph 15, which states:

Among the 28 compounds identified in the [Holz-Schietinger Declaration dated December 2, 2016 as filed in U.S. Application Serial No. 14/797,006] experiments, two in particular are well-known to possess meaty sensory characteristics and to be important and potent odorants in cooked meat. Specifically, 2-methyl-3-furanthiol (MFT) and its dimerized form bis(2-methyl-3-furyl)disulfide (MFT-MFT) are important aroma compounds in cooked meat (Tang et al., 2013). Both compounds have extremely low odor detection thresholds of 0.7 parts-per-trillion (pptr) and 0.02 pptr, respectively (Belitz et al. 2006), and are usually only present in low abundance (pptr range) in cooked meat (Tang et al., 2013). Despite their low abundance in meat they are still among the most impactful aroma compounds because of their extremely low odor detection thresholds.

³ See also the enclosed Declaration under 37 CFR § 1.132 by Dr. Devin Grant Peterson ("the Peterson Declaration"), attached as **Exhibit D**, paragraph 21, which states:

Additionally, the [Holz-Schietinger Declaration dated December 2, 2016 as filed in U.S. Application Serial No. 14/797,006] 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.

thiazoleethanol nonanal, undecanal, furfural, decanal, 2-acetyl-1-pyrroline, methyl-pyrazine, and 2,3-dimethyl-pyrazine have been identified as being very important for meaty aroma.

11. The presence of LegH in the flavor reaction mixture with 10 mM ribose and 1.5 mM thiamine also resulted synergistic and dramatic increase in the amount at least **twelve (12)** meat associated aroma compounds produced upon heating compared to the amount of the same aroma compounds produced upon heating in the absence of LegH (i.e., in the ribose + thiamine 1.5 mM sample) or in the LegH only sample. For example, in the presence of LegH, the amount of:

- 2,3-butanedione was increased **36-fold**;
- 2,3-pentanedione was increased **13-fold**;
- dimethyl trisulfide was increased **40-fold**;
- carbon disulfide was increased **19-fold**;
- acetaldehyde was increased **36-fold**;
- 2-methyl-butanal was increased **312-fold**; and
- benzaldehyde was increased **161-fold**.

Significantly, 2,3-butanedione and 2,3-pentanedione have been strongly associated with meaty aroma. See, for example, the references cited in **Exhibit B**.

12. The presence of LegH in combination with cysteine resulted in the production of **twenty two (22)** novel aroma compounds that were not produced in the absence of LegH. The 22 different novel meat associated aroma compounds can be divided into six (6) different classes of compounds as follows.

- *Ketones*: three (3) novel ketones were produced (i.e., 2-heptanone, 3-octanone, 2,3-pentanedione);
- *Sulfur (S)-containing heterocyclic*: five (5) novel S-containing heterocyclic compounds were produced (i.e., 2-methyl-3-furanthiol, 2-methyl-thiazole, dihydro-3-(2H)-thiophenone, 3-thiophenemethanol, 3-methyl-thiophene);
- *Sulfides*: no (0) novel sulfide compounds were produced;
- *Aldehydes*: seven (7) novel aldehydes were produced (i.e., pentanal, butanal, nonanal, furfural, decanal, 3-methyl-2-butanal, octanal);
- *Alcohols*: one (1) novel alcohol was produced (i.e., 1-hexanol).
- *Oxygen (O)- and/or Nitrogen (N)-containing heterocyclic*: six (6) novel O- and/or N-containing heterocyclic compounds were produced (i.e., 2-acetyl-1-pyrroline,

methyl-pyrazine, 2-methyl-furan, 2-pentyl-furan, 2,5-dimethyl-pyrazine, 2,3-dimethyl-pyrazine).

Significantly, each of the twenty two (22) different novel aroma compounds have been associated, directly or indirectly, with meaty aroma; see, for example, the references provided in Exhibit B. For example, 2,3-pentanedione, 2-methyl-3-furanthiol, 2-methyl-thiazole, dihydro-3-(2H)-thiophenone, 3-thiophenemethanol, nonanal, furfural, decanal, 2-acetyl-1-pyrroline, methyl-pyrazine, and 2,3-dimethyl-pyrazine have been identified as being very important for meaty aroma.

13. The presence of LegH in the flavor reaction mixture with 10 mM ribose and 1.5 mM cysteine also resulted synergistic and dramatic increase in the amount at least seventeen (17) meat associated aroma compounds produced upon heating compared to the amount of the same aroma compounds produced upon heating in the absence of LegH (i.e., in the ribose + cysteine 1.5 mM sample) or in the LegH only sample. For example, in the presence of LegH, the amount of:

- 2,3-butanedione was increased 33-fold;
- 2-butanone was increased 23-fold;
- thiazole was increased 47-fold;
- 2-acetylthiazole was increased 35-fold;
- thiophene was increased 30-fold;
- carbon disulfide was increased 23-fold;
- 2-methyl-propanal was increased 46-fold;
- benzaldehyde was increased 29-fold;
- undecanal was increased 11-fold;
- 3-methyl-butanal was increased 56-fold;
- 2-methyl-2-butenal was increase 31-fold; and
- pyrazine was increased 21-fold.

Significantly, 2,3-butanedione, 3-methyl-butanal, 2-acetylthiazole and thiophene have been strongly associated with meaty aroma. See, for example, the references cited in **Exhibit B**.

15. 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: 8/17/17

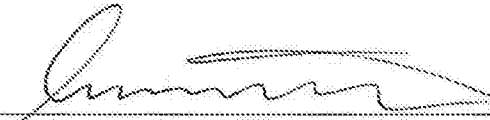

Celeste Holz-Schietinger

EXHIBIT A

Celeste Holz-Schietinger, PhD

Principal Scientist, Impossible Foods

Redwood City, CA
celeste.holz@impossiblefoods.com

Innovative R&D Scientist and Product Developer responsible for leading the Flavor Department at Impossible Foods. Technical skills include enzymology, flavor chemistry, sensory science, lipid and protein biochemistry, protein purification, analytical chemistry, metabolic pathways and small molecule identification.

Education

Ph.D. Biochemistry, **University of California** at Santa Barbara 2007-2012
from the Biomolecular Science and Engineering Program

B.S. Molecular Biology & Evolution, minor in Chemistry, 2001- 2005
The Evergreen State College, Olympia, WA

Research

Principal Scientist, *Impossible Foods, Dr. Pat Brown* 2015- Current

Senior Scientist, *Impossible Foods, Dr. Peg Scott, Dr. Chris Davis* 2013- 2015

Scientist, *Impossible Foods, Dr. Chris Davis* 2012-2013

- R&D scientist and flavor department leader at Impossible Foods, directing flavor technology research and flavor product integration.
- Manage a team of scientists to understand the fundamental biology and chemistry of flavor perception and flavor creation. We identify the catalysis and precursors that create aroma compounds, determine how aroma compounds create particular flavors profiles, and then how to recreate these flavors in products.
- Lead chemist on the identification, characterization and manipulation of biological flavor catalysis, including heme-proteins, microbes, and enzymes.
- Support off flavor identification and removal in products and feedstock with product developers, protein biochemist and process engineers.
- Flavor product integration, leading efforts for creating meat replicas from plants that taste and smell like animal based meats, working closely with manufacturing to scale the process, and marketing to meet consumers desires.
- Previous roles also included but not limited to sensory panel leader, lipid group leader, and product developer creating nut-base cheese (Kite Hill Cheese).

Enzymology Research Associate, *UC Santa Barbara, Dr. Norbert Reich* 2007 –2012

- Researched the underlying chemical mechanisms that regulate how the genetic code is translated to creates phenotypic variation in human cells; particularly, understanding the developmental enzymes (DNMT3B and DNMT3A) and their modulators.
- Identified the mechanisms and structural changes that occurred with mutated forms of DNMT3A, highly associated with diseased states that change gene expression.

- Particular specialty was the development and execution of kinetic and biophysical assays to characterize the enzymology and regulation of the *de novo* DNA methyltransferase (DNMT3A).
- Mentored and lead 6 undergraduate researchers.

Biochemistry Visiting Associate, Stanford University, Dr. Pat Brown (collaboration 2008-2010) 06-11/2009

- Global identification of RNA associated with developmental enzymes (DNMT3B and DNMT3A) through cellular differentiation and correlated to gene expression and DNA methylation patterns. Developed RNA library construction protocols for next generation sequencing, and became skilled in microarray, hES cell culture, and fluorescence microscopy.

Scientist in Endocrinology, U.S. Geological Survey 05-09/2006

- Conducted research on the stress response of translocation on the desert tortoise, though ELISA-based assays, leading team of 5 interns.

Molecular Biology NSF Fellow, University of Georgia, Dr. Travis Glenn 05-09/2005

- Conducted independent research on pair bonding in alligators and determining mutation rates by carrying out ABI Prism sequencing for SNP and microsatellites, multiplexed PCR, and sequence analyzes for genotyping.

Genetics Research Assistant, Evergreen State College, Dr. Erik Thusen 2004–2005

- Independent research on the population genetics of moon jellies, gaining experience in genomic DNA purification, PCR for species identification, and phylogenetic analyzes.

Developmental Biology Research Assistant, Evergreen State College, Dr. Donald Morisato 2003–2004

- Investigated the molecular mechanisms of generating the anterior-posterior axis in *Drosophila*, by carrying out *lac z* staining, *in situ* hybridization, and genetic crosses.

Environmental Science Research Assistant, Pacific Crest Community School, Dr. Tigerin Peare 2000–2001

- Independent research on the *E. coli* levels in the Willamette River, Portland, OR. Data collected results in new city testing and regulations on the city water treatment facilities.

Teaching/Mentoring Experience

Research Mentor for Undergraduate Students, UC Santa Barbara 2008-2011

- Lead undergraduate students in research projects; taught techniques and guided experiments.

Teaching Assistant for epigenetics, developmental & biology laboratory, UCSB, intermittently 2007-2010

- Prepared lectures, gave lectures, and instructed laboratory sections.

Teaching Assistant, Santa Barbara City College, 2006 - 2007

- Molecular biology, botany and general biology laboratories.

Scientific Computing Specialist, Evergreen State College 2003–2006

- Taught and helped students with science software - including biological modeling, statistics, phylogenetic, mathematical, and graphical programs.

Publications

- Holz-Schietinger, C.,** Matje, D.M., Reich, N.O. Mutations in DNA methyltransferase (DNMT3A) observed in Acute Myeloid Leukemia patients disrupt methylation of clustered CpG sites. *J. Biol. Chem.* 287: 30941-30951 (2012).
- Holz-Schietinger, C.,** and Reich, N.O. RNA modulation of the human *de novo* methyltransferase 3A (DNMT3A). *Nucleic Acids Res.* 40: 8550-8557 (2012).
- Holz-Schietinger, C.,** Matje, D.M., Flexer Harrison, M. and Reich, N.O. DNA oligomerization controls the mechanism of *de novo* DNA methylation. *J. Biol. Chem.* 286, 41479-41488 (2011).
- Holz-Schietinger, C. &** Reich, N.O. The inherent processivity of the human *de novo* methyltransferase 3A (DNMT3A) is enhanced by DNMT3L. *J. Biol. Chem.* 285, 29091–29100 (2010).
- Holz-Schietinger, C.,** Matje, D.M. and Reich, N.O. *De novo* DNA methyltransferase DNMT3A: regulation of oligomeric state and mechanism of action in response to physiological pH changes. *Biochimica et Biophysica Acta (BBA)*. 1850:1131-1139 (2015).
- Purdy, M.M., **Holz-Schietinger, C. &** Reich, N.O. Identification of a second DNA binding site in human DNA methyltransferase 3A by substrate inhibition and domain deletion. *Arch. Biochem. Biophys.* 498, 13-22 (2010).
- Lance, S., Tuberville, T., Dueck, L., **Holz-Schietinger, C.,** Trosclair, P., Elsey, R., Glenn, T. Multi-year multiple paternity and mate fidelity in the American alligator, *Alligator mississippiensis*. *Molecular Ecology*. 2009. 18, 4508-4520 (2009).

Awards/Fellowships

- 2010-2011 Philip & Aida Siff Fellowship for excellent undergraduate student mentoring
- 2010, 2011 Best graduate presentation for USCB BMSE and MCDB departments
- 2009 Honorable Mention NSF Graduate Research Fellowship
- 2007-2008 Amgen Fellowship
- 2005 NFS Undergraduate Research Fellowship
- 2005 Evergreen foundation grant (research funding)
- 2002-2004 Evergreen State College foundation scholarship for academic excellence
- 2001-2002 USAA All-American Scholar, Philip S. Wilder Award for Academic Distinction, National Deans List

Training Courses /Research Conferences

- 14th Weurman Flavour Research Symposium, (2014) Cambridge, UK
- FKS Flavor Reaction Course, (2014), University of Missouri St Louis
- American Oil Chemistry Society (AOCS) conference, Lecithin Functions in Technology and Nutrition Course, (2013) AOCS Montreal QC, Canada
- Enzymes, Coenzymes & Metabolic Pathways Gordon conference, (2011, 2010) Waterville Valley, NH

Compound	Signal Intensity: 100 = Not detected		Signal Intensity: 50 = Not detected		Signal Intensity: 15,000 = Not detected		Fold Increase or		References
	Light	ribose +	Light + ribose +	ribose +	Light + ribose +	Novel	Novel		
		thiamine 1.5 mM	thiamine 1.5 mM	cysteine 1.5 mM	cysteine 1.5 mM	(Light + ribose + thiamine) -	(Light + ribose + cysteine) -		
Ketones									
2,3-Butanedione		2,161,257	78,563,251	1,958,985	65,540,109	36	33	Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)	
2-Butanone			25,239,728	557,993	12,758,206	Novel	23	Moon et al. (2006, Food Res. Int., 39:294-308); Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85)	
2-Heptanone			415,292		371,172	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)	
3-Octanone			40,094		76,618	Novel	Novel	Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)	
2,3-Pentanedione		67,534	896,415		523,473	13	Novel	www.thegoodscentscompany.com	
Heterocyclic compounds									
2-Methyl-3-furanthiol			14,315		236,221	Novel	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); www.thegoodscentscompany.com; Mottram (1998, Food Chem., 62:415-24)	
2-methyl-Thiazole			25,979		163,919	Novel	Novel	Burdock (2010, Fenaroli's handbook of flavor ingredients, 6th Ed.)	
Thiazole			21,109	229,262	10,868,037	Novel	#NAME?	Burdock (2010, Fenaroli's handbook of flavor ingredients, 6th Ed.)	
2-Acetylthiazole				14,712	512,095	ND	#NAME?	Song et al. (2012, J. Chromatog. A, 1254:115-24); McGorin et al. (2001, Special Publication - Royal Soc. Chem., 274:67-84); www.thegoodscentscompany.com; Kerth et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8)	
Dihydro-3-(2H)-thiophenone					112,049	ND	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); McGorin et al. (2001, Special Publication - Royal Soc. Chem., 274:67-84); www.thegoodscentscompany.com; Kerth et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8)	
3-Thiophenemethanol					282,483	ND	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Farmer 1991 cited	
3-methyl-Thiophene		217,425	1,376,548		205,936	#NAME?	Novel	www.thegoodscentscompany.com; Burdock (2010, Fenaroli's handbook of flavor ingredients, 6th Ed.)	
Thiophene		149,193	683,022	90,079	2,702,671	#NAME?	#NAME?	www.thegoodscentscompany.com; Burdock (2010, Fenaroli's handbook of flavor ingredients, 6th Ed.)	
4-methyl-5-Thiazoleethanol			39,748			Novel	ND	www.thegoodscentscompany.com	
Sulfides									
dimethyl-Disulfide	15,378	36,281	237,336	33,880	69,922	6	2	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)	
Dimethyl trisulfide		1,264	51,164	24,473	46,195	40	2	Rochat; Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)	
Carbon disulfide		1,000,948	18,591,830	1,209,153	28,040,380	19	23	Jayasena et al. (2013, Trends in Food Sci. & Technol., 34:96-108)	
Aldehydes									
2-methyl-Propanal			18,511,383	405,613	18,666,330	Novel	46	Guth & Grosch (1995, Z. Lebensm. Unters. Forsch., 201:25-6)	
Acetaldehyde	2,118,924	1,845,887	67,952,925	5,414,934	51,772,491	36	9	Moon et al. (2006, Food Res. Int., 39:294-308); Guth & Grosch (1995, Z. Lebensm. Unters. Forsch., 201:25-6)	
Pentanal			159,313		381,708	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308)	
Benzaldehyde	37,128	86,183	13,953,129	248,601	7,208,946	161	29	Moon et al. (2006, Food Res. Int., 39:294-308)	
Butanal					49,139	ND	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)	
Nonanal			231,989		525,261	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)	
Undecanal			361,596	27,895	316,482	Novel	11	Moon et al. (2006, Food Res. Int., 39:294-308); Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Morita et al. (2003, J. Sci. Food Agric., 83:289-97)	
3-methyl-Butanal			22,944,561	406,703	22,645,420	Novel	56	Moon et al. (2006, Food Res. Int., 39:294-308); Rochet & Chaintreau (2005, J. Agric. Food Chem., 53:9578-85)	
Furfural			367,866		211,815	Novel	Novel	Resconi et al. (2013, Molecules, 18:6748-81)	

Decanal			124,311		107,277	Novel	Novel	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Morita et al. (2003, J. Sci. Food Agric., 83:289-97); Moon et al. (2006, Food Res. Int., 39:294-308); Song et al. (2012, J. Chromatog. A, 1254:115-24); Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8); www.thegoodscentscompany.com
3-methyl-2-Butenal			41,782		36,857	Novel	Novel	www.thegoodscentscompany.com; Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2-methyl-Butanal		45,898	14,311,236	232,394	7,137,316	312	31	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
Octanal			158,036		124,554	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2-Propenal		542,416	1,579,560	459,857	3,596,886	3	8	
Alcohols								
1-Hexanol			107,946		133,744	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2-Ethyl-1-hexanol	49,233	56,239	199,266	53,611	169,030	3	2	Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2-Furanmethanol			15,707			Novel	ND	Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher); Farmer & Patterson (1991, Food Chem., 40:201-5)
N or O heterocyclic								
2-Acetyl-1-pyrroline			19,866		22,489	Novel	Novel	Moon et al. (2006, Food Res. Int., 39:294-308); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
methyl-Pyrazine			214,505		160,388	Novel	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Morita et al. (2003, J. Sci. Food Agric., 83:289-97)
2-methyl-Furan			56,185		110,826	Novel	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); Kerth et al. (2013, White Paper: Product Quality, Texas A&M University Dept of Animal Sciences); Moon et al. (2006, Food Res. Int., 39:294-308); www.thegoodscentscompany.com
Pyrazine			438,672	18,939	402,961	Novel	21	Song et al. (2012, J. Chromatog. A, 1254:115-24); Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Morita et al. (2003, J. Sci. Food Agric., 83:289-97)
2-pentyl-Furan			23,414		21,576	Novel	Novel	Song et al. (2012, J. Chromatog. A, 1254:115-24); Moon et al. (2006, Food Res. Int., 39:294-308); www.thegoodscentscompany.com; Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Karahadian & Lindsay (1989, J. Amer. Oil Chem. Soc., 66:1302-8); Leduc et al. (2012, J. Sci. Food Agric., 92:2560-8)
2,5-dimethyl-Pyrazine			22,739		27,137	Novel	Novel	www.thegoodscentscompany.com; Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Morita et al. (2003, J. Sci. Food Agric., 83:289-97); Van Ba et al. (2012, "Principle of meat aroma flavors and future prospect," Ch 7 in Latest Research into Quality Control, InTech Open Access Publisher)
2,3-dimethyl-Pyrazine			25,077		34,400	Novel	Novel	www.thegoodscentscompany.com; Brewer (2006, "The Chemistry of Beef Flavor – Executive Summary," National Cattlemen's Beef Association, #12814); Morita et al. (2003, J. Sci. Food Agric., 83:289-97)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Patrick O'Reilly Brown	Art Unit :	1792
	M.D., Ph.D.	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

First Named Inventor : Patrick O'Reilly Brown M.D., Attorney's Docket No.: 38767-0012001 / M1
Ph.D.
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Filed : January 10, 2014
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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

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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

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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).

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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

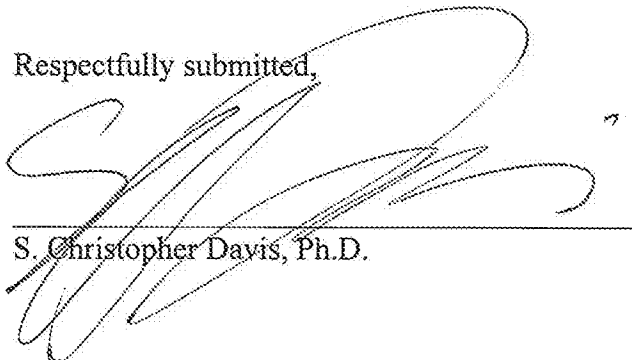

S. Christopher Davis, Ph.D.

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

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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, Gjalb W. *Nature Biotechnology* (2007), 25(3), 338-344.

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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.

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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

**SILLIKER, Inc.****Northern California Laboratory**

5262 Pirrone Court, Salida, CA 95368

Tel. 209/ 549 7508 Fax. 209/ 545 0245

CERTIFICATE OF ANALYSIS

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	Laboratory ID:	362549446		
Desc. 2:	500g Powder	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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Except as otherwise stated, Mérieux NutriSciences Terms and Conditions for Testing Services apply.

**SILLIKER, Inc.****Northern California Laboratory**

5262 Pirrone Court, Salida, CA 95368

Tel. 209/ 549 7508 Fax. 209/ 545 0245

CERTIFICATE OF ANALYSIS

COA No:	CCA-39255182-0
Supersedes:	None
COA Date	8/9/16
Page 2 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.
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 991.20.1	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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Except as otherwise stated, Mérieux NutriSciences Terms and Conditions for Testing Services apply.



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
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Page 3 of 3	

TO:

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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	Laboratory ID: 362549446		
Desc. 2:		500g Powder	Condition Rec'd: NORMAL		
			Temp Rec'd (°C): 27		
<u>Analyte</u>		<u>Result</u>	<u>Units</u>	<u>Method Reference</u>	<u>Test Date Loc.</u>
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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EXHIBIT C

Amino Acid Analysis Report

Order 16967



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.

Amino Acid Analysis Report

Order 16967



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

Amino acids	ANALYSIS INFO	ANALYSIS DATA		CALCULATIONS	
	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
---	-------------

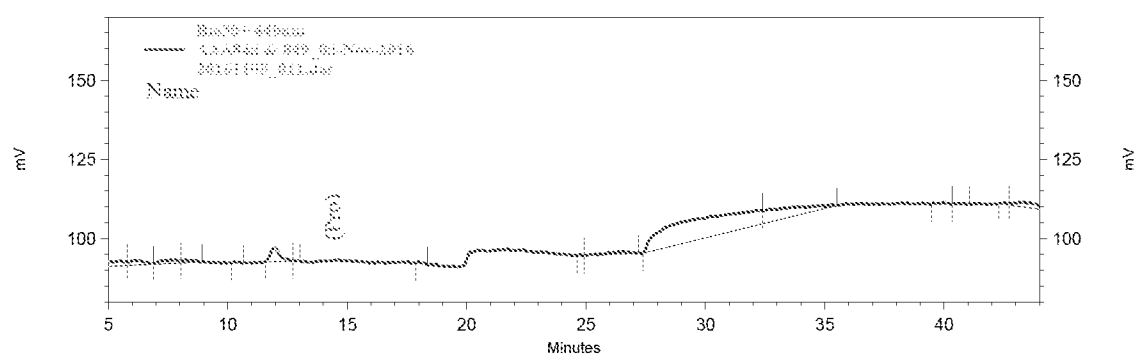
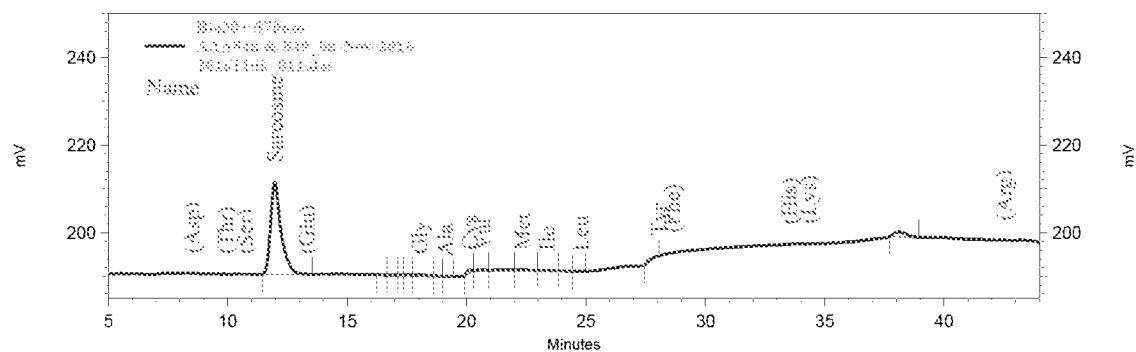
AlphaLyse - Biochrom 30+

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

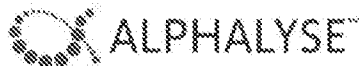
Regarding order 16967

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



AlphaLyse Inc. • 7300 Piedmont Road, Suite 100 • Palo Alto, CA 94306 • USA
Tel: (650) 942-3183 • email: info@alphalyse.com
www.alphalyse.com

EXHIBIT E

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 B₁₂, 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

MS 20160507 Submitted 31/3/2016, Accepted 18/5/2016. Authors are with Dept. of Nutritional Sciences, Univ. of Wisconsin-Madison, Madison, WI, 53706, USA. Direct inquiries to author Tanumihardjo (E-mail: sherry@nutrisci.wisc.edu).

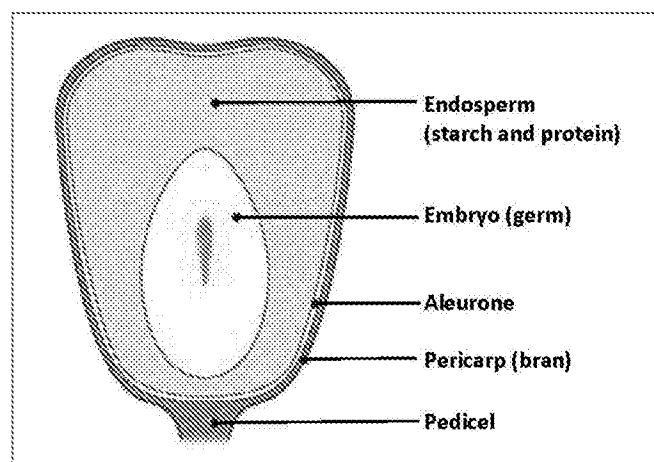


Figure 1—Major components of a maize kernel. Adapted from: Micklos, <http://www.carolina.com/teacher-resources/Interactive/using-carolina-corn-ears-to-teach-genetic-imprinting/tr28902.tr>.

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 pre-formed 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 μ g retinol from a mixed diet in healthy people. The Inst. of Medicine (IOM) set RAEs at 12 μ g for β -carotene, and 24 μ g 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 μ g RAE and 700 μ g 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 μ g 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 μ g provitamin A carotenoids/g in yellow varieties to a target of 15 μ g/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 μ g β -carotene/g, 0.95 μ g α -carotene/g and 1.62 μ g/g β -cryptoxanthin (Li and others 2007). A randomized controlled study with high- β -carotene maize (about 20 μ g 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

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

	Nutrient content/100 g ^a										
	USDA NDB no.	Calcium (mg)	Copper (mg)	Iron (mg)	Magnesium (mg)	Manganese (mg)	Phosphorus (mg)	Potassium (mg)	Selenium (mg)	Sodium (mg)	Zinc (mg)
Maize product											
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

^aSource: USDA Nutrient Database (USDA 2011).^bRecommended daily allowances are for adults 19 to 30 y of age (Ottan and others 2006).^cNonpregnant, nonslactating.

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

Maize product	Nutrient content/100 g ^a												
	USDA NDB no.	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

^aSource: USDA Nutrient Database (USDA 2011); biotin content not available; N/A, data not available.^bThe recommended daily allowances listed are for adults 19 to 30 y of age (Orren and others 2006).^cNonpregnant, nonlactating.

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 <i>masa</i>	Tortillas
Other traditional methods: soaking, germination, fermentation	Traditional maize preparation may involve one or more of these methods	<i>Ogi</i> , a fermented porridge eaten in Nigeria

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

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

Maize product	Nutrient content/100 g ^a								
	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

^aSource: 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

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-blanching 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

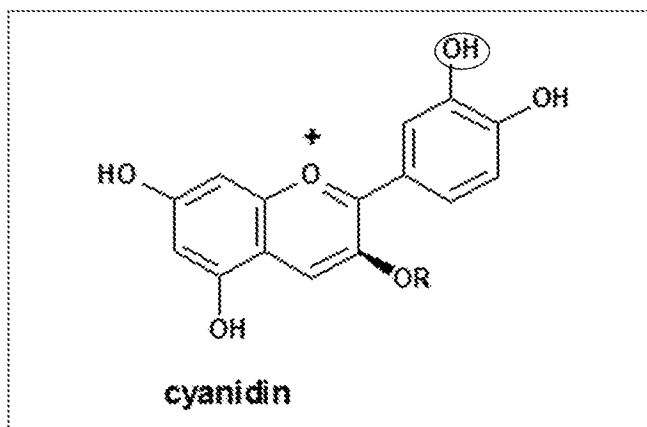


Figure 2—Polyphenols that are commonly quantified in purple, blue, or red maize include 7 anthocyanins (Collison and others 2015). The basic anthocyanin backbone is called cyanidin that contains the circled hydroxyl group. The circled functional group is not present in pelargonidin or it is methylated 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.

(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-Urbe 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.

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, pelargonidins, 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).

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

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

Sources:

^aDe La Parra and others (2007).^bLopez-Martinez and others (2009).^cCortés and others (2006).^dLopez-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, B₆, folate, and biotin, but not B₁₂ 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 B₁ (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 B₂ (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 B₃ (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 μ g 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).

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 pellagrigenic effect as maize (Goldsmith and others 1961; Prinzo 2000).

Vitamin B₅ (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 B₆. Vitamin B₆ is present in food in free or bound forms of pyridoxine, pyridoxal, and pyridoxamine. In maize, more than half of its B₆ content occurs as pyridoxal (Roth-Maier and others 2002). Dry maize contains 0.62 mg vitamin B₆/100 g, almost half of the RDA for adults (1.3 mg). This amount decreases in processed maize. Prececal digestibility of B₆ from maize was found to be 67% in pigs (Roth-Maier and others 2002); however, B₆ 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 B₇ (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 μ g/100 g in whole-grain maize flour and 1.4 μ g/100 g in degermed maize flour. Accordingly, 100 g whole-grain flour provides about 4.1 to 10.8 μ g of biotin, or 14% to 36% of the RDA (30 μ g), with degermed flour providing only 1.4 μ g/100 g, about 5% of the RDA.

Vitamin B₉ (folate). Dry maize contains little folate, about 19 μ g/100 g. The RDA for folate is 400 μ g for nonpregnant, nonlactating females, and for male adults. Whole grain maize flour contains 28 μ g/100 g, which is reduced by 64% to 10 μ g/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 ± 0.02 μ mol 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 μ g/d for adults 19 to 30 y of age (Otten and others 2006). Whole, dry maize contains 314 μ g/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 μ g 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 (Rochefford 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).

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% (Rochefford 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 $\mu\text{g/g}$ 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; Rochefford 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 non-pregnant, 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 K₁ (phyloquinone) is 120 $\mu\text{g/d}$ for men and 90 $\mu\text{g/d}$ for nonpregnant, nonlactating women (Otten and others 2006). Maize contains little phyloquinone, about 0.3 $\mu\text{g}/100\text{ g}$ (USDA 2011). As a fat-soluble vitamin, the vitamin K content of maize oil is higher, at about 2.9 $\mu\text{g}/100\text{ g}$; this amount is low when compared with other oils such as rapeseed and soybean oils that contain >140 $\mu\text{g}/100\text{ g}$ (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 phyloquinone, with the exception of popcorn, which has 1 to 4 $\mu\text{g}/100\text{ g}$ (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 phyloquinone to dihydrophyloquinone (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 dihydrophyloquinone in processed maize foods, but no increases in phyloquinone 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

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 (Davidson 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 μ g/100 g (USDA 2011), which provides almost a third of the adult RDA for selenium at 55 μ g/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 μ g/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

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) Tortillas: 25% to 46% Fried chips: 19% to 25% (Del Pozo-Insfran and others, 2006) Extrusion: 43% (Mora-Rochin and others, 2010)
Anthocyanins and polyphenols	Endosperm			34% to 63% (Del Pozo-Insfran and others, 2006)	
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) 39% to 49% (USDA, 2011)
Selenium	Germ	68% to 90% (Ferretti and Levander, 1974; USDA, 2011)			
Xanthophyll carotenoids	Endosperm	91% to 136% (Pillay and others, 2014)	100% (Li and others, 2007)	33% to 14-fold increase (Gutiérrez-Urbe and others, 2014; De La Parra and others, 2007)	78% to 118% (porridge) (Pillay and others, 2014) 50% to 71% (bread, extruded puffs) (Kear and others, 2008) 27% to 116% (tortilla) (Gutiérrez-Urbe 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.

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-blanching 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; Hidvégi 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, Kivistö 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 (Lommerdal 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

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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Disclaimer

The views expressed in this manuscript are those of the authors and do not necessarily reflect the position or policy of PepsiCo, Inc.

Author Contributions

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

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EXHIBIT F



**Donald Danforth Plant Science Center
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 600 μ L of water: chloroform: methanol (3:5:12 v/v) extraction solvent, collect the supernatant, the residue was re-extracted with another 600 μ L of water: chloroform: methanol extraction solvent. Supernatants were combined then 300 μ L of chloroform and 450 μ L of water were added. The upper phase was collected and dried. The pellets were resuspended with 100 μ L of 20 mM HCl. During derivatization, the samples were diluted ten times; 10 μ L was used for a reaction volume totaling 100 μ L, 1 μ L 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/ μ L 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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	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

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

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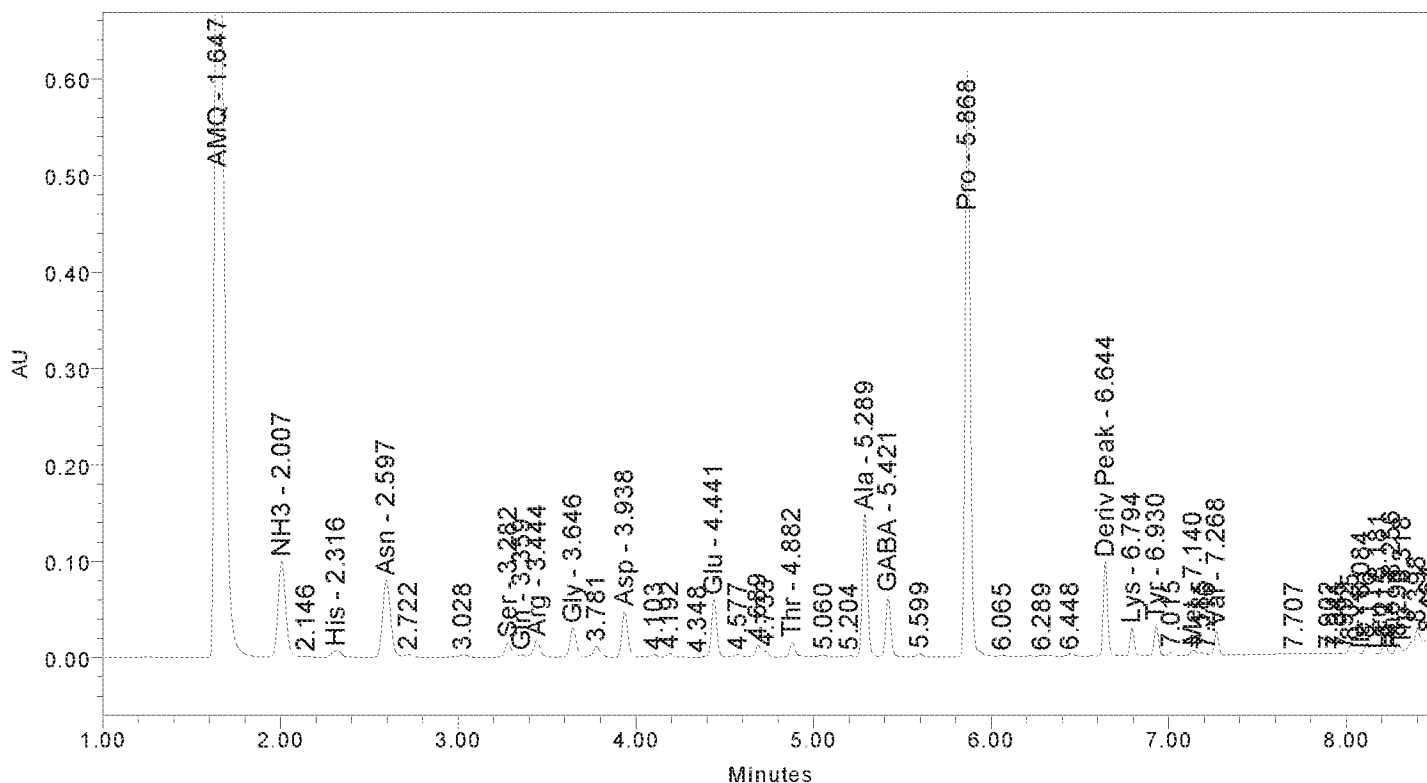
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AccQ-Tag Ultra Sample Information

SampleName	Flour-2	Sample Type	Unknown
MW	0	Run Time	9.5 Minutes
Matrix	None	Dilution	1.00000
Vial	1:A,7	Channel	ACQ-2996 260.0nm-4.8
Injection	1	Date Acquired	1/9/2017 1:14:55 PM CST
Injection Volume	1.00 ul	Date Processed	1/12/2017 12:06:46 PM CST
Project Name	AccQTag_Ultra_Template	Current Date	1/12/2017
Acq Method Set	AccQTag Cell Culture 2996		
Processing Method	20150701_FAA		
Report Method Name	Mole Percent 2_Free AA		

Auto-Scaled Chromatogram





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		4.669		
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		



Peak Results

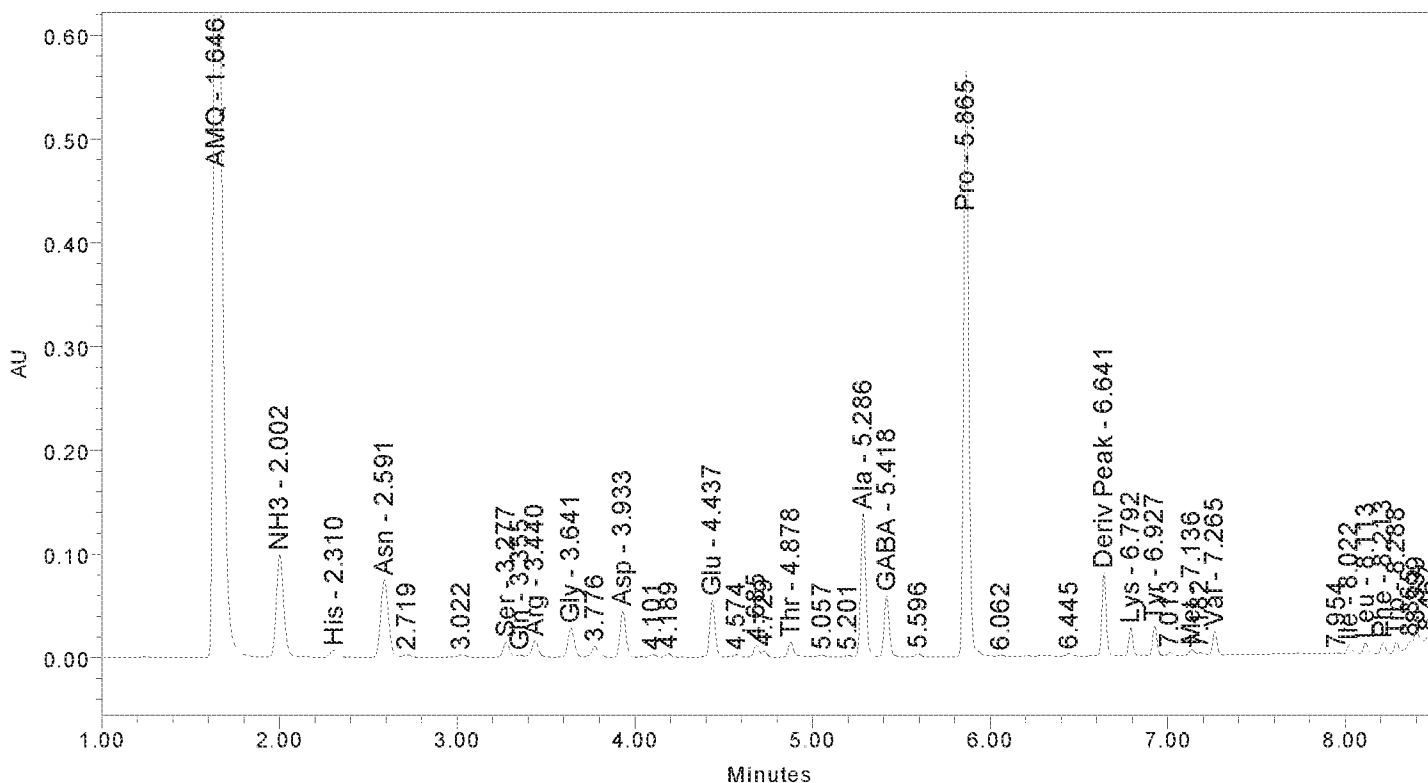
	Amino Acid	RT	pmole	Mole%
34	Lys	6.794	8.892	1.45
35	Tyr	6.930	10.918	1.78
36		7.015		
37	Met	7.140	2.689	0.44
38		7.185		
39		7.216		
40	Val	7.268	9.407	1.54
41	NVa	7.396		
42		7.707		
43		7.902		
44		7.941		
45		7.992		
46		8.025		
47	Ile	8.084	3.561	0.58
48		8.116		
49	Leu	8.181	6.479	1.06
50		8.215		
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	Sample Type	Unknown
MW	0	Run Time	9.5 Minutes
Matrix	None	Dilution	1.00000
Vial	1:A,8	Channel	ACQ-2996 260.0nm-4.8
Injection	1	Date Acquired	1/9/2017 1:26:02 PM CST
Injection Volume	1.00 ul	Date Processed	1/9/2017 3:24:58 PM CST
Project Name	AccQTag_Ultra_Template	Current Date	1/9/2017
Acq Method Set	AccQTag Cell Culture 2996		
Processing Method	20150701_FAA		
Report Method Name	Mole Percent 2_Free AA		

Auto-Scaled Chromatogram





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		



Peak Results

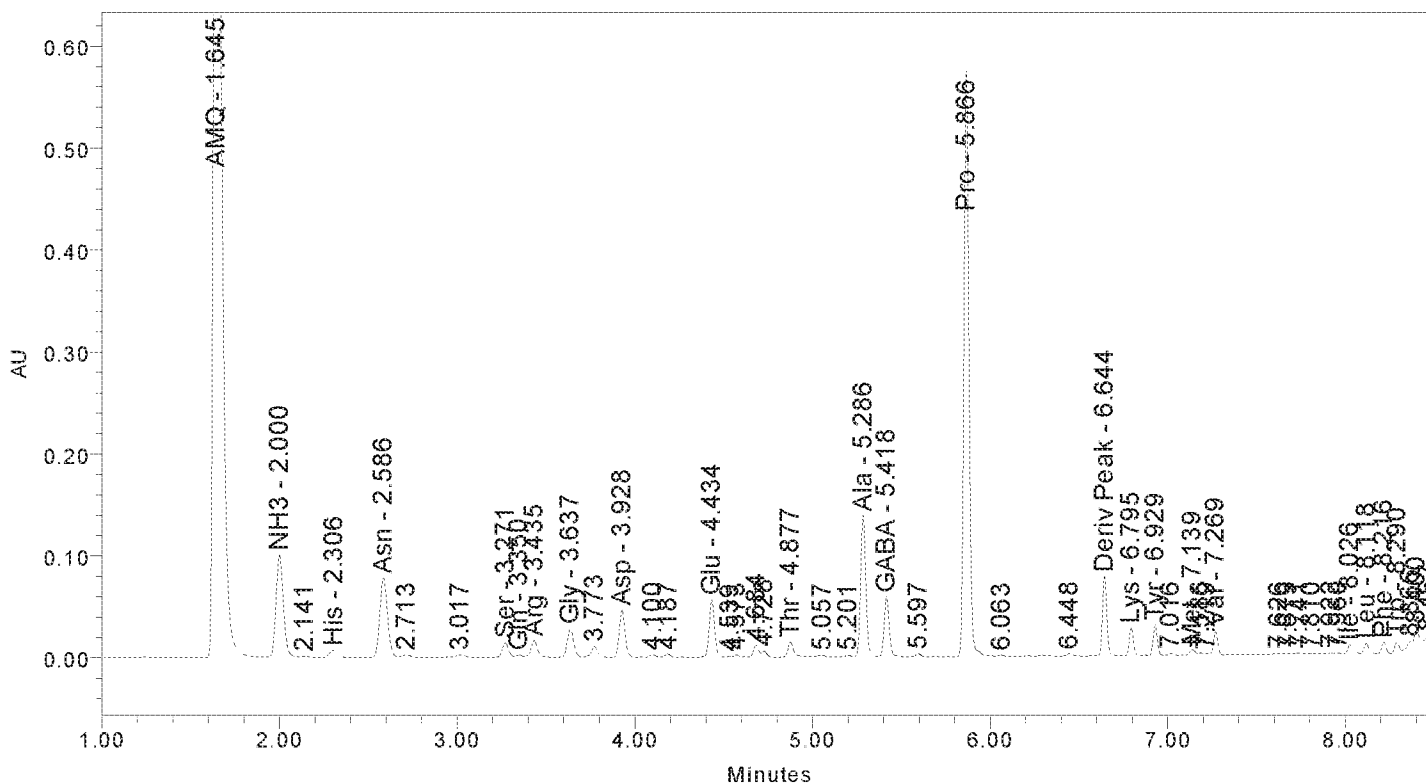
	Amino Acid	RT	pmole	Mole%
34	Met	7.136	2.527	0.45
35		7.182		
36	Val	7.265	8.746	1.55
37	NVa	7.403		
38		7.954		
39	Ile	8.022	3.609	0.64
40	Leu	8.113	4.671	0.83
41	Phe	8.213	4.624	0.82
42	Trp	8.288	5.514	0.97
43		8.365		
44		8.399		
45		8.467		
46		8.527		
47		8.572		
48		8.681		
49		8.715		
50		8.802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



AccQ-Tag Ultra Sample Information

SampleName	Flour-4	Sample Type	Unknown
MW	0	Run Time	9.5 Minutes
Matrix	None	Dilution	1.00000
Vial	1:B,1	Channel	ACQ-2996 260.0nm-4.8
Injection	1	Date Acquired	1/9/2017 1:37:09 PM CST
Injection Volume	1.00 ul	Date Processed	1/9/2017 3:24:58 PM CST
Project Name	AccQTag_Ultra_Template	Current Date	1/9/2017
Acq Method Set	AccQTag Cell Culture 2996		
Processing Method	20150701_FAA		
Report Method Name	Mole Percent 2_Free AA		

Auto-Scaled Chromatogram





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		4.664		
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



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://www.epicurious.com/recipes/food/views/iroquois-stew-with-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
<i>Aquifex aeolicus</i>	SEQ ID NO:3	100%	25%	19%	21%
Leghemoglobin	SEQ ID NO:4	25%	100%	36%	19%
Barley (<i>Hordeum vulgare</i>)	SEQ ID NO:5	19%	36%	100%	20%
<i>Bovin Myoglobin</i>	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

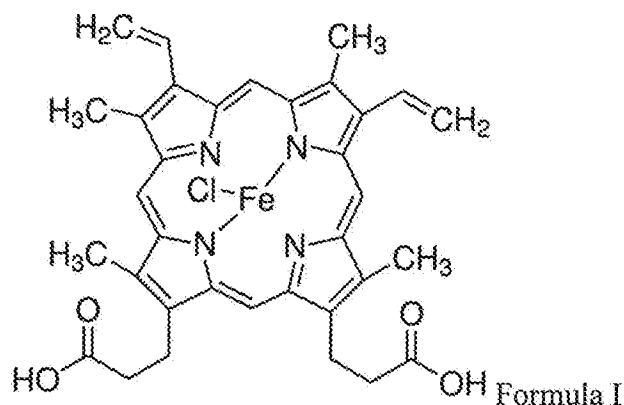
Name	LegH	Barley	<i>B. myoglobin</i>	<i>A. aeolicus</i>
Furan	x	x	x	x
Thiazole	x	x	x	x
benzaldehyde	x	x	x	x
2-acetylthiazole	x	x	x	x
2-methyl-propanal	x	x	x	x
furfural	x	x	x	x
2,3-butanedione	x	x	x	x
2-pentyl-furan	x	x	x	x
2-pentanone	x	x		
pyrazine	x	x	x	x
dimethyl trisulfide	x	x	x	x
3-methyl-butanal	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.



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 hemein 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 hemein 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

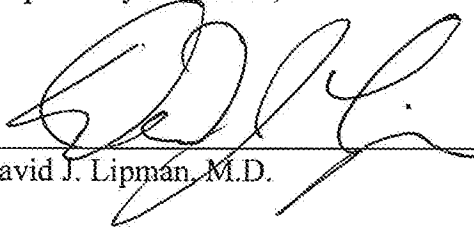

David J. Lipman, M.D.

EXHIBIT A

July 2017

David J. Lipman, M.D.

Home Address:

1818 Greenplace Terrace
Rockville, MD 20850
(301) 762-0003

Office Address:

Impossible Foods
525 Chesapeake Dr.
Redwood City, CA 94063-4724
(650) 461-4380

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*]

Rossi, J.J., Ross, W., Egan, J., Lipman, D.J., Landy, A. (1979) "Structural Organization of Escherichia coli tRNA Tyr gene clusters in four different transducing bacteriophages." J. Mol. Biol. 128, 21-47.

Lipman, D.J., Maizel, J. (1982) "Comparative analysis of nucleic acid sequences by their general constraints", Nuc. Acids Res. 10, 2723-2739.

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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.

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, A_j is the j th amino acid of protein A and B_i is the i th amino acid of protein B . The A_j represent the columns and the B_i the rows of the two-dimensional array, MAT. Then the cell, MAT ij , represents a pair combination that contains A_j and B_i .

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 MAT mn is part of a pathway including MAT ij , 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 MAT $ab \dots MATyz$, where $a \geq 1$, $b \geq 1$, the i and j of all subsequent cells of MAT are larger than the running indices of the previous cell and $y \leq K$, $z \leq M$, 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 MAT ij and MAT mn 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, MAT ij is assigned the value, one, if A_j is the same kind of amino acid as B_i ; if they are different amino acids, MAT ij 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

SIMILARITIES IN AMINO ACID SEQUENCE

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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.

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

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.*,

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 A_i and B_j referenced the correspondence array to determine the type of amino acid pair constituted by A_i and B_j . 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

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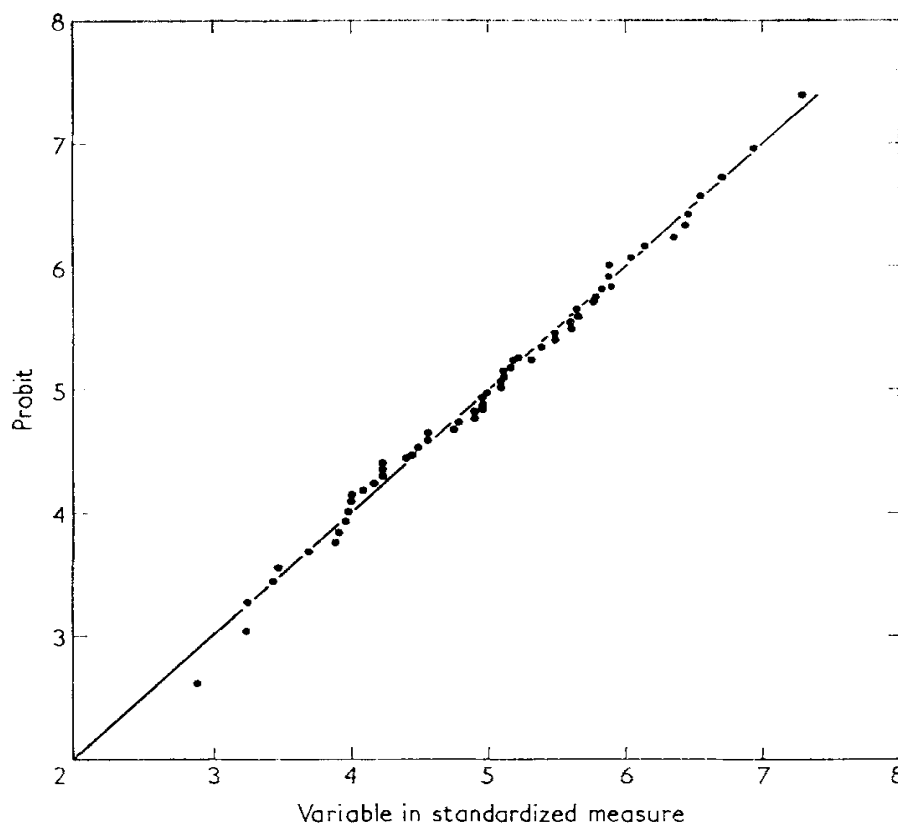


FIG. 3. Probit plot for six grouped random samples.

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		Penalty	Maximum-match value sum		<i>s</i>	Real <i>X</i>	Minimum deletions	
	2	1		Real	Random†			Real	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.

TABLE 2
Ribonuclease-lysozyme maximum matches

Variable set	Match values for pair types		Penalty	Maximum-match value sum		<i>s</i>	Real <i>X</i>	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

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

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 (Zuckerkindl & 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.

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◆Database sequences non-default value

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☐ Organism ☐ BioProjectID ☐ WGS Project

Organism

Optional

Enter organism name or id--completions will be suggested

☐ Exclude

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown

Exclude

Optional

☐ Exclude Models (XM/XP) ☐ Exclude Uncultured/environmental sample sequences

Limit to

Optional

☐ Sequences from type material

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☐ Show results in a new window

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Linear ▾		Existence: 5 Extension: 2 ▾		Linear ▾
Existence: 5 Extension: 2 ▾				

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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=Methylobacterium inferorum (isolate V4) GN=hmp PE=1 SV=1
Q66586	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
Q04985	HBL2_ORYSJ Non-symbiotic hemoglobin 2 OS=Oryza sativa subsp. japonica GN=HB2 PE=2 SV=1
Q24520	HBL1_ARATH Non-symbiotic hemoglobin 1 OS=Arabidopsis thaliana GN=AHB1 PE=1 SV=1
Q48668	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_benthiana	Nicotiana_benthiana non symbiotic hemoglobin
A0A063XGS7	A0A063XGS7_BACIU Group 2 truncated hemoglobin Yjbl OS=Bacillus subtilis GN=yjbl 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 Gln OS=Synechocystis sp. (strain PCC 6803 / Kazusa) GN=gln 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 Gln OS=Nostoc commune GN=gln 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 <i>Vigna radiata</i>
B3DUZ7	SEQ ID NO:2 <i>Methylophilum inferorum</i>
O66586	SEQ ID NO:3 <i>Aquifex aeolicus</i>
P02236	SEQ ID NO:4 <i>Glycine max</i>
Q42831	SEQ ID NO:5 <i>Hordeum vulgare</i>
G4N2X9	SEQ ID NO:6 <i>Magnaporthe oryzae</i>
N4UFN7	SEQ ID NO:7 <i>Fusarium oxysporum</i>
Q4ING3	SEQ ID NO:8 <i>Fusarium graminearum</i>
Q08753	SEQ ID NO:9 <i>Chlamydomonas eugametos</i>
P17724	SEQ ID NO:10 <i>Tetrahymena pyriformis</i>
P15160	SEQ ID NO:11 <i>Paramecium caudatum</i>
Q5DVM4	SEQ ID NO:12 <i>Aspergillus niger</i>
Q9FY42	SEQ ID NO:13 <i>Zea mays</i>
O04985	SEQ ID NO:14 <i>Oryza sativa subsp. japonica</i>
O24520	SEQ ID NO:15 <i>Arabidopsis thaliana</i>
O48668	SEQ ID NO:16 <i>Pisum sativum</i>
Q43236	SEQ ID NO:17 <i>Vigna unguiculata</i>
P02192	SEQ ID NO:18 <i>Bos taurus</i>
P02189	SEQ ID NO:19 <i>Sus scrofa</i>
P68082	SEQ ID NO:20 <i>Equus caballus</i>
Nicotiana_benthiana	SEQ ID NO:21 <i>Nicotiana benthamiana</i>
A0A063XGS7	SEQ ID NO:22 <i>Bacillus subtilis</i>
Q8NMW6	SEQ ID NO:23 <i>Corynebacterium glutamicum</i>
P73925	SEQ ID NO:24 <i>Synechocystis PCC6803</i>
B4WLV9	SEQ ID NO:25 <i>Synechococcus sp. PCC 7335</i>
Q00812	SEQ ID NO:26 <i>Nostoc commune</i>
G2RXE0	SEQ ID NO:27 <i>Bacillus megaterium</i>

	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_benthiana	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%
Q04985	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

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

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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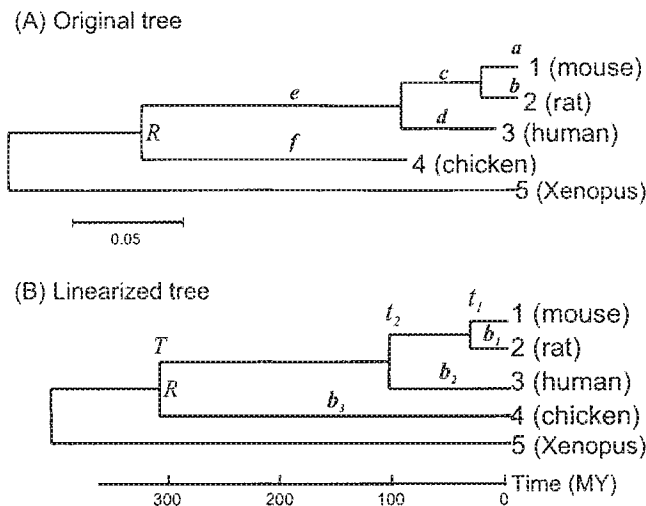


Fig. 1. Phylogeny of the five species used. (A) NJ tree constructed by using distance d_2 for 104 protein sequences. (B) Linearized tree of the above NJ tree. R, root of the four species under consideration.

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. 1A 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 $\hat{t}_{1i}$ for all the proteins used, as mentioned above. That is,

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

where $\hat{t}_{1i}$ is the estimate of t_1 obtained by the i th 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 i th 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 $[(\sum_i \hat{b}_{1i})/(\sum_i \hat{b}_{3i})]T$ rather than by $\hat{t}_1 = \sum_i (\hat{b}_{1i}/\hat{b}_{3i})T/k$. If we assume that b_1 and b_3 are random variables (subscript i dropped), the expectation of $\hat{b}_1/\hat{b}_3$ is approximately given by

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

where $V(\hat{b}_3)$ is the variance of $\hat{b}_3$, and $\text{Cov}(\hat{b}_1, \hat{b}_3)$ 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. 1B and want to estimate T by $\hat{T} = \sum_i (\hat{b}_{3i}/\hat{b}_{1i})t_1/k$. In this case, $\hat{T} = (\hat{b}_{3i}/\hat{b}_{1i})t_1$ 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 $\hat{b}_{3i}$, $\hat{T}$ 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

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

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

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

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 (d_s) 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 d_s 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 ($\hat{a}_1$, $\hat{a}_2$, and $\hat{a}_3$) 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 $\hat{a}_3$, 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

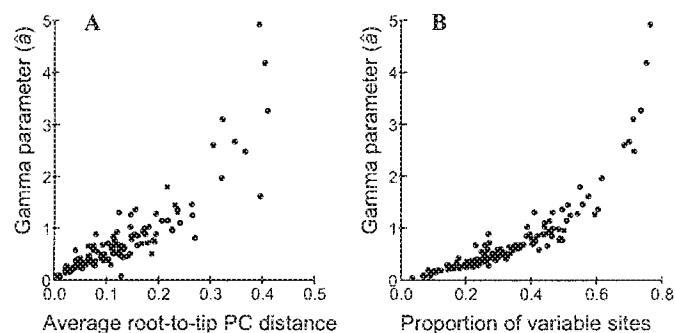
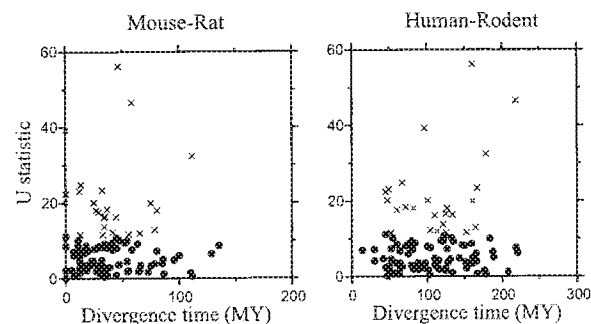


Fig. 2. (A) Relationship between estimated gamma parameter ($\hat{a}$) and the average root-to-tip distance (b_R) for 104 nuclear proteins from the five species used. (B) Relationships between $\hat{a}$ and the proportion of variable sites (p_v) among the five sequences.

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.

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 freedom) 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

(A) PC distance



(B) PC gamma distance

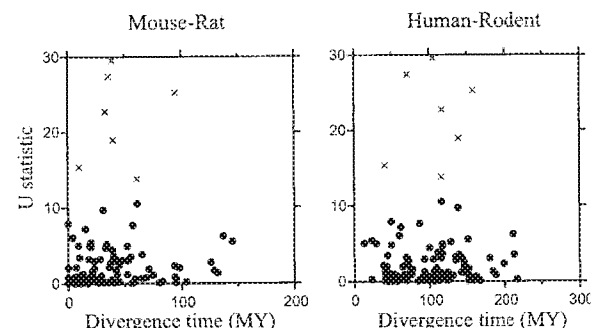


Fig. 3. Relationships between estimated divergence times and U statistic values for each of 104 proteins. The molecular clock hypothesis was rejected for the proteins indicated with the $\times$ symbol.

Table 1. Estimates ($\pm$ 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 $\pm$ 3.4	38.5 $\pm$ 3.2	113.4 $\pm$ 5.0	102.9 $\pm$ 5.0
CD approach				
d_1 ($\bar{a} = 0.76$)	40.7 $\pm$ 3.0	34.4 $\pm$ 2.8	112.3 $\pm$ 5.4	99.9 $\pm$ 5.3
d_2 ($\bar{a} = 0.76$)	39.1 $\pm$ 2.3	33.0 $\pm$ 2.0	110.0 $\pm$ 4.4	97.6 $\pm$ 4.4
d_3 ($a = 0.57$)		32.9 $\pm$ 2.3		95.5 $\pm$ 4.2
d_3 ($a = 0.28$)		(25.2 $\pm$ 2.0)		(82.0 $\pm$ 4.0)
d_L ($a = 0.5$)		32.0 $\pm$ 5.0		90.0 $\pm$ 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. 1A 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. 1A, 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. 1B) 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.

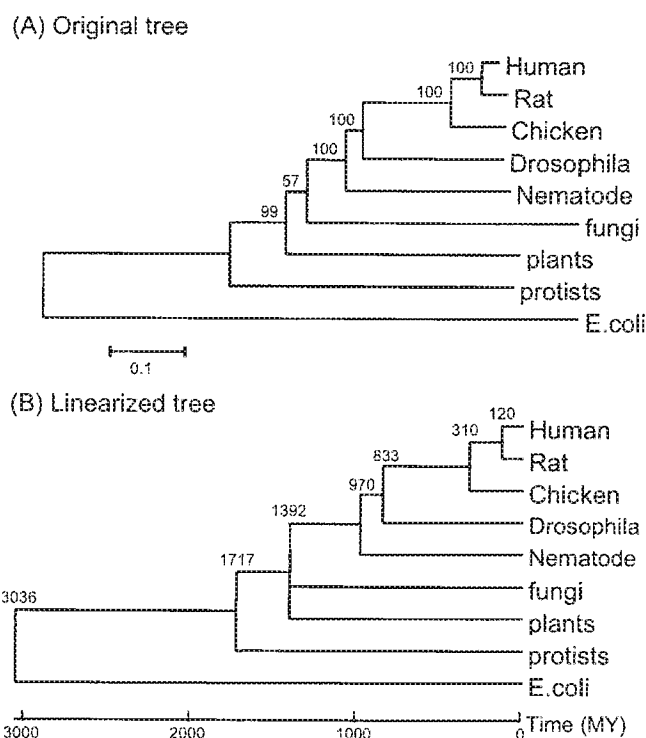


Fig. 4. (A) NJ tree constructed by using distance d_3 with $a = 1.24$ for 11 protein sequences. The numbers given for this tree stand for the bootstrap values (500 replications). (B) Linearized tree of the above NJ tree. The numbers given for this tree represent the estimates of divergent times.

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 archeobacterial 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 ($\pm$ standard errors) of divergence times (MY) of various organisms from the human lineage

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

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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