



08 May 2023

Dr. Paulette Gaynor
OFFICE OF FOOD ADDITIVE SAFETY (HFS-200)
CENTER FOR FOOD SAFETY AND APPLIED NUTRITION (CFSAN)
FOOD AND DRUG ADMINISTRATION
5001 CAMPUS DRIVE
COLLEGE PARK, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice for Miracle Fruit Powder

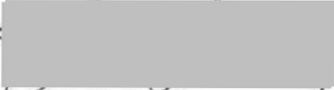
In accordance with 21 CFR §170 Subpart E consisting of § 170.203 through 170.285, Miracle Fruit Farm, LLC, as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the conclusion that Miracle Fruit Powder, is GRAS on the basis of scientific procedures, for use in water-based beverages. The proposed food uses of Miracle Fruit Powder are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*.

Information setting forth the basis for Miracle Fruit Farm's GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the Agency.

I certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection 12.1.5.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,


Erik Tietig
CEO
Miracle Fruit Farm, LLC

Email: erik@miraclefruitfarm.com
Tel: 305-345-8422

GRAS NOTICE FOR MIRACLE FRUIT POWDER

SUBMITTED TO:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:

Miracle Fruit Farm LLC
16300 SW 184th Street
Miami, FL
33187 USA

DATE:

08 May 2023

GRAS Notice for Miracle Fruit Powder

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GRAS Notice for Miracle Fruit Powder

PART 1 §170.225 SIGNED STATEMENTS AND CERTIFICATION

In accordance with Title 21 of the *Code of Federal Regulations* (CFR) §170 Subpart E consisting of §170.203 through 170.285, Miracle Fruit Farm, LLC ("Miracle Fruit Farm") hereby informs the United States (U.S.) Food and Drug Administration (FDA) that miracle fruit powder, as manufactured by Miracle Fruit Farm, is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Miracle Fruit Farm's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Miracle Fruit Farm, the undersigned hereby certifies that all data and information presented in this Notice represent a complete, representative, and balanced submission, and considered all unfavorable as well as favorable information known to Miracle Fruit Farm and pertinent to the evaluation of the safety and GRAS status of miracle fruit powder as a food ingredient for use in water-based beverages, as described herein.

Signed,



Erik Tietig
CEO
Miracle Fruit Farm, LLC

Date

5/5/23

1.1 Name and Address of Notifier

Miracle Fruit Farm LLC
16300 SW 184th Street
Miami, FL
33187 USA

1.2 Common Name of Notified Substance

Miracle fruit powder

1.3 Conditions of Use

Miracle fruit powder is intended to be added as an ingredient to water-based beverages at a use level of 50 ppm (0.005%). This use falls under the "Beverages and Beverage Bases" food category as defined under 21 CFR §170.3 (U.S. FDA, 2022a). Miracle fruit powder is not intended for use in infant formula or infant food products, and the proposed food category does not include food uses that are subject to the oversight by the United States Department of Agriculture (USDA) and its Food Safety Inspection Service (FSIS).

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30(a)(b) (U.S. FDA, 2022b), Miracle Fruit Farm has concluded that the intended use of miracle fruit powder as described herein is GRAS on the basis of scientific procedures.

1.5 Availability of information

The data and information that serve as the basis for this GRAS Notice will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Miracle Fruit Farm LLC
16300 SW 184th Street
Miami, FL
33187 USA

Should the U.S. FDA have any questions or additional information requests regarding this GRAS Notice, Miracle Fruit Farm will supply these data and information upon request.

1.6 *Freedom of Information Act, 5 U.S.C. 552*

It is Miracle Fruit Farm's view that all data and information presented in Parts 2 through 7 of this GRAS Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential; therefore, all data and information presented herein are not exempted from the *Freedom of Information Act, 5 U.S.C. 552*.

PART 2 §170.230 IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity and Composition of the Ingredient

Synsepalum dulcificum is an evergreen bush or tree native to tropical West Africa that grows up to 18 ft. tall (Chen *et al.*, 2006). The bright red fruit of this tree is small (2 to 3 cm) and is referred to as “miracle fruit,” “miraculous berry,” “sweet berry,” or “miracle berry” (Lipatova and Campolattaro, 2016). The first historical record of human consumption of miracle fruit dates back to the early 1700s in Ghana (Roeklein and Leung, 1987). Miracle fruit was introduced to the U.S. from Africa by the USDA in 1917, and since then, cultivation of this fruit and its uses in the U.S. has steadily grown. Miracle fruit is commercially available in the U.S. in different forms, as a fresh berry, a freeze-dried powder, or tablet in a variety of dietary supplement products.

The notified ingredient, miracle fruit powder, is minimally processed and produced by pulping, maceration, and freeze-drying of de-seeded miracle fruit berries without the use of any solvents or chemical processing aids. Miracle fruit powder is a red/red-brown to pink powder with an odor that is characteristic of the fruit. Miracle fruit powder has been fully characterized and is primarily comprised of carbohydrates (~87% on dry basis), protein (~6.6% on dry basis), ash (~3.9% on dry basis), and moisture (~3.6%). The ingredient is intended for use in water-based beverages for its taste-modifying effects, attributed to the active glycoprotein, miraculin, which accounts for approximately 0.1% (on dry basis) of the miracle fruit powder ingredient.

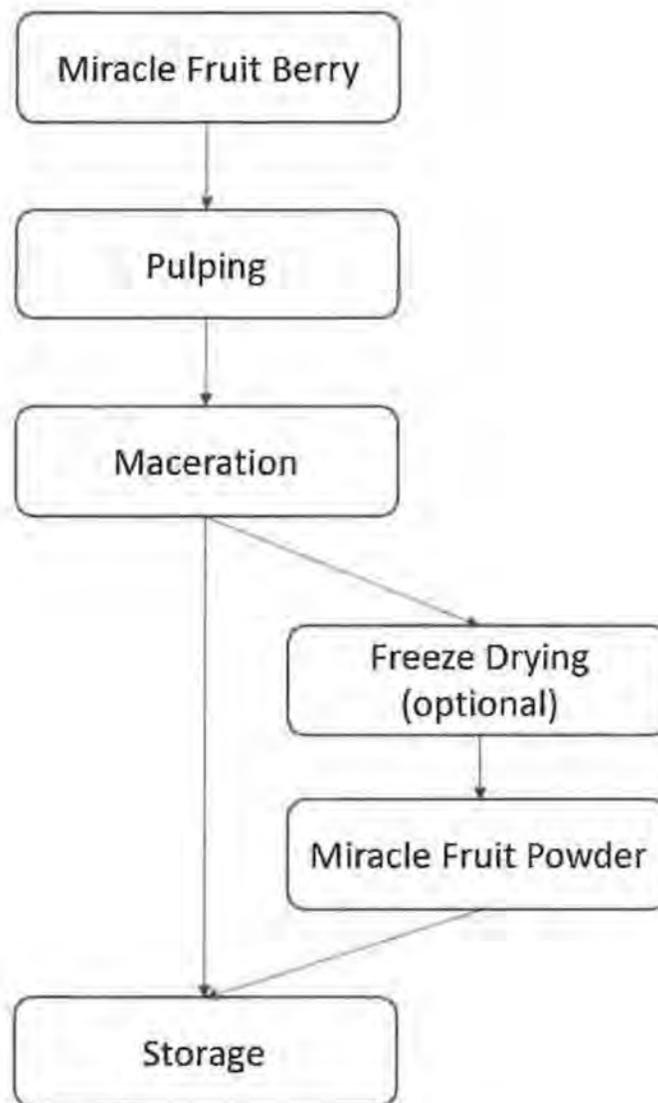
Miraculin is responsible for the taste-modifying effect of the miracle fruit by binding to the sweet receptors of the tongue, turning sour tastes into sweet (Morris, 1976). Miraculin is the largest known macromolecule that can affect taste perception (Lipatova and Campolattaro, 2016). Miraculin was first isolated in 1968 by researchers at Florida State University (Kurihara and Beidler, 1969), and was later purified and characterized by Theerasilp and Kurihara (1988). Miraculin exists naturally as a homodimer connected through a single interchain disulfide bond at Cys-138 and has a molecular weight of 24,600 Da (Theerasilp and Kurihara, 1988; Theerasilp *et al.*, 1989). Miraculin is expressed as a single polypeptide with 220 amino acids, containing 29 amino acid residues that are removed by post-translational processing. The peptide sequence of miraculin is publicly available on the UniProt/SwissProt database under Accession No. P13087.

2.2 Method of Manufacture

Miracle fruit powder is manufactured in accordance with current Good Manufacturing Practice (cGMP) and complies with the principles of Hazard Analysis and Critical Control Points (HACCP). A schematic overview of the production process is provided in Figure 2.2-1 below. The miracle fruit is grown on a bushy shrub maintained at a height of approximately 6 ft. Plants are grown for 3 to 4 years before they produce commercial volumes of miracle fruit. The fruit is grown year-round and harvested by hand picking under good agricultural practices. Following picking, the miracle fruit is washed using chlorinated municipal water and a food-grade vegetable wash (Regal Veggie Wash from Chem-tel, Inc.).

In the first step of the production process, the seed is removed from miracle fruit. The fruit is then pulped, macerated, freeze-dried, milled into a powder, and packaged. The production process includes quality control steps throughout to ensure that physical, chemical, and biological hazards are not introduced into the final product. For example, the process includes analysis during the freeze-drying step to ensure a reduction in water activity to control for microbiological hazards. Analytical data on potential impurities that may be introduced from the manufacturing process or carried over from the starting material demonstrate the absence of any chemical, toxicological, or microbiological hazards arising from the production process of miracle fruit powder that would have an adverse effect on human health (see Sections 2.4 and 2.5). The production process involves the use of physical processing steps without the use of any chemical solvents or processing aids. The final product, miracle fruit powder, is essentially the miracle fruit berry with water removed.

Figure 2.2-1 Flowchart for the Production Process of Miracle Fruit Powder



2.3 Product Specifications

Food-grade specifications for physical, chemical, heavy metal, and microbiological parameters have been established for miracle fruit powder (see Table 2.3-1). All methods of analysis are internationally recognized (e.g., Association of Official Analytical Collaboration [AOAC], U.S. FDA *Bacteriological Analytical Manual*) or have been developed internally and validated. The microbiological specifications for miracle fruit powder include control for standard microbial contaminants (e.g., total plate count, yeast and mold, *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes*), as well as additional specification limits for thermophilic acidophilic bacteria, guaiacol-producing bacteria, and heat-resistant mold for high acidic still beverages that are subject to heat treatment.

Table 2.3-1 Product Specifications for Miracle Fruit Powder

Specification Parameter	Specification Limit	Method of Analysis
Physical Parameters		
Appearance	Red/red-brown to pink powder	Visual
Odor	Characteristic of fruit	Sensory
Sensory (sweetness)	Sweetness induction of 0.15% citric acid solution equivalent to 3 to 7 Brix sucrose in water	Internal Method
Particle size	<420 µm	USP
Chemical Parameters		
Miraculin (dry basis)	≥0.048%	Internal Method (ELISA)
Carbohydrates (dry basis)	≥80%	Calculated
Total dietary fiber (dry basis)	≥5.5%	AOAC 991.43
Total fatty acids (dry basis)	≥0.4%	AOAC 996.06 AOCS Ce 2-66/Ce2b-11
Protein (dry basis)	≥4.5%	AOCS Ac 4-91
Ash (dry basis)	<6%	AOAC 923.03
Moisture	<6%	AOAC 934.03
Heavy Metals		
Arsenic	<0.1 ppm	AOAC 2011.19 (ICP-MS) AOAC 993.14 (ICP-MS)
Cadmium	<0.2 ppm	AOAC 2011.19 (ICP-MS) AOAC 993.14 (ICP-MS)
Lead	<0.1 ppm	AOAC 2011.19 (ICP-MS) AOAC 993.14 (ICP-MS)
Mercury	<0.1 ppm	AOAC 2011.19 (ICP-MS) AOAC 993.14 (ICP-MS)
Microbiological Parameters		
Total count	<3,000 CFU/g	FDA BAM
Mold	<300 CFU/g	FDA BAM
Yeast	<300 CFU/g	FDA BAM
<i>Escherichia coli</i>	Negative/g	FDA BAM
<i>Salmonella</i>	Negative/25 g	FDA BAM
<i>Listeria monocytogenes</i>	Negative/25 g	FDA BAM
Thermophilic acidophilic bacteria ^a	<1,000 CFU/g	IFU Method No. 12
Guaiacol-producing bacteria ^{a,b}	Absent	IFU Method No. 12

Table 2.3-1 Product Specifications for Miracle Fruit Powder

Specification Parameter	Specification Limit	Method of Analysis
Heat-resistant mold ^a	Absent	Chapter 22, Compendium of Methods for Microbiological Examination of Foods, 5th Ed, (2015).

AOAC = Association of Official Analytical Collaboration; AOCS = American Oil Chemists' Society; BAM = *Bacteriological Analytical Manual*; CFU = colony-forming units; ELISA = enzyme-linked immunosorbent assay; FDA = Food and Drug Administration; ICP-MS = inductively coupled plasma–mass spectrometry; IFU = International Fruit and Vegetable Juice Association; ppm = parts per million; USP = *United States Pharmacopela*.

^a Additional microbiological specification limit for high acidic still beverages subject to heat treatment.

^b Testing performed only if there is positive thermophilic acidophilic bacteria growth.

2.4 Product Analysis of Miracle Fruit Powder

Three to 9 non-consecutive lots of miracle fruit powder were analyzed to determine conformance to the established physical, chemical, heavy metal and microbiological parameters, as presented in Section 2.3. As summarized in the sections that follow, the results demonstrate that the manufacturing process, as described in Section 2.2, produces a consistent product that meets the established product specifications.

2.4.1 Physical Parameters

Analysis of 3 non-consecutive lots of miracle fruit powder (Lot Nos. 2019-09-0060, 2019-11-0060, and 2019-13-0060) demonstrates conformance to the established physical specification parameters (see Table 2.4.1-1).

Table 2.4.1-1 Analysis of Physical Parameters for 3 Non-Consecutive Batches of Miracle Fruit Powder

Specification Parameter	Specification Limit	Manufacturing Lot No.		
		2019-09-0060	2019-11-0060	2019-13-0060
Appearance	Red/red-brown to pink powder	Pink granular powder with black specks	Pink granular powder with black specks	Pink granular powder with black specks
Odor	Characteristic of fruit	Conforms	Conforms	Conforms
Sensory (sweetness)	Sweetness induction of 0.15% citric acid solution equivalent to 3 to 7 Brix sucrose in water	Conforms	Conforms	Conforms
Particle size	<420 µm	Conforms	Conforms	Conforms

2.4.2 Chemical Parameters

2.4.2.1 Proximates and Miraculin

Analysis of 9 non-consecutive lots of miracle fruit powder (Lot Nos. 2019-09-0060, 2019-11-0060, 2019-13-0060, KVS20200506AD_Powder 2020-19-001A, KVS20200506AE_Powder 2020-19-002B, KVS20200506AF_Powder 2020-19-003C, 20220608-A, 20220608-B, and 20220608-C) demonstrates conformance to the established specifications for proximates and miraculin content (see Table 2.4.2.1-1).

Table 2.4.2.1-1 Proximate Analysis of 9 Non-Consecutive Batches of Miracle Fruit Powder

Specification Parameter	Specification Limit	Manufacturing Lot No.								
		2019-09-0060*	2019-11-0060*	2019-13-0060*	KVS2020 0506AD Powder 2020-19-001A	KVS2020 0506AE Powder 2020-19-002B	KVS2020 0506AF Powder 2020-19-003C	202206 08-A	202206 08-B	202206 08-C
Carbohydrates (dry basis)	≥80%	90.8	90.7	90.8	86.2	85.4	86.9	83.91	86.06	84.21
Total dietary fiber (dry basis)	≥5.5%	13.0	13.6	13.3	10.9	11.8	10.2	16.00	16.40	16.30
Total fatty acids (dry basis)	≥0.4%	0.672	0.674	0.673	0.788	0.872	0.811	0.89	0.86	0.91
Protein (dry basis)	≥4.5%	5.16	5.23	5.14	7.56	8.34	7.22	7.01	6.98	6.92
Ash (dry basis)	<6%	3.38	3.38	3.40	5.45	5.41	5.11	3.66	3.27	3.22
Moisture	<6%	1.82	1.87	1.78	5.63	5.91	4.53	3.37	4.25	3.08
Miraculin (dry basis)	≥0.048%	0.055	0.052	0.054	0.28	0.24	0.32	0.12	0.14	0.13

*Values for Lots 2019-09-0060, 2019-11-0060, and 2019-13-0060 are corrected for moisture from "as is" data.

The carbohydrate profile of 3 non-consecutive batches of miracle fruit powder (Lot Nos. 20220608-A, 20220608-B, and 20220608-C) was analyzed (see Table 2.4.2.1-2). Most of the carbohydrate fraction is comprised of monosaccharides and disaccharides such as glucose, fructose, and sucrose (average of ~51.5%) and an average total dietary fiber content of ~16.2%. The average total organic acids content is ~16.4% and includes citric acid, malic acid, quinic acid, and pyruvic acid.

Table 2.4.2.1-2 Carbohydrate Profile and Organic Acid Content of 3 Non-Consecutive Batches of Miracle Fruit Powder

Parameter	Manufacturing Lot No.		
	20220608-A	20220608-B	20220608-C
Total Carbohydrate* (%)	83.91	86.06	84.21
Mono-/di-saccharide (% dry basis)	51.70	51.30	51.40
Glucose (% dry basis)	21.40	20.90	20.30
Fructose (% dry basis)	23.80	23.00	22.40
Sucrose (% dry basis)	6.50	7.40	8.70
Starch incl. maltodextrin (% dry basis)	0.70	0.60	0.60
Total dietary fiber (% dry basis)	16.00	16.40	16.30
Insoluble IDF (% dry basis)	10.50	11.30	11.20
Soluble SDFP+LMWSDF (% dry basis)	5.40	5.11	5.19
Total organic acid (% dry basis)	15.51	17.76	15.91
Citric acid	12.8	13.8	13
Pyruvic acid	0.0058	0.0057	0.0049
Malic acid	1.83	2.29	2.05
Quinic acid	0.875	0.888	0.851

Table 2.4.2.1-2 Carbohydrate Profile and Organic Acid Content of 3 Non-Consecutive Batches of Miracle Fruit Powder

Parameter	Manufacturing Lot No.		
	20220608-A	20220608-B	20220608-C
Succinic acid	ND ^{2a}	0.737	ND ^{2a}
Glycolic acid	ND ^{3a}	0.044	ND ^{4a}

LMWSDF = low molecular weight soluble dietary fiber; LOQ = limit of quantitation; ND = not detected; SDFP = soluble dietary fiber, which precipitates in the presence of 76% ethanol.

^a Sum of mono-/di-saccharide, starch incl. maltodextrin, total dietary fiber, and total organic acid.

ND¹: LOQ is 0.4950%; ND²: LOQ is 0.4840%; ND³: LOQ is 0.0289%; ND⁴: LOQ is 0.0320%.

2.4.2.2 Heavy Metals

Analysis of 3 non-consecutive lots of miracle fruit powder (Lot Nos. 2019-09-0060, 2019-11-0060, and 2019-13-0060) demonstrates conformance to the established heavy metal specification parameters (see Table 2.4.2.2-1).

Table 2.4.2.2-1 Analysis of Heavy Metals of 3 Non-Consecutive Batches of Miracle Fruit Powder

Specification Parameter	Specification Limit	Manufacturing Lot No.		
		2019-09-0060	2019-11-0060	2019-13-0060
Arsenic	<0.1 ppm	0.0359	0.0351	0.0353
Cadmium	<0.2 ppm	0.0480	0.0482	0.0476
Lead	<0.1 ppm	0.0110	0.00723	0.0105
Mercury	<0.1 ppm	<0.005	<0.005	<0.005

ppm = parts per million.

2.4.3 Microbiological Parameters

Analysis of 3 non-consecutive lots of miracle fruit powder (Lot Nos. 2019-09-0060, 2019-11-0060, and 2019-13-0060) demonstrates conformance to the established microbiological specification parameters (see Table 2.4.3-1).

Table 2.4.3-1 Microbiological Analysis of 3 Non-Consecutive Batches of Miracle Fruit Powder

Specification Parameter	Specification Limit	Manufacturing Lot No.		
		2019-09-0060	2019-11-0060	2019-13-0060
Total count	<3,000 CFU/g	30	35	70
Yeast	<300 CFU/g	ND ^a	40	40
Mold	<300 CFU/g	80	70	90
<i>Escherichia coli</i>	<3 MPN/g	<3 MPN/g	<3 MPN/g	<3 MPN/g
<i>Salmonella</i>	Negative/25 g	Negative/10 g	Negative/10 g	Negative/10 g
<i>Listeria monocytogenes</i>	Negative/25 g	Negative	Negative	Negative
Thermophilic acidophilic bacteria ^b	<1,000 CFU/g	Negative	Negative	Negative
Heat-resistant mold ^b	Absent	ND	ND	ND

CFU = colony-forming units; LOQ = limit of quantitation; MPN = most probable number; ND = not detected.

^a Additional microbiological analyses for high acidic still beverages subject to heat treatment.

^b LOQ is 10 CFU/g.

2.5 Additional Chemical Characterization of Miracle Fruit Powder

2.5.1 Polyphenols

Three non-consecutive lots of miracle fruit powder (Lot Nos. 20220608-A, 20220608-B, and 20220608-C) were analyzed for total polyphenol content spectrophotometrically using the Folin-Ciocalteu method (Singleton *et al.*, 1999) (see Table 2.5.1-1). The results demonstrate that miracle fruit powder contains small amounts of total polyphenols ranging from 1.13 to 1.25% as gallic acid equivalents (GAE). The polyphenol composition of miracle fruit powder was further characterized by high-performance liquid chromatography, which provided a detailed and comprehensive breakdown of the polyphenol composition of the miracle fruit. As presented in Table 2.5.1-1, the polyphenol content of miracle fruit powder is primarily comprised of anthocyanidins and glycosides, anthocyanosides, and catechins.

Table 2.5.1-1 Analyses and Characterization of 3 Non-Consecutive Batches of Miracle Fruit Powder for Total Polyphenols and Polyphenol Composition

Polyphenol*	Manufacturing Lot No.		
	20220608-A	20220608-B	20220608-C
Total Polyphenols (% as GAE)	1.17	1.2	1.25
Total anthocyanidins and total glycoside (%)	0.059	0.057	0.062
Total anthocyanidins (%)	0.034	0.032	0.033
Cyanidin (%)	0.033	0.031	0.032
Delphinidin (%)	0.0005	0.001	0.001
Total anthocyanosides (%)	0.025	0.025	0.03
Cyanidin-3-O-galactoside (%)	0.01	0.009	0.012
Cyanidin-3-O-glucoside (%)	0.016	0.016	0.018
Total catechins (%)	0.171	0.228	0.268
Catechin (%)	0.035	0.041	0.051
Epicatechin (%)	0.042	0.053	0.062
Gallocatechin (%)	0.011	0.016	0.015
Catechin gallate (%)	ND ^a	0.012	0.013
Epigallocatechin gallate (%)	0.015	0.022	0.026
Gallocatechin gallate (%)	0.069	0.084	0.101

GAE = gallic acid equivalents; HPLC = high-performance liquid chromatography; LOQ = limit of quantitation; ND = not detected.
^a LOQ is 0.010%.

*The total polyphenol content was analyzed spectrophotometrically using the Folin-Ciocalteu method and expressed as % GAE, while the polyphenol composition was determined by HPLC and expressed as %.

2.5.2 Antinutrients

Three non-consecutive lots of miracle fruit powder (Lot Nos. KVS20200506AD_Powder 2020-19-001A, KVS20200506AE_Powder 2020-19-002B, and KVS20200506AF_Powder 2020-19-003C) were analyzed for antinutrient content (phytic acid, oxalic acid, trypsin inhibitors). As demonstrated in Table 2.5.2-1, phytic acid and trypsin inhibitor content were below the limit of quantitation (LOQ) across the 3 lots tested, while the oxalic acid content ranged from 1,170 to 1,350 ppm.

Table 2.5.2-1 Analyses of 3 Non-Consecutive Batches of Miracle Fruit Powder for Antinutrients

Parameter	Manufacturing Lot No.		
	KVS20200506AD_Powder 2020-19-001A	KVS20200506AE_Powder 2020-19-002B	KVS20200506AF_Powder 2020-19-003C
Oxalic acid (ppm, dry basis) ^a	1,180	1,350	1,170
Phytic acid (mg/g, dry basis) ^b	ND ¹	ND ¹	ND ²
Trypsin inhibitor (TIU/mg, dry basis) ^c	ND ³	ND ⁴	ND ⁵

AOAC = Association of Official Analytical Collaboration; AOCS = American Oil Chemists' Society; LOQ = limit of quantitation; ND = not detected; ppm = parts per million; TIU = trypsin inhibitor units.

^a Method of analysis: AOAC 986.13 (modified).

^b Method of analysis: Lehrfeld (1989, 1994).

^c Method of analysis: AOCS Ba 12-75, Hamerstrand *et al.* (1981) (modified).

ND¹: LOQ is 1.06 mg/g; ND²: LOQ is 1.05 mg/g; ND³: LOQ is 0.530 TIU/mg; ND⁴: LOQ is 0.531 TIU/mg; ND⁵: LOQ is 0.524 TIU/mg.

Three non-consecutive lots of miracle fruit powder (Lot Nos. KVS20200506AD_Powder 2020-19-001A, KVS20200506AE_Powder 2020-19-002B, and KVS20200506AF_Powder 2020-19-003C) were analyzed for the presence of secondary plant compounds, specifically pyrrolizidine alkaloids or tropane alkaloids using liquid chromatography with tandem mass spectrometry (LC-MS/MS). The reporting limit ranged from 1 to 2 µg/kg. The levels of each compound, including the sum of all pyrrolizidine alkaloids or tropane alkaloids, were below the reporting limit (see Appendix A for a complete list of analyzed alkaloids).

2.5.3 Pesticides

Analysis for residual pesticides was conducted on 3 non-consecutive lots of miracle fruit powder (Lot Nos. 2019-09-0060, 2019-11-0060, and 2019-13-0060) using a method based on AOAC 2007.01 and CEN Standard method EN 15662. The samples were prepared and analyzed by gas chromatography with tandem mass spectrometry or LC-MS/MS. The typical LOQs were in the range of 0.01 and 0.05 mg/kg. The pesticide content was below the LOQ across all tested batches, indicating the absence of pesticides in the final product.

2.5.4 Mycotoxins and Other Secondary Metabolites

Analysis for mycotoxins and other secondary metabolites was conducted on 3 non-consecutive lots of miracle fruit powder (Lot Nos. 2019-09-0060, 2019-11-0060, and 2019-13-0060) using ultra-high-performance liquid chromatography with tandem mass-spectrometry (UHPLC-MS/MS). The UHPLC-MS/MS method was based on the method described by Varga *et al.* (2012). The results are summarized in Table 2.5.4-1 and demonstrate that the levels of these mycotoxins and other secondary metabolites were below each respective LOQ in the final product.

Table 2.5.4-1 Analysis for Mycotoxins and Other Secondary Metabolites of 3 Non-Consecutive Batches of Miracle Fruit Powder

Mycotoxin/Secondary Metabolite (ng/g)	LOQ (ng/g)	Manufacturing Lot No.		
		2019-09-0060	2019-11-0060	2019-13-0060
Aflatoxin B1	0.5	ND	ND	ND
Aflatoxin B2	0.5	ND	ND	ND
Aflatoxin G1	0.5	ND	ND	ND
Aflatoxin G2	0.5	ND	ND	ND
Aflatoxin M1	0.5	ND	ND	ND

Table 2.5.4-1 Analysis for Mycotoxins and Other Secondary Metabolites of 3 Non-Consecutive Batches of Miracle Fruit Powder

Mycotoxin/Secondary Metabolite (ng/g)	LOQ (ng/g)	Manufacturing Lot No.		
		2019-09-0060	2019-11-0060	2019-13-0060
Aflatoxin M2	0.5	ND	ND	ND
Deoxynivalenol	100	ND	ND	ND
T-2 Toxin	10	ND	ND	ND
HT-2 Toxin	100	ND	ND	ND
Fumonisin B1	25	ND	ND	ND
Fumonisin B2	25	ND	ND	ND
Ochratoxin A	1	ND	ND	ND
Zearalenone	30	ND	ND	ND

LOQ = limit of quantitation; ND = not detected.

2.6 Stability of Miracle Fruit Powder

The stability of miracle fruit powder (Lot Nos. 2019-09-0060, 2019-11-0060, and 2019-13-0060) was investigated in a 52-week shelf-life stability study. Samples of miracle fruit powder were stored at $25 \pm 2^\circ\text{C}$ and 60% relative humidity in metalized barrier pouches for 52 weeks. The miraculin content and moisture content of each sample were measured at 0, 2, 4, 8, 14, 26, 39, and 52 weeks. The data indicate that miracle fruit powder is stable for up to 52 weeks when stored at ambient temperature and humidity.

Table 2.6-1 Shelf-life Stability Results of Miracle Fruit Powder (Lot Nos. 2019-09-0060, 2019-11-0060, and 2019-13-0060) Stored at $25 \pm 2^\circ\text{C}/60\%$ Relative Humidity for 52 Weeks

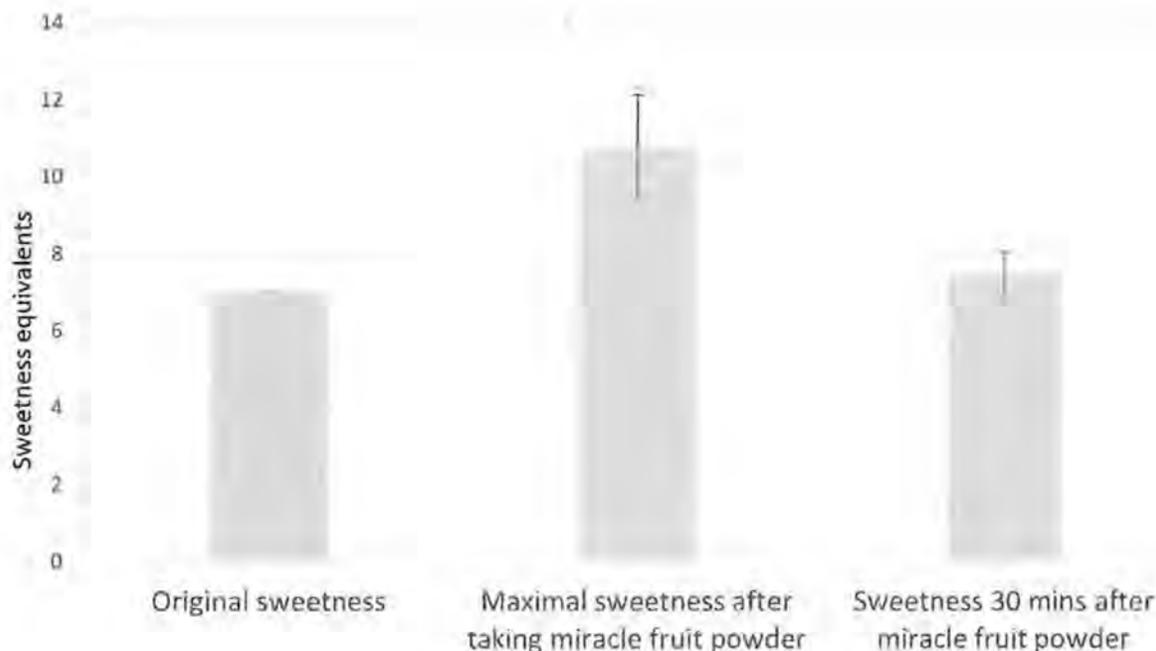
Parameter	Manufacturing Lot No.		
	2019-09-0060	2019-11-0060	2019-13-0060
Moisture (%)			
0	1.82	1.87	1.78
2	1.75	1.78	1.76
4	1.54	1.58	1.53
8	2.06	2.02	1.84
14	2.43	2.26	2.10
26	3.36	3.39	2.80
39	4.13	4.20	4.23
52	4.78	4.69	4.50
Miraculin ($\mu\text{g/g}$)^a			
0	545	519	538
2	554	517	543
4	555	531	550
8	684	614	627
14	645	595	612
26	581	592	679
39	524	545	557
52	487	495	519

^a Average miraculin content based on 3 replicates. All values are on as-is basis.

2.7 Technical Effect

Miracle fruit powder will be marketed in powdered form and added to beverage products for its ability to impart sweetness by modifying taste from sour to sweet due to the active glycoprotein, miraculin. The sweetness profile of miracle fruit was evaluated by 6 trained panelists (Tafazoli *et al.*, 2019). A baseline sweetness intensity was established with lemonade juice with a sweetness intensity of 7 Brix. Following establishment of a baseline sweetness intensity, each panelist consumed 0.08 g of miracle fruit powder and was instructed to hold the powder in the mouth for 1 minute before swallowing. Each panelist then consumed 60 mL of the original lemonade juice every 5 minutes for 30 minutes, and the sweetness of each cup was recorded. The results expressed as sweetness equivalency are summarized in Figure 2.7-1 below. The results indicate that miracle fruit significantly increased the perceived sweetness of lemonade juice, and sweetness of the juice returned to baseline levels in all subjects after 30 minutes. These results indicate the taste-modifying effect of miraculin is rapid with no lasting desensitization effect. It is noted that this sensory study was conducted with a miracle fruit powder amount (0.08 g) that is expected to be much greater than the amounts proposed for use in water-based beverages (50 ppm) and would reflect a "worst-case" effect scenario. In another published study evaluating the taste-modifying effect of miraculin, the maximum relative sweetening effect was achieved within 3 minutes of consumption, and rapidly declined after 30 minutes. These effects were concentration-dependent (Kurihara and Beidler, 1969).

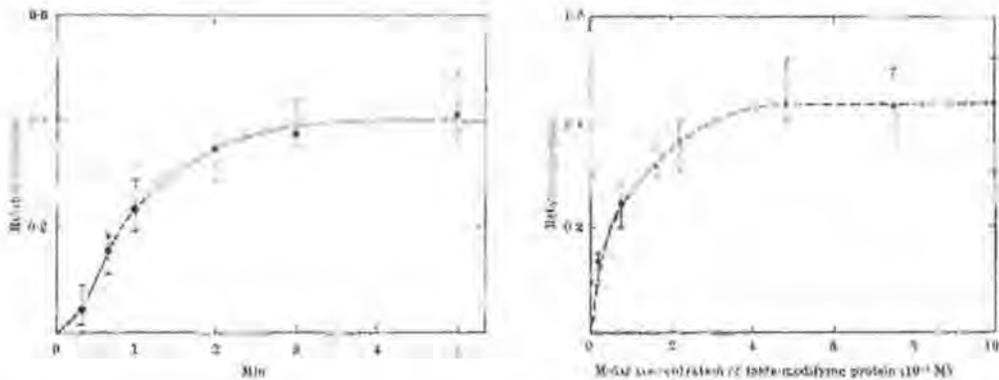
Figure 2.7-1 Sweetness Intensity of Miracle Fruit Powder by Trained Panelists (n=6)



2.7.1 Effect of Concentration and Time on Miraculin's Perceived Sweetness in Humans

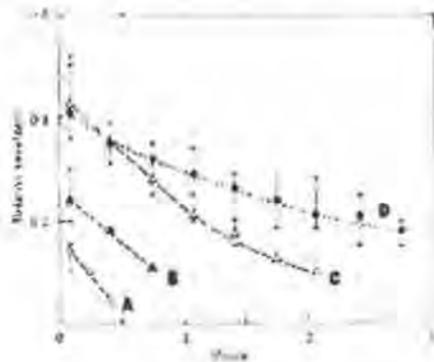
Studies by Kurihara and Beidler (1969) confirmed miraculin's taste-modifying properties of any sour substance over a wider range of concentrations reaching a maximum level of sweetness at 4×10^{-7} M (400 nM) equal to the sweetness of a 0.4 M sucrose solution. Higher miraculin concentrations had no further impact of the perceived sweetness of a 0.02 M citric acid solution. Almost half-maximal sweetness was already reached after holding miraculin for only 1 minute in the mouth followed by a rinse with a 0.02-M citric acid solution. This demonstrates that brief exposure to miraculin is sufficient to perceive its taste-modifying property (see Figure 2.7.1-1, adapted from Kurihara and Beidler, 1969).

Figure 2.7.1-1 Concentration- and Time-dependent Effect of Miraculin on Relative Sweetness



Similar to the perceived sweetness, the persistency of the effect is a function of miraculin concentration. While high concentrations of miraculin induce long-lasting sweetness for several hours, concentrations as low as 2×10^{-8} M (20 nM) do not elicit any effect after 20 minutes. It can therefore be assumed that even lower concentrations, well below 20 nM, would lead to a marked less long-lasting sweetness induced by miraculin. Importantly, the authors stated that induced secretion of saliva led to an even faster decline of the sweetening effect, arguing against a strong persistence of miraculin in the oral cavity (see Figure 2.7.1-2, adapted from Kurihara and Beidler, 1969).

Figure 2.7.1-2 Latency of Sweetening Effect of Miraculin Based on Concentration A (2×10^{-8} M), B (8×10^{-8} M), C (4.8×10^{-7} M), and D (2.3×10^{-6} M)



2.7.2 Effect of Miraculin on Other Taste Qualities

Kurihara and Beidler (1969) also investigated the effect of miraculin on the taste thresholds of other taste qualities such as bitterness, saltiness, sweetness, and sourness. Taste thresholds of different subjects were determined before and after application of 2×10^{-6} M (2 μ M) taste-modifying protein in the mouth. No impact of miraculin on the different taste qualities was observed. The perceived sweetness of sucrose was also not impacted by miraculin. Using gymnemic acid, a sweet taste blocker, Kurihara and Beidler (1969) demonstrated that the sourness of citric acid is not blocked by miraculin. Gymnemic acid blocked the taste-modifying effect of miraculin, resulting in a still perceivable sour taste of citric acid even after application of 2×10^{-6} M (2 μ M) taste-modifying protein.

The possible impact of miraculin on other taste qualities has also been investigated by other groups. Capitanio *et al.* (2011) investigated the changes induced by 25 mg of a freeze-dried miraculin extract on the gustatory sensations evoked by citric acid 0.01 M (sour), caffeine 0.005 M (bitter), NaCl 0.034 M (salty), and sucrose 0.037 M (sweet). Consistent with the previously reported data, Capitanio *et al.* (2011) demonstrated that the pre-exposure to miraculin had no effect on bitter, salty, or sweet taste. In addition, the effect of miraculin on the taste sensation of binary and ternary mixtures was investigated in the presence of citric acid (Capitanio *et al.*, 2011). In all mixtures, a reduction in sourness and an increase in sweetness were observed, thus demonstrating the typical effect of miraculin also on mixtures containing a sour stimulus. A decrease of saltiness and bitter was observed in binary mixtures containing NaCl or caffeine. This effect was likely due to an increase in sweet taste which masks other taste qualities. Importantly, the binary mixture containing citric acid and sucrose showed a marked increase in sweetness after exposure to miraculin, demonstrating that the taste-modifying activity of miraculin does not negatively impair sweet perception but rather gives additional sweetness to an already sweet stimulus.

The taste-modifying effect of miracle fruit berries was also investigated as part of a *Sensation and Perception* course offered at Christopher Newport University (Lipatova and Campolattaro, 2016). Authors reported the findings from 1 semester involving 19 students. Each individual was provided 1 to 2 fresh berries obtained from Miracle Fruit Farm. One berry was reported to elicit an effect lasting approximately 30 minutes. Individuals were instructed to chew the berry thoroughly to coat the entire membrane of their tongue with the pulp of the fruit (approximately 30 seconds of chewing). The seed was discarded. Following consumption of the berries, each individual was provided 4 food products targeted for different taste receptors (sweet, sour, salty, and bitter). These included jellybeans (sweet), lemon wedges (sour), crackers (salty), and raw broccoli (bitter). In addition to these food items, grapefruit, limes, green apple, sour candy, and apple cider were provided to further investigate the effect on the sour receptor. The taste perception was scored on a 0 to 10 scale, with 0 being not sour, bitter, or sweet, and 10 being very sour, bitter, or sweet. The palates of each individual were rinsed with water after each tasting. The authors reported 1 individual not experiencing any change in taste perception following consumption of miracle fruit. In the other individuals, miracle fruit did not significantly alter the perception of salty or bitter tastes. The perceived sweetness of each acidic food item (grapefruit, limes, green apple, sour candy, apple cider and lemon) were significantly increased. The authors also reported a significant decrease in perception of sour intensity of the same food items following consumption of miracle fruit, an effect similar to what was previously observed by Capitanio *et al.* (2011). The authors reported that miracle fruit does not have any physical effect on the sour taste receptor, and the change in perception of sourness is due to the psychophysical nature of the human senses.

Another study investigated the effect of miracle fruit on 12 typical foods that have sweet, sour, salty, bitter, or umami as their primary taste (Okamoto *et al.*, 2018). In all 8 sour foods, the sweet taste intensity significantly increased, and the sour taste intensity significantly decreased after tasting miracle fruit. In 5 sour foods, a decrease in bitter taste intensity and an increase in umami taste intensity were observed, as well as a decrease in sour taste intensity and an increase in sweet taste intensity. A further increase in sweet taste intensity was also observed in non-sour foods. Palatability increased in the 8 sour foods, whereas no changes in palatability were observed in sweet, salty, bitter and umami foods. A reduced bitterness of bitter tasting foods and an increase of sweetness in already sweet tasting products after consumption of miraculin/miracle fruit is similar to the findings by Capitanio *et al.* (2011).

Taken together, these human sensory studies confirmed the increase of sweetness and simultaneous decrease of sourness of sour tastants after pre-exposure with miraculin. However, there is striking evidence that miraculin has no effect on single bitter, salty, and sweet stimuli rendering it very unlikely that miraculin acts on the biological targets responsible for the detection of these taste qualities. Nevertheless, an intense increase of sweetness after exposure to miraculin combined with a lack of an effect on sourness can cause a diversion of the overall flavor from the expected traditional flavor in complex food products.

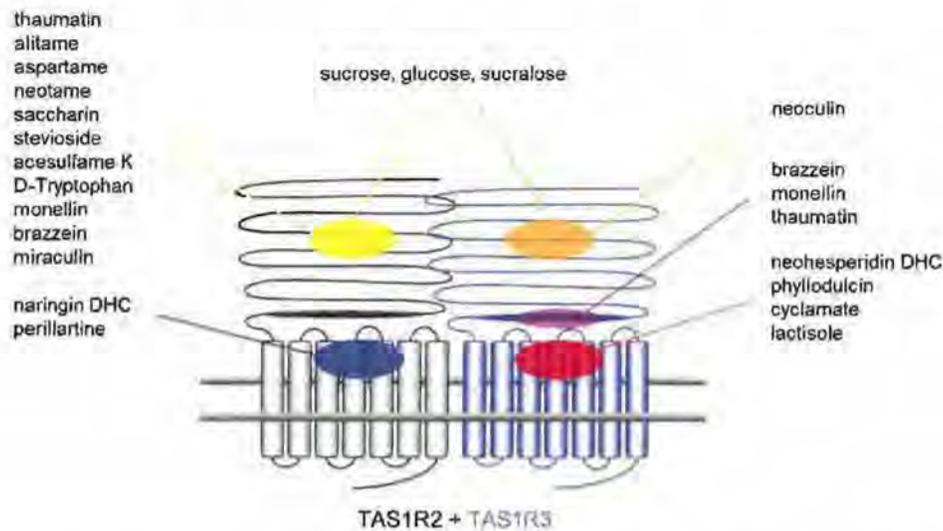
In summary, the above-described human sensory studies indicate that miraculin does not impact the overall taste profile and does not interfere with other taste qualities such as bitter and salty and has little or no impact on the taste profile of foods consumed with or following miraculin consumption. Those human sensory studies conducted by Kurihara and Beidler (1969) indicated that the taste-modifying effect of miraculin dissipates quickly at low concentrations of 20 nM and knowing the correlation between concentration and affinity at the receptor, it may be assumed that the residence time of miraculin will be even shorter at concentrations of between 0.4 and 4 nM that are aligned with the proposed uses of miracle fruit powder in the water-based beverages.

2.7.3 Interaction of Miraculin with Human Sweet Taste Receptor, Biological Mechanism, and Effect

The human sweet taste receptor is a large heterodimeric protein, consisting of 2 different class C G-protein coupled receptors (GPCRs): TAS1R2 and TAS1R3. This receptor has been studied and characterized at a molecular level by functional expression of both subunits in heterologous cell systems. It has been demonstrated that this single receptor responds to all sweet stimuli tested, having multiple different binding sites facilitating allosteric interactions among them.

The human sweet taste receptor is known to recognize a broad spectrum of chemically distinct compounds ranging from sugars and low-molecular-weight sweeteners, such as sucrose and aspartame, to large, sweet-tasting proteins, such as brazzein and thaumatin (Temussi, 2002; Masuda *et al.*, 2012). Using both molecular modeling-based docking simulations and site-directed mutagenesis studies, it was possible to identify that the sweet receptor orthosteric-binding site exists in the cleft between the 2 lobes of the VFD, in this case on TAS1R2 (Masuda *et al.*, 2012; Neiers *et al.*, 2016; Chéron *et al.*, 2017; Kashani-Amin *et al.*, 2019). This TAS1R2 subunit was also found to be crucial for the activation of low-calorie sweeteners such as saccharin, acesulfame K, aspartame, and stevioside (Xu *et al.*, 2004; Zhang *et al.*, 2010; Masuda *et al.*, 2012). To date, the modeling experiments have identified at least 5 different sweet receptor allosteric binding sites as being involved in sweetener recognition (Jiang *et al.*, 2004; Xu *et al.*, 2004; Koizumi *et al.*, 2007; Winnig *et al.*, 2007; Masuda *et al.*, 2012) (see Figure 2.7.3-1).

Figure 2.7.3-1 Binding Mechanism of Various High-Intensity Sweeteners

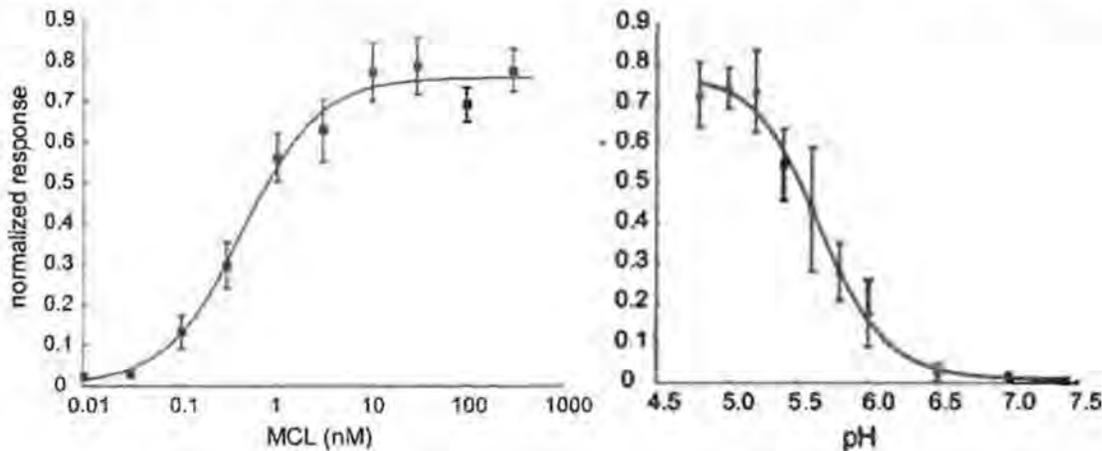


The N-terminal domain of the TAS1R2 receptor is considered important in the recognition of many chemically diverse sweeteners, including carbohydrate sweeteners, low-calorie sweeteners, and sweet proteins, including brazzein, monellin, and the flavor modifier, miraculin. Although these proteins are large-mass molecules, they do not share sequence similarity among each other, which suggests that they do not bind in a comparable way to the sweet taste receptor. Overall, the binding data indicate that despite a general activation of the sweet taste receptor, the site of interaction of sweet proteins with the receptor is very different.

Because of the high molecular weight of miraculin, it is unlikely that the protein penetrates taste cells but rather interacts with membrane-bound proteins. Koizumi *et al.* (2011) reported that the human sweet taste receptor mediates acid-induced sweetness of miraculin. In their experiments, Koizumi *et al.* (2011) replicated previously done human sensory studies. They functionally expressed TAS1R2/TAS1R3 in heterologous cells and incubated these cells with different concentrations of miraculin. After 30 minutes of incubation, the cells were washed, and citric acid was applied at different concentrations. The application of citric acid after pre-incubation with miraculin led to a pronounced sweetness response in cells expressing TAS1R2/TAS1R3. Cells not expressing a functional sweet receptor did not respond. These findings mimicked the human sensory studies and demonstrated that the taste-modifying effect of miraculin is mediated by the human sweet taste receptor TAS1R2/TAS1R3. These findings were later corroborated by Sanematsu *et al.* (2016).

Koizumi *et al.* (2011) also investigated the effect of miraculin concentrations and pH on the activation strength of the sweet receptor under *in vitro* conditions. Similar to the existing human sensory data, the authors demonstrated that activation of the receptor is dependent on the concentration of miraculin and pH (see Figure 2.7.3-2, adapted from Koizumi *et al.*, 2011). No activation of the sweet taste receptor was observed at neutral pH. As shown in Figure 2.7.3-2, miraculin concentrations as low as 0.1 nM are sufficient to activate the receptor to approximately 15 to 20% (as measured by cellular response to the protein), whereas activity saturation is reached at 10 nM.

Figure 2.7.3-2 Effect of Miraculin Concentration and pH on Activation of the Sweet Taste Receptor



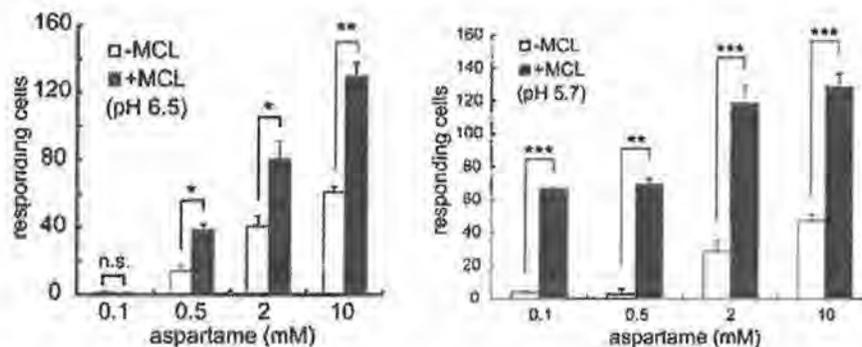
In sensory studies, the taste-modifying effect of miraculin begins from a few seconds to several minutes after consumption until the taste buds are amply coated by miraculin. The effect then typically lasts from 30 minutes to 2 hours with potency decreasing over time, or until the miraculin is eliminated by saliva (Faus, 2000; Asakura *et al.*, 2011). Koizumi *et al.* (2011) mimicked this effect by pre-incubating TAS1R2/TAS1R3 expressing cells with miraculin, washing it out and repeatedly stimulating the cells with acidic buffer for about 15 to 20 minutes, thereby always observing receptor activation. These results indicate that miraculin remains bound and can activate the receptor repeatedly. However, it is noteworthy that the authors used 100 nM miraculin to demonstrate this effect. As their own data implies, this is 10x higher than the observed saturation at the receptor level (see Figure 2.7.3-2). This finding demonstrates that concentration is a key determinant of affinity and residence time of compound/receptor complexes (de Witte *et al.*, 2018). The concentration-response curve of miraculin from Figure 2.7.3-2 indicates a reduced receptor activation/reduced affinity at concentrations below 10 nM. These lower concentrations of miraculin, leading to a lower affinity of miraculin at the receptor, are suggestive of a significantly reduced or even absent receptor activation.

Koizumi *et al.* (2011) also studied the influence of miraculin on the activation of the sweet receptor by several diverse sweeteners. To do so, sweet receptor expressing cells were stimulated with 30 nM miraculin in the presence of the investigated sweeteners. The experiments were performed at neutral pH 7.4 where miraculin itself is not active (see Figure 2.7.3-3). In all cases, significantly reduced receptor responses to the sweeteners were observed in the presence of miraculin. These findings suggest that although not active at pH 7.4, miraculin may interact with the sweet receptor thereby attenuating the response to other sweeteners. These data contrast with human sensory data by Kurihara and Beidler (1969) and Capitano *et al.* (2011), which show no effect, or a positive effect, on the perceived sweetness of other sweeteners and implies differences between *in vivo* and *in vitro* situations.

It is known that the pH of human saliva differs depending on the individual. Saliva has a pH normal range of 6.2 to 7.6 with 6.7 being the average pH (Baliga *et al.*, 2013). Therefore, a difference in pH between a well standardized *in vitro* buffer at pH 7.4 and slightly acidic conditions of saliva present in human sensory tests might explain the discrepancies observed between the receptor studies and human sensory data.

Using aspartame as an example, Koizumi *et al.* (2011) demonstrate that a slight shift into acidic conditions (pH 6.5) transforms miraculin from a sweet taste “blocker” into a sweet taste “enhancer” (see Figure 2.7.3-3, adapted from Koizumi *et al.*, 2011). This positive modulatory activity is further increased by lowering the pH to 5.7. In summary, at slightly acidic conditions (pH 6.5) miraculin not only does mildly activate the sweet receptor, but also acts in strong synergy with other sweeteners as shown for aspartame.

Figure 2.7.3-3 Effect of Aspartame on Sweetening Effect of Miraculin



While those *in vitro* studies conducted by Koizumi *et al.* (2011) reported repeated receptor activation, even after washing the cells, resulting in a high persistence of miraculin at the receptor, it should be noted that the authors used 10-fold higher concentrations (100 nM) than what was required to elicit full receptor activation (10 nM). In addition, the concentrations used in the *in vitro* studies by Koizumi *et al.* (2011) are around 40-fold higher than those which will be used in the water-based beverages and therefore are not considered relevant or appropriate for establishing receptor binding effects following the consumption of a water-based beverage.

2.7.4 Sensory Studies Conducted with Miracle Fruit Powder in Humans

As discussed in Sections 2.7.1 through 2.7.3, the available published human sensory studies and *in vitro* receptor studies conducted with miraculin indicate that its flavor-modifying effects (conversion of sour to sweet) are both concentration and time dependent. However, these studies were conducted at concentrations that were much higher than the proposed uses of miracle fruit powder in water-based beverages and therefore were not considered relevant to the conditions of use of miracle fruit powder. Considering this—and to fully investigate the sensory effects of miraculin in miracle fruit powder under the conditions of intended use—a series of 3 human sensory studies were undertaken to investigate the following:

1. Duration of any taste-modifying effects of miraculin in miracle fruit powder and whether the effects are dose-dependent (Experiment 1);
2. Impact of repeated stimulation with miracle fruit powder on the taste-modifying effects of citric acid, and the duration of this effect, if any (Experiment 2); and
3. Receptor binding of miraculin in miracle fruit powder and if the binding would impact sweetness perception of other sweeteners at neutral pH (Experiment 3).

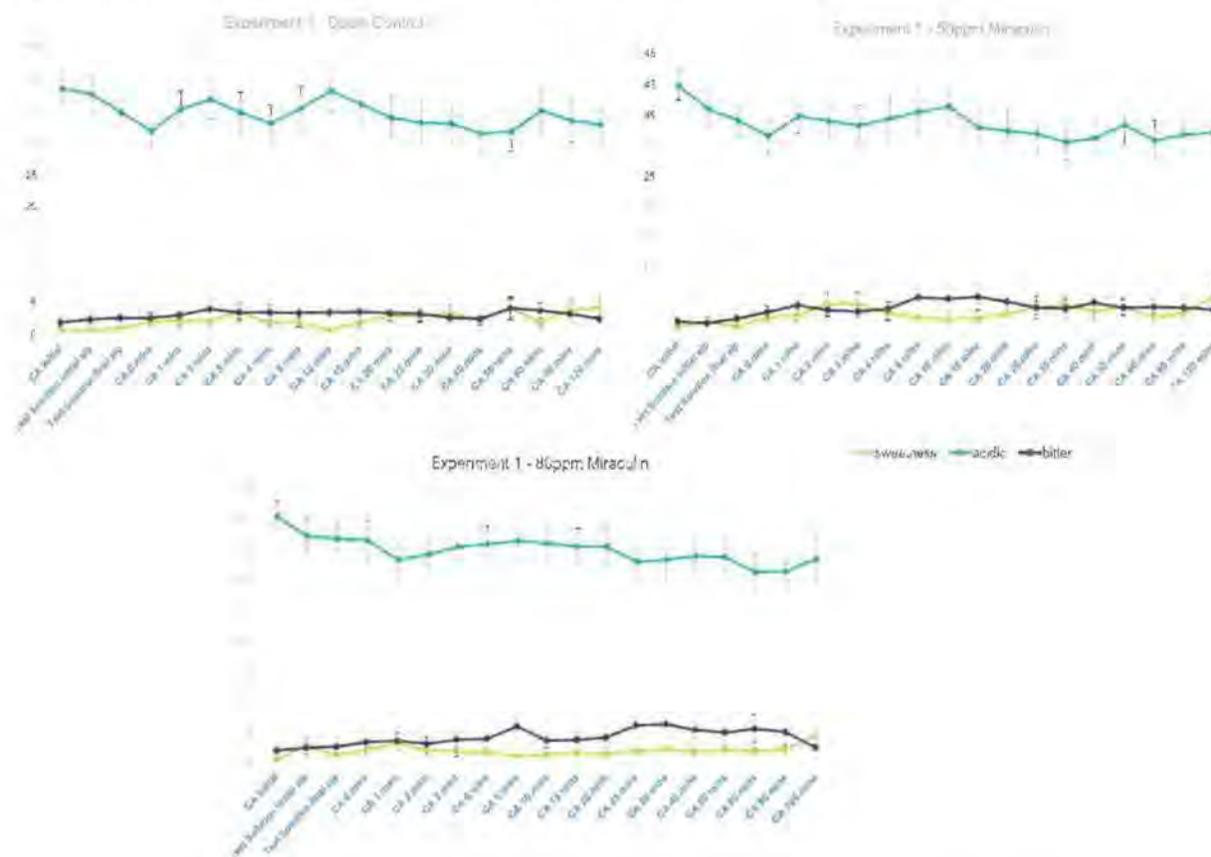
These experiments were performed with miracle fruit powder in a water-based beverage at concentrations of 50 ppm or 80 ppm, consistent with the proposed conditions of use of the miracle fruit powder. The experiments are described in the following sections

2.7.4.1 Duration and Dose Dependency of Taste-modifying Effects of Miracle Fruit Powder

Two concentrations (50 ppm and 80 ppm) of miracle fruit powder were added to a model soft drink formulation (citric acid/sodium citrate buffer) to represent a future water-based beverage. A control beverage (not containing miracle fruit powder) was included to assess for the potential degree of effect. The panelists (N=14) provided basic taste ratings of sweet, acidic, and bitter following consumption of a pre-test solution of a 0.1% citric acid solution. The panelists then consumed 100 mL of the miracle fruit powder-containing beverage over 2 minutes to approximate typical soft drink consumption behavior. They were asked to rate basic tastes (sweetness, acidity, or bitterness) at the beginning and end of the 2-minute consumption period. Following consumption of the miracle fruit powder-containing beverage, post-test solutions of 0.1% citric acid were assessed immediately (time 0) and after 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, and 120 minutes. Panelists were asked to provide basic taste ratings (sweetness, acidity, or bitterness). Three replicate sessions were carried out for each miracle fruit powder concentration in the test solution.

The results clearly demonstrate that the mean sweetness and bitterness ratings of citric acid solutions tested immediately and up to 2 hours after tasting the buffer test solution remained low (under 6 on the 0–100-point scale) for all 3 concentrations of miracle fruit powder (0, 50, and 80 ppm) (see Figure 2.7.4.1-1). There was no significant difference between the ratings for sweetness or bitterness when miracle fruit powder present in the model soft drink was compared with the corresponding control ratings throughout the testing period. Furthermore, mean acidity ratings of the citric acid solutions varied between 30 and 40 on the 100-point scale. There was no significant difference between the ratings for acidity when miracle fruit powder present in the model soft drink was compared with the corresponding control ratings throughout the testing period. In addition, at all 16 time points for each basic taste, there were no significant difference between miracle fruit powder concentrations. The results clearly demonstrate no detectable taste-modifying effects of citric acid solutions when consumed after ingestion of miracle fruit powder at either 50 or 80 ppm in a model soft drink formulation.

Figure 2.7.4.1-1 Mean Scores for Sweetness, Bitterness, and Acidity



2.7.4.2 Effects of Continued Stimulation with Miracle Fruit Powder on Taste Modification

Miracle fruit powder was added to a model soft drink formulation (citric acid/sodium citrate buffer) at a concentration of 80 ppm. The beverage containing miracle fruit powder was evaluated in comparison to a control beverage containing 0 ppm miracle fruit powder in buffer. Similar to Experiment 1, prior to tasting the test solution, panelists (N=12) were asked to assess a 0.1% citric acid solution (pre-test solution) and rate the intensity of sweetness, acidity, or bitterness. Similarly, panelists were presented with 100 mL of the first test solution and asked to consume it over a 2-minute period. They were asked to rate the same basic taste qualities at the beginning and end of the 2-minute period. Following consumption of the miracle fruit powder beverage, post-test solutions of 0.1% citric acid were then assessed immediately (time 0) and after 5, 10, 15, 20, 30, and 40 minutes for the same basic taste ratings. This same procedure was adopted after 45 minutes, when the panel was presented with a second test solution. Again, basic taste ratings were given for the test solution and for post-test citric acid solutions after 5, 10, 15, 20, 30, and 40 minutes. The same procedure was further conducted at 90 minutes, although the basic taste ratings were conducted after 5, 10, 15, 20, 30, 40, 50, 60, 90, and 120 minutes. Triplicate sessions were carried out for both the control and test beverage.

The mean sweetness rating results following the consumption of citric acid solutions after the first, second, and third ingestions of the buffer test solution remained low (under 3 on the 0–100-point scale) for both 0 ppm (control) and 80 ppm miracle fruit powder (see Figure 2.7.4.2-2). Similarly, the mean bitterness ratings of citric acid solutions tested after the first, second, and third ingestions of the buffer test solution also remained low (under 10 on the 0–100-point scale) for both 0 ppm (control) and 80 ppm miracle fruit powder. Likewise, the mean acidity ratings of the citric acid solutions varied between 33 and 45 on the 100-point scale for the control and miracle fruit powder beverage.

Given the high number of time points tested over 3 phases of repeated administration, 1 or 2 significant differences in mean sweetness, bitterness, and acidity ratings to post-test citric acid solutions were observed following analysis of variance (ANOVA) at the individual time points, comparing the control and 80 ppm miracle fruit powder concentrations. However, these findings were not consistent across the 3 repeated administrations and are therefore considered to be spurious, especially as out of the 204 different tests conducted, only 13 (6.4%) were significant; a little above 5% which would be expected by chance alone. Overall, the results indicate that repeated stimulation of the receptor, with 80 ppm miracle fruit powder in a model soft drink, has no taste-modifying effects on citric acid solutions tested post-ingestion.

Figure 2.7.4.2-2 Effect of Miracle Fruit Powder (80 ppm) on Acidity, Sweetness, and Bitterness

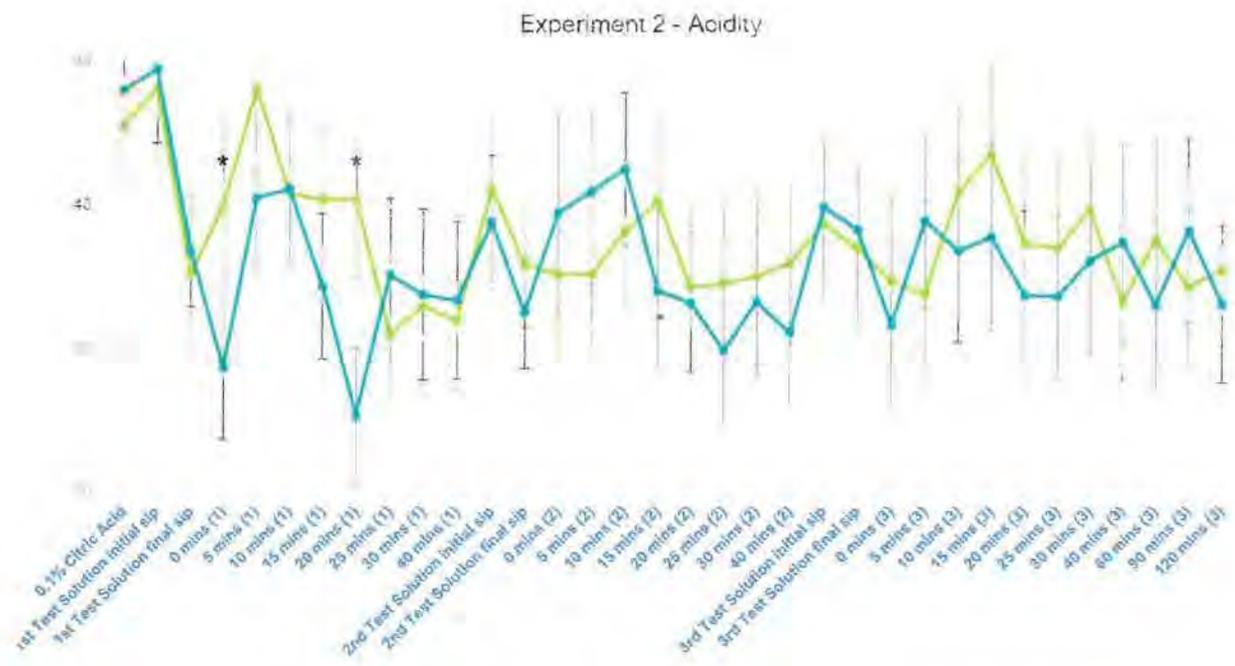
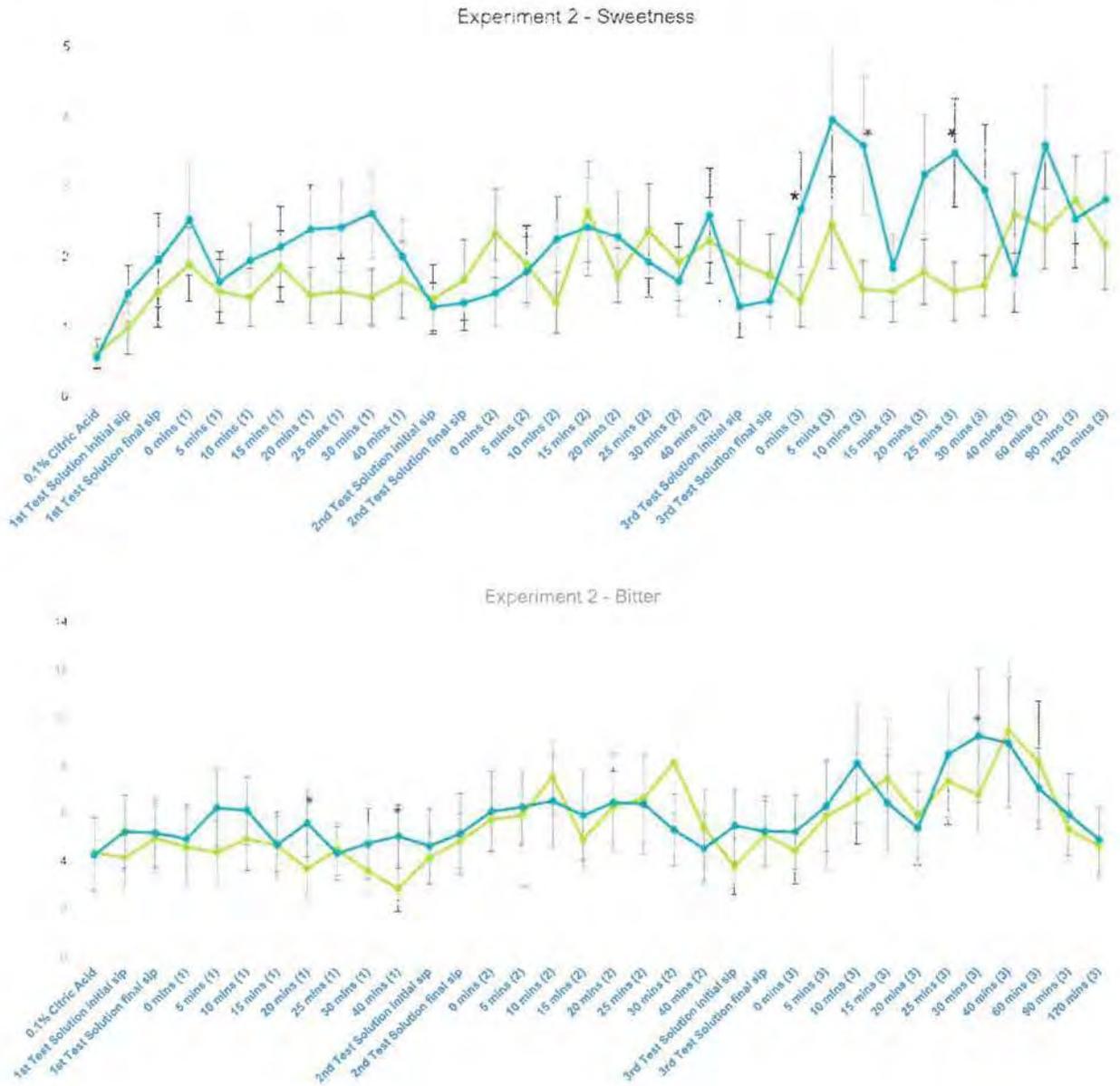


Figure 2.7.4.2-2 Effect of Miracle Fruit Powder (80 ppm) on Acidity, Sweetness, and Bitterness



2.7.4.3 Impact of Sweetness Perception at Neutral pH

Miracle fruit powder was added to a model soft drink formulation (citric acid/sodium citrate buffer) at a concentration of 80 ppm. The miracle fruit powder-containing beverage was evaluated in comparison to a control beverage containing 0 ppm miracle fruit powder in buffer. The methodology used for the sensory panel testing was very similar to that employed in Experiment 1, except that the pre-test and post-test solutions were sweetener solutions rather than citric acid solutions. Three different sweeteners, aspartame, sucralose, and sucrose were evaluated in 3 separate phases of this experiment, each phase testing an individual sweetener. The pre- and post-test solutions evaluated in each phase were 250 ppm aspartame in water, 112 ppm sucralose in water, and 6% sucrose in water. Prior to tasting the test solution, panelists (N=12) were asked to assess the sweetener solution (pre-test solution) and provide the 3 basic taste ratings (sweetness, acidity, or bitterness). They were then presented with 100 mL of the test solution, which was a citric acid/sodium citrate buffer containing either 0 ppm or 80 ppm miracle fruit powder. Panelists consumed the test solution over the course of 2 minutes to approximate typical soft drink consumption behavior with the 3 basic tastes (sweet, acid, and bitter) assessed at the beginning and end of the 2-minute period. Post-test solutions of the sweetener solution were then assessed immediately (time 0) and after 5, 10, 15, 20, 25, 30, 40, 50, and 60 minutes for those basic taste ratings. Triplicate sessions were carried out at each miracle fruit powder concentration.

Following either the 80 ppm miracle fruit powder or the control beverage, the panel mean score for sweetness of 250 ppm aspartame, 120 ppm sucralose, or 6% sucrose all followed the same pattern of returning to, or close to, its pre-test level immediately after test consumption and showed a slight lowering at 5 minutes and at subsequent tasting points for aspartame and sucralose, while sucrose remained approximately at the pre-test level at subsequent tasting points (see Figure 2.7.4.3-1). There was no significant difference between the ratings for sweetness when miracle fruit powder was present in the model soft drink in comparison to the corresponding control ratings throughout the testing period. Statistically significant differences at individual time points were observed in the 120 ppm sucralose and 6% sucrose groups, although these were considered spurious, as only 10 of 234 (4.3%) individual time point comparisons being conducted were significant a value below the 5% that would be expected by chance alone. Overall, results of Experiment 3 indicate that the receptor binding of miraculin in miracle fruit powder at a typical concentration (80 ppm) in soft drinks does not impact the sweetness perception of other sweeteners at neutral pH over time following miracle fruit powder consumption.

Figure 2.7.4.3-1 Effect of Miracle Fruit Powder or Control Beverage on Sweetness of Aspartame, Sucralose, or Sucrose

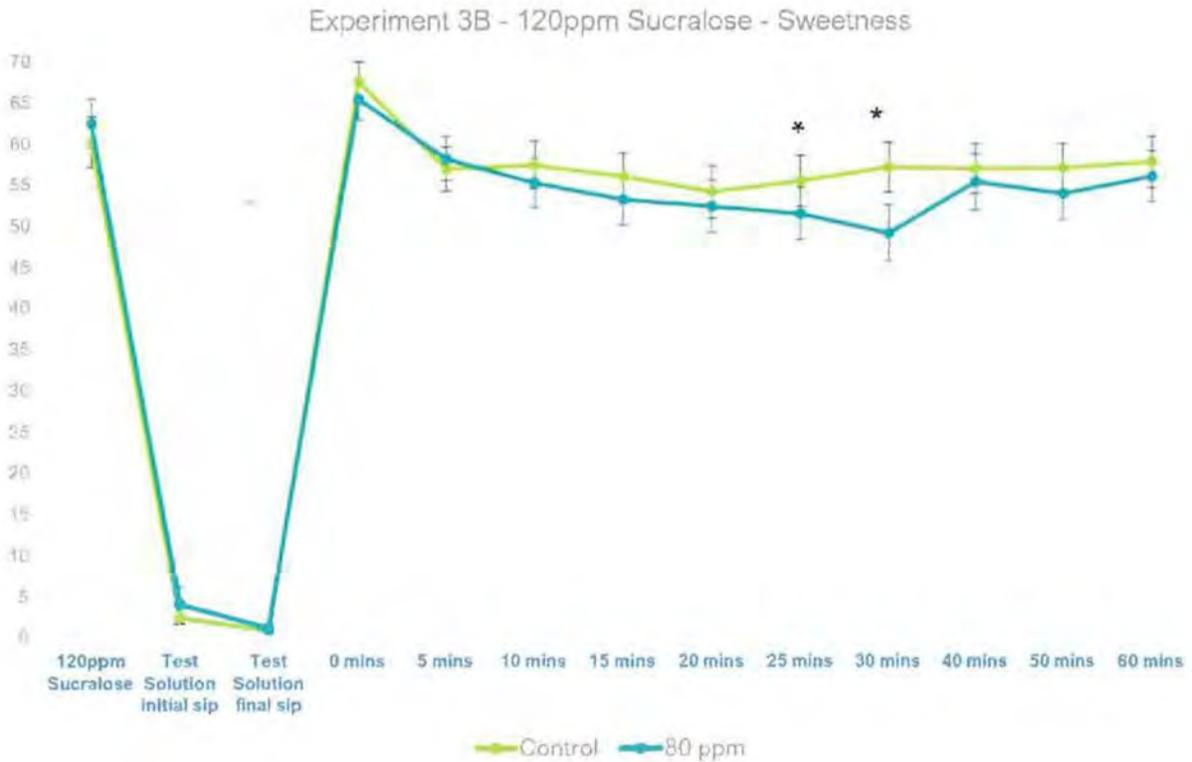
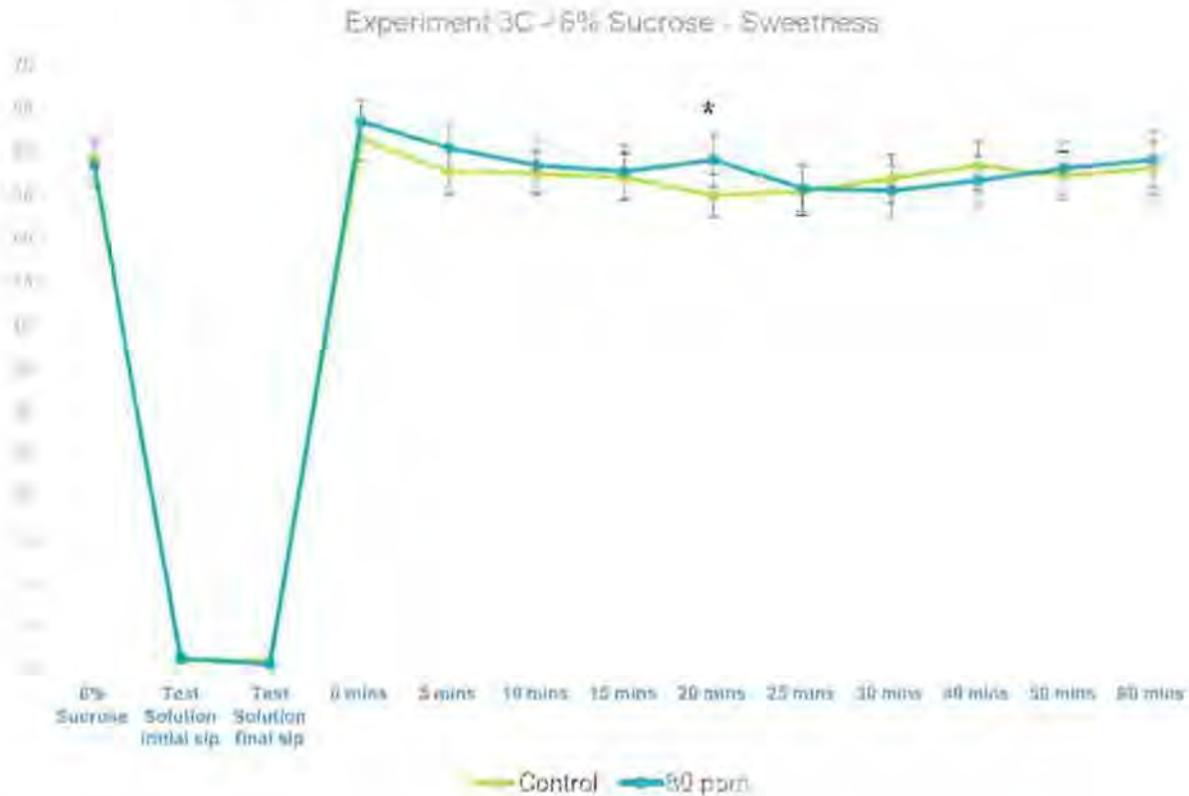


Figure 2.7.4.3-1 Effect of Miracle Fruit Powder or Control Beverage on Sweetness of Aspartame, Sucralose, or Sucrose



2.7.4.4 Summary of Sensory Studies

In summary, the human sensory panel experiments were conducted at miracle fruit powder concentrations of either 50 ppm or 80 ppm to represent the levels that are proposed for use within water-based beverages. The results from Experiment 1 support the findings from previously published studies that a miracle fruit powder-containing beverage does not interfere with taste qualities such as sweet, bitter, and acid (sour) following subsequent administration of an acid solution. In fact, the low levels of incorporation within the test beverage clearly demonstrate that there are no detectable taste-modifying effects of citric acid solutions consumed post-ingestion of miracle fruit powder at either 50 or 80 ppm (approximately 3 to 4 nM) in a model soft drink formulation. This lack of effect was anticipated on the basis that a concentration of 20 nM of miraculin resulted in the disappearance of a taste-modifying effect after 20 minutes (Kurihara and Beidler, 1969). A lower concentration would, therefore, be expected to result in less of an effect. The results of Experiment 2 further confirm that repeated stimulation of an 80-ppm miracle fruit powder model soft drink every 45 minutes, over a 90-minute period, also has no taste-modifying effects (sweet, bitter, and acid), on citric acid solutions consumed post-ingestion of miracle fruit powder solutions. The repeated exposure analysis further supports that repeated administration of an 80-ppm miracle fruit powder-containing mock beverage has no additive effect at the receptor level when consumed close together. Lastly, the results of Experiment 3 indicate that the receptor binding of miraculin in miracle fruit powder at a typical concentration of 80 ppm in a mock beverage does not impact the sweetness perception of other sweeteners at neutral pH over time following miracle fruit powder consumption. These results corroborate the human sensory study findings of Lipatova *et al.* (2016) while contradicting the published findings of

Koizumi *et al.* (2011) who reported that miraculin protein, when incubated with the sweet taste receptor at neutral pH, reduced the sweet taste response to many diverse sweeteners including carbohydrate, protein, and “artificial” sweeteners. Therefore, it is likely that there were some limitations in the *in vitro* study design.

Overall, the human sensory experiments corroborate that low levels of miraculin protein from the proposed uses of miracle fruit powder in water-based beverages will have a sweetening effect, but has a little to no taste-modifying effects on foods and beverages that are subsequently consumed.

PART 3 §170.235 DIETARY EXPOSURE

3.1 Functionality

Miracle fruit powder will be marketed in powdered form and added to water-based beverage products for its sweetening and taste-modifying properties due to the active glycoprotein, miraculin. Products to which miracle fruit powder will be added will not carry any structure/function or health claims and will be marketed similar to conventional products that do not contain this ingredient. The only difference between the products containing Miracle Fruit Farm's miracle fruit powder and the conventional products will be in the ingredient list. Therefore, no increases or changes in the consumption pattern of food products containing miracle fruit powder are expected compared to that from the conventional food products that do not contain the ingredient.

While addition of miracle fruit powder to water-based beverages is expected to change the sweetness profile of the beverages, this ingredient will not have an impact on the pH of the beverage to which it is added. Considering that the miracle fruit powder-containing product will be marketed in a similar manner as any other conventional water-based beverage, there will be no risk to consumers that suffer from conditions such as acid reflux or other digestive disorders; therefore, consumers who may experience digestive disorders such as acid reflux would be expected to self-regulate products that contain Miracle Fruit Farm's miracle fruit powder in the same manner as any other acidic products and refrain from consuming them.

3.2 Estimated Dietary Intake of Miracle Fruit Powder

3.2.1 Methods

An assessment of the anticipated intake of miracle fruit powder as an ingredient under the intended conditions of use (see Section 1.3) was conducted using data available in the 2015-2016 cycle of the U.S. National Center for Health Statistics' National Health and Nutrition Examination Survey (NHANES) (USDA, 2019; CDC, 2020a,b). This assessment was primarily conducted to evaluate exposure to antinutrients present in miracle fruit powder that could negatively affect the bioavailability of other nutrients in foods to which the ingredient is added (see Section 6.4). A summary of the results is presented herein.

The NHANES data are collected and released in 2-year cycles with the most recent cycle containing data collected in 2015-2016. Information on food consumption was collected from individuals *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2). Sample weights were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative (USDA, 2019; CDC, 2020a,b). The NHANES data were employed to assess the mean and 90th percentile intake of miracle fruit powder for each of the following population groups:

- Children, 2 to 5 years;
- Children, 6 to 11 years;
- Female teenagers, 12 to 19 years;
- Male teenagers, 12 to 19 years;
- Female adults, 20 years and up;
- Male adults, 20 years and up; and
- Total population (2 years and older, gender groups combined).

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of miracle fruit powder by the U.S. population.¹ Estimates for the daily intake of miracle fruit powder represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2015-2016; these average amounts comprised the distribution from which mean and percentile intake estimates were determined. Mean and percentile estimates were generated by incorporating survey weights in order to provide representative intakes for the entire U.S. population. “*Per capita*” intake refers to the estimated intake of miracle fruit powder averaged over all individuals surveyed, regardless of whether they consumed food products in which miracle fruit powder is proposed for use, and therefore includes individuals with “zero” intakes (*i.e.*, those who reported no intake of food products containing miracle fruit powder during the 2 survey days). “Consumer-only” intake refers to the estimated intake of miracle fruit powder by those individuals who reported consuming food products in which the use of miracle fruit powder is currently under consideration. Individuals were considered “consumers” if they reported consumption of 1 or more food products in which miracle fruit powder is proposed for use on either Day 1 or Day 2 of the survey.

Estimates for the intake of miracle fruit powder were generated using the maximum use level indicated for the water-based beverage together with food consumption data available from the 2015-2016 NHANES dataset. The resulting intake estimates of miracle fruit powder are presented in Section 3.2.2.

3.2.2 Estimated Daily Intake of Miracle Fruit Powder

A summary of the estimated daily intake of miracle fruit powder from proposed food uses is provided in Table 3.2.2-1 on an absolute basis (mg/person/day), and in Table 3.2.2-2 on a body weight basis ($\mu\text{g}/\text{kg}$ body weight/day).

The percentage of consumers was low among all age groups evaluated in the current intake assessment; less than 3.6% of the population groups consisted of consumers of food products in which miracle fruit powder is currently proposed for use. Children ages 6 to 11 had the greatest proportion of consumers at 3.6%. The consumer-only estimates are more relevant to risk assessments as they represent exposures in the target population; consequently, only the consumer-only intake results are discussed in detail herein.

Among the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of miracle fruit powder were determined to be 16.5 and 36.0 mg/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest statistically reliable mean consumer-only intakes of miracle fruit powder on an absolute basis at 22.1 mg/person/day. Female adults had the lowest statistically reliable mean consumer-only intakes of 14.9 mg/person/day. The 90th percentile value was not considered to be statistically reliable in any of the age groups, as the number of consumers within the individual population groups were all below 80. Similarly, the mean intakes of miracle fruit powder by individual populations ages ≤ 19 years were not statistically reliable based on low sample size ($n < 30$).

¹ Statistical analysis and data management were conducted in DaDiet Software (Dazult Ltd., 2018). DaDiet Software is a web-based software tool that allows accurate estimate of exposure to nutrients and to substances added to foods, including contaminants, food additives and novel ingredients. The main input components are concentration (use level) data and food consumption data. Data sets are combined in the software to provide accurate and efficient exposure assessments.

Based on an alternative serving-size approach for estimating exposure, the use of miracle fruit powder at a use level of 0.005% in water-based beverages with a serving size of 360 mL would equate to intakes of 18 mg/day at the mean (assuming consumption of 1 bottle of water-based beverage per day). At the 90th percentile and based on the assumption that the heavy consumers will be consuming 2 bottles of the water-based beverages per day, the intakes of miracle fruit powder would equate to 36 mg/day. This is aligned with the intakes of miracle fruit powder from the proposed food uses by the total population of consumers.

Table 3.2.2-1 Summary of the Estimated Daily Intake of Miracle Fruit Powder from Proposed Food Uses in the United States by Population Group (2015-2016 NHANES Data)

Population Group	Age Group (Years)	Per Capita Intake (mg/day)		Consumer-only Intake (mg/day)			
		Mean	90 th Percentile	%	n	Mean	90 th Percentile
Children	2 to 5	0.2*	na	3.0	14	6.3*	12.4*
Children	6 to 11	0.3*	na	3.6	24	8.5*	17.6*
Female teenagers	12 to 19	0.5*	na	2.0	13	22.7*	48.7
Male teenagers	12 to 19	0.2*	na	1.3	7	14.9*	17.8*
Female adults	20 and up	0.4	na	2.6	52	14.9	30.0*
Male adults	20 and up	0.5	na	2.4	46	22.1	38.0*
Total population	2 and up	0.4	na	2.5	156	16.5	36.0

n = sample size; na = not available; NHANES = National Health and Nutrition Examination Survey.

* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements (mean n<30; 90th percentile n<80).

On a body weight basis, the total population (ages 2 years and older) mean and 90th percentile consumer-only intakes of miracle fruit powder were determined to be 253 and 519 µg/kg body weight/day, respectively. Among the individual population groups, male adults were identified as having the highest statistically reliable mean consumer-only intakes of any population group at 258 µg/kg body weight/day. Female adults had the lowest statistically reliable mean consumer-only intake of 194 µg/kg body weight/day, respectively. The 90th percentile value was not considered to be statistically reliable in any of the age groups, as the number of consumers within the individual population groups were all below 80. Similarly, the mean intakes of miracle fruit powder by individual populations ages ≤19 years were not statistically reliable based on low sample size (n<30).

Table 3.2.2-2 Summary of the Estimated Daily Intake Per Kilogram Body Weight of Miracle Fruit Powder from Proposed Food Uses in the United States by Population Group (2015-2016 NHANES Data)

Population Group	Age Group (Years)	Per Capita Intake ($\mu\text{g}/\text{kg bw}/\text{day}$)		Consumer-only Intake ($\mu\text{g}/\text{kg bw}/\text{day}$)			
		Mean	90 th Percentile	%	n	Mean	90 th Percentile
Children	2 to 5	11*	na	3.1	14	341*	646*
Children	6 to 11	13*	na	3.6	24	354*	704*
Female teenagers	12 to 19	7*	na	1.7	12	388*	795*
Male teenagers	12 to 19	3*	na	1.3	7	209*	239*
Female adults	20 and up	5	na	2.5	51	194	374*
Male adults	20 and up	6	na	2.4	45	258	519*
Total population	2 and up	6	na	2.5	153	253	519

bw = body weight; n = sample size; na = not available; NHANES = National Health and Nutrition Examination Survey.

* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements (mean $n < 30$; 90th percentile $n < 80$).

3.2.3 Estimated Daily Intake of Miraculin from the Proposed Food Uses of Miracle Fruit Powder

The resulting miraculin exposure based on the proposed food uses of miracle fruit powder was estimated based on the highest miraculin content of 0.32% across 9 tested batches (see Table 2.4.2.1-1). In the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of miraculin were determined to be approximately 52.8 and 115.2 $\mu\text{g}/\text{person}/\text{day}$, respectively. In the female teenagers, the highest miraculin exposure was approximately 72.64 $\mu\text{g}/\text{day}$ (mean) and 155.84 $\mu\text{g}/\text{day}$ (90th percentile).

Table 3.2.3-1 Summary of the Estimated Daily Intake of Miraculin from the Proposed Food Uses of Miracle Fruit Powder in the United States by Population Group (2015-2016 NHANES Data)

Population Group	Age Group (Years)	Per Capita Intake ($\mu\text{g}/\text{day}$)		Consumer-Only Intake ($\mu\text{g}/\text{day}$)	
		Mean	90 th Percentile	Mean	90 th Percentile
Children	2 to 5	0.64	na	20.16	39.68
Children	6 to 11	0.96	na	27.2	56.32
Female teenagers	12 to 19	1.6	na	72.64	155.84
Male teenagers	12 to 19	0.64	na	47.68	56.96
Female adults	20 and up	1.28	na	47.68	96
Male adults	20 and up	1.6	na	70.72	121.6
Total population	2 and up	1.28	na	52.8	115.2

n = sample size; na = not available; NHANES = National Health and Nutrition Examination Survey.

3.2.4 Summary

Consumption data and information pertaining to the intended food uses of miracle fruit powder were used to estimate the *per capita* and consumer-only intakes of this ingredient for specific demographic groups and for the total U.S. population. There were a number of assumptions included in the assessment that render exposure estimates conservative. For example, it has been assumed in this exposure assessment that all food products within a food category contain miracle fruit powder at the maximum specified level of use. In reality, the levels added to specific foods will vary depending on the nature of the food product and it is unlikely that miracle fruit powder will have 100% market penetration in all identified food categories.

In summary, on a consumer-only basis, the resulting mean and 90th percentile intakes of miracle fruit powder by the total U.S. population (ages 2 years and older) from proposed food uses in the U.S. were estimated to be 16.5 mg/person/day (253 µg/kg body weight/day) and 36.0 mg/person/day (519 µg/kg body weight/day), respectively. Among the individual population groups, the highest statistically reliable mean intake of miracle fruit powder was determined to be 22.1 mg/person/day (258 µg/kg body weight/day) as identified for male adults. Notably, the number of consumers within the individual population groups were all below 80 and, therefore, the 90th percentile value was not considered to be statistically reliable in any of the age groups.

Miracle Fruit Farm has determined that the average miracle fruit weighs approximately 1.6 g, while 1.1 g is the edible portion (*i.e.*, after removal of the seed). After freeze-drying the edible portion, the total solids (*i.e.*, miracle fruit powder) was estimated to be approximately 11.5% or 0.13 g. Therefore, the highest 90th percentile consumer-only intake of miracle fruit powder of 48.7 mg/day would equate to the consumption of approximately 40% of a single miracle fruit. The proposed food uses of the miracle fruit powder would result in a dietary exposure that is well below the exposures to miracle fruit extract from its uses in dietary supplements (see Section 6.1). Furthermore, the European Food Safety Authority (EFSA) concluded the daily intake of 0.7 g/day of dried miracle berry, which were obtained from depitted and lyophilized *S. dulcificum* berries, do not pose a safety concern based on the results of a 90-day toxicity study in rats (EFSA, 2021).

Taking into account the highest miraculin content of 0.32% across 9 tested batches (see Table 2.4.2.1-1), the mean and 90th percentile consumer-only intakes of miraculin were determined to be approximately 52.8 and 115.2 µg/person/day, respectively, with female teenagers having the highest miraculin exposure at approximately 72.64 µg/day (mean) and 155.84 µg/day (90th percentile).

The information on the intakes of miracle fruit powder has been used to evaluate exposure to antinutrients present in the ingredient. The results of this assessment are provided in Section 6.4.

It should be noted that none of the ingredients are intended for use in food products consumed by infants and children up to 2 years of age.

PART 4 §170.240 SELF-LIMITING LEVELS OF USE

Miracle fruit powder is intended to be used as an ingredient for addition to water-based beverage products as a result of its ability to impart sweetness by modifying taste from sour to sweet due to the active glycoprotein, miraculin. The taste-modifying effects of miraculin are limited by the capacity of the interaction with receptors on the tongue.

PART 5 §170.245 EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Not applicable.

PART 6 §170.250 NARRATIVE AND SAFETY INFORMATION

The subject of this GRAS Notice is miracle fruit powder obtained by mechanical processing steps (*i.e.*, pulping, maceration, freeze-drying, milling) from miracle fruit as the starting material. Analytical composition data (see Section 6.3) demonstrated that there are no compositional differences between the miracle fruit powder and the starting material, aside from the moisture content considering that the miracle fruit powder undergoes freeze-drying to obtain the powder form. Mean values from the analysis of 9 production batches of miracle fruit powder (see Table 2.4.2.1-1) demonstrate that the ingredient is primarily composed of carbohydrates (~88% on dry basis), protein (~6% on dry basis), ash (~4% on dry basis), and moisture (~3.5%). The active ingredient, miraculin, is present at $\geq 0.048\%$ on a dry weight basis.

Miracle Fruit Farm's conclusion on the GRAS status of miracle fruit powder under its conditions of intended use, as an ingredient in various beverage products, is based on scientific procedures supported by the following:

1. Long history of consumption of miracle fruit globally;
2. The low intakes of miracle fruit powder under the intended conditions of use that equates to consuming approximately only 40% of a single miracle fruit;
3. Publicly available data related to the safety of miracle fruit, miracle fruit powder, and miraculin;
4. Compositional data on miracle fruit powder and pulp, including individual polyphenols, and comparison with other commonly consumed fruits;
5. Exposure to antinutrients from proposed uses of miracle fruit powder;
6. Sensory studies on miracle fruit powder demonstrating that the sensory profile of foods and beverages consumed after consumption of the ingredient is not adversely impacted;
7. Consensus among a panel of experts (the GRAS Panel), qualified by scientific training and experience to evaluate the safety of food ingredients, namely the following scientific experts: Professor Emeritus I. Glenn Sipes, Ph.D., Fellow AAAS and ATS (University of Arizona College of Medicine); Professor Emeritus George C. Fahey, Jr. (University of Illinois); and Professor Emeritus Stephen L. Taylor (University of Nebraska).

6.1 Long History of Global Consumption of Miracle Fruit

Miracle fruit powder is minimally processed and has been demonstrated through analytical data to be compositionally similar to miracle fruit pulp, which is used as a starting material, with the only difference being the moisture content. The first historical record of human consumption of miracle fruit dates back to the early 1700s in Ghana (Roeklein and Leung, 1987). In 1917, miracle fruit was introduced to the U.S. from Africa by the USDA. Since then, cultivation of miracle fruit and its use in the U.S. has steadily grown. Currently, a number of different products containing miracle fruit/miracle berry, and derivatives thereof, are marketed over the internet by various companies. Web searches for "miracle berry," "miracle fruit," and "miraculin" identified several dietary supplement-type products containing miracle berry or miracle fruit extract (mberry Miracle Fruit Tablets, My M Fruit LLC; MiraBurst Easy Melt Miracle Berry Tablets, MiraBurst; Miraculous Miracle Fruit Tablets, Miracle Fruit Farm; Miracle Frooties Miracle Fruit Tablets, Ruby Forest LLC)

that are currently available on the U.S. market. The recommended serving size appears to be 100 to 175 mg miracle fruit extract/day. A Miracle Berry Diet Cookbook is also available instructing how to cook with and use the miracle berry within the daily diet, containing over 150 recipes to incorporate miracle berry in breakfast, lunch, and dinner options as well as cocktail recipes (Cantu, 2013). To date, there have been no adverse events resulting from consumption of miracle berry, miracle fruit, or miraculin products reported through the U.S. FDA Adverse Event Reporting System or Center for Food Safety and Applied Nutrition Adverse Event Reporting System, suggesting that there is a history of safe use of miracle fruit in the U.S. Historical sales data from Miracle Fruit Farm over 10 years indicate that the Farm has supplied approximately 24.8 million single uses of miracle fruit; of this, 19,839,600 fresh fruit, 3,636,000 uses in the powder form, and 1,394,648 uses in a processed freeze-dried form other than powder. The proposed food uses of the miracle fruit powder would result in a dietary exposure that is well below the exposures to miracle fruit extract from its uses in dietary supplements. In the European Union, dried miracle berry was concluded to not pose a safety concern at consumption levels up to 0.7 g/day in dietary supplement products (EFSA, 2021).

6.2 Publicly Available Data Related to Safety of Miracle Fruit and Miracle Fruit Powder

A comprehensive search of the scientific literature was conducted through January 2023 to identify publications related to the metabolism and safety of miracle fruit powder. The search was limited to full text articles within peer-reviewed scientific journals from the following literature databases: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile®. The search identified several repeated-dose studies on miracle fruit powder and fruits and leaf extracts of miracle fruit, which evaluated the effects on blood glucose, glucose tolerance, insulin resistance, hematology and blood chemistry of diabetic and non-diabetic rodents, and anti-hyperuricemic effects in mice. While these studies mainly evaluated efficacy-related endpoints of miracle fruit or extracts of the plant on diabetic and non-diabetic rats or rats consuming a high-fructose diet, the lack of adverse findings on the limited safety-related endpoints provide supporting evidence of the safety of miracle fruit powder. The results of these studies are summarized in Table 6.2-1 below.

Table 6.2-1 Summary of Repeated-Dose Studies of Miracle Fruit Powder or Extracts of Miracle Fruit and Miracle Fruit Leaf

Details of the Study Methodology	Reported Findings	Reference
Miracle Fruit Powder		
Test Animal: Male Wistar Rat (N=8/group) Route of Administration: Oral (gavage) Duration: Single dose ^b Doses: 0, 0.02, 0.04, or 0.2 mg/kg	<ul style="list-style-type: none"> Significant decrease in plasma glucose in all treatment groups (over 90 min) 	Chen <i>et al.</i> (2006)
Test Animal: Male Wistar Rat (N=8/group) Route of Administration: Oral (gavage) Duration: 3 days ^c Doses: 0, 0.02, 0.04, or 0.2 mg/kg	<ul style="list-style-type: none"> Significant decrease in plasma glucose in IPGTT Significant decrease in total AUC for glucose response and plasma insulin in all treatment groups Significant decrease in glucose-insulin index in 0.2 mg/kg group 	
Test Animal: Male Wistar Rat (N=8/group) Route of Administration: Oral (gavage) Duration: 28 days ^d Doses: 0 or 0.2 mg/kg	<ul style="list-style-type: none"> Amelioration of plasma glucose lowering effect of tolbutamide in treatment groups compared to control 	
Test Animal: Male Wistar Rat (N=8/group) ^e Route of Administration: Oral (gavage)	<ul style="list-style-type: none"> Significant increase in plasma glucose lowering activity Reversal of hyperphagia effects 	

Table 6.2-1 Summary of Repeated-Dose Studies of Miracle Fruit Powder or Extracts of Miracle Fruit and Miracle Fruit Leaf

Details of the Study Methodology	Reported Findings	Reference
<p>Duration: 10 days^f Doses: 0 or 0.2 mg/kg</p>	<ul style="list-style-type: none"> • Significant decrease in food and water intake • No significant effect on body weight 	
Extract of Miracle Fruit and Miracle Fruit Leaf		
<p>Test Animal: Male albino rats^a (N=5/group) Route of Administration: Oral (gavage) Duration: 4 weeks Doses: 0 or 200 mg/kg bw/day^h</p>	<ul style="list-style-type: none"> • Significant decrease in blood glucose 	Diosa <i>et al.</i> (2016)
<p>Test Animal: Rat (sex and strain were not reported) (N=7/group) Route of Administration: Oral (drinking water) Duration: 21 days Doses: 0, 30, or 60 mg/kg methanolic (MSD) or flavonoid-rich (FSD) <i>Synsepalum dulcificum</i> leaf extract</p>	<ul style="list-style-type: none"> • Significant increase in plasma Ca, Na, and K concentrations in diabetic control compared to non-diabetic control • Significant decrease in plasma calcium and potassium concentrations in all MSD and FSD groups compared to diabetic control but ↑ compared to non-diabetic control • Significant decrease in plasma sodium concentration in MSD groups compared to diabetic control but ↑ compared to non-diabetic control • Significant decrease in plasma sodium concentration in FSD groups compared to diabetic control; no significant effect compared to non-diabetic control • Significant increase in WBC and neutrophil count in diabetic control compared to non-diabetic control • Significant decrease in PCV, hemoglobin concentration, and RBC in diabetic control compared to non-diabetic control • Significant decrease in WBC and neutrophil count in all MSD and FSD groups compared to diabetic control but ↑ compared to non-diabetic control • Significant increase in PCV, hemoglobin concentration, and RBC in all MSD and FSD groups compared to diabetic control but ↓ compared to non-diabetic control 	Obafemi <i>et al.</i> (2016)
<p>Test Animal: Rat (sex and strain were not reported) (N=7/group) Route of Administration: Oral (drinking water) Duration: 21 days Doses: 0, 30, or 60 mg/kg MSD or FSD <i>Synsepalum dulcificum</i> leaf extract</p>	<ul style="list-style-type: none"> • Significant decrease in body weight in diabetic control compared to non-diabetic control • Significant increase in body weight in all MSD and FSD groups • Amelioration of serum glucose levels in all MSD and FSD diabetic animals • Significant decrease in ALP, AST, ALT, urea, and creatinine in all MSD and FSD diabetic groups compared to diabetic control; no significant effect in ALP and creatinine compared to non-diabetic control • Significant increase in AST and ALT in all MSD and FSD diabetic groups compared to normal control • Significant decrease in urea in high-dose MSD group compared to non-diabetic control • Significant increase in urea in high-dose FSD group compared to non-diabetic control • Significant decrease in ALP, AST, ALT, creatinine, urea in non-diabetic control compared to diabetic control • Significant increase in total protein in non-diabetic control compared to diabetic control • NSD in ALP or creatinine in non-diabetic MSD and FSD groups compared to non-diabetic control • NSD in AST or urea in non-diabetic MSD group compared to non-diabetic control • Significant increase in AST, ALT, urea in non-diabetic FSD group compared to non-diabetic control 	Obafemi <i>et al.</i> (2017)

Table 6.2-1 Summary of Repeated-Dose Studies of Miracle Fruit Powder or Extracts of Miracle Fruit and Miracle Fruit Leaf

Details of the Study Methodology	Reported Findings	Reference
	<ul style="list-style-type: none"> Significant increase in total plasma protein in all MSD and FSD diabetic groups compared to diabetic control; ↓ compared to non-diabetic control Amelioration of liver, kidney, and pancreas lipid peroxidation biomarkers in all MSD and FSD groups 	
Test Animal: Rat (sex and strain were not reported) Route of Administration: Oral Duration: 21 days Doses: 0, 30, or 60 mg/kg MSD or FSD <i>Synsepalum dulcificum</i> leaf extract	<ul style="list-style-type: none"> Significant decrease in HbA1c, IL-6, TNF-α Significant increase in serum insulin levels, hepatic hexokinase activity 	Obafemi <i>et al.</i> (2019)
Test Animal: ICR Mice Route of Administration: Oral Duration: 7 days Doses: 0, 500, or 1,000 mg/kg body weight/day	<ul style="list-style-type: none"> No significant effect relative-to-body liver and kidney weights, serum creatinine, or blood urea nitrogen 	Shi <i>et al.</i> (2016)

↑ = increased; ↓ = decreased; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; AUC = area under the curve; bw = body weight; Ca = calcium; FSD = flavonoid-rich *Synsepalum dulcificum* leaf extract; HbA1c = hemoglobin A1c; IL-6 = interleukin-6; IPGTT = intraperitoneal glucose tolerance test; K = potassium; M = males; min = minutes; MSD = methanolic *Synsepalum dulcificum* leaf extract; Na = sodium; NSD = no significant difference; PCV = packed cell volume; RBC = red blood cells; TNF-α = tumor necrosis factor-α; WBC = white blood cells.

^a All reported findings were statistically significant compared to respective controls unless otherwise noted.

^b Animals were administered a single dose of miracle fruit powder and then provided a fructose-rich diet (60%) for 4 weeks.

^c Animals were provided a fructose-rich diet (60%) for 4 weeks and were administered 3 doses per day of miracle fruit powder.

^d Miracle fruit powder was administered every 8 hours, 3 doses per day, and 10 mg/kg tolbutamide was administered at 5 hours after miracle fruit powder treatment.

^e Diabetes was induced by streptozocin injection.

^f Miracle fruit powder was administered every 8 hours, 3 doses per day for 10 days, and then challenged with insulin injection.

^g Diabetes was induced by alloxan injection.

^h Extracted with ethanol.

Overall, the studies described in Table 6.2-1 are of limited relevance to the safety of Miracle Fruit Farm’s miracle fruit powder considering that they are mainly efficacy-focused studies with some limited toxicological endpoints. In the study by Chen *et al.* (2006), the effects of lyophilized miracle fruit powder on insulin resistance of male Wistar rats consuming a fructose-rich diet was evaluated, which mainly focused on changes to the plasma glucose levels in rats fed a high-fructose diet, as compared to their respective control and were not reflective of such effects in comparison to animals that were fed standard rat chow alone. The potential effect of the lyophilized powder on dietary intake due to palatability was highlighted in the last experiment conducted in STZ-diabetic rats, wherein the authors reported that treatment with miracle fruit powder significantly reduced both food and water intake in comparison to those animals only receiving the dose vehicle (saline). If this reduction in food intake similarly occurred in the fructose-rich fed group that was fed miracle fruit powder, then this would have been a confounding factor in the overall results analysis thereby impacting the ability to accurately interpret the outcome of miracle fruit powder administration. The effect could have been amplified in the experiments with 60% fructose in the diet. Based upon the fact that the lyophilized miracle fruit powder used in the study included all parts of the miracle fruit, including seed, an argument can be made regarding the potential difference in material composition between the test material used by Chen *et al.* (2006) and Miracle Fruit Farm’s miracle fruit powder. Furthermore, based upon a clear lack of detailed information on the study design and methodology, including the results from the control animals and the lack of information related to food and water intake and body weight data, it is difficult to interpret the impact of miracle fruit powder on insulin

resistance. Based on these study limitations, the study by Chen *et al.* (2006) is of limited value to the risk assessment of Miracle Fruit Farm’s miracle fruit powder.

In the studies conducted by Dioso *et al.* (2016), Obafemi *et al.* (2016, 2017, 2019), and Shi *et al.* (2016), the test articles were extracted from the leaf and fruits of miracle fruit through the use of various solvents including butanol, ethanol and methanol. These studies are considered of limited relevance to the safety of Miracle Fruit Farm’s miracle fruit powder, which undergoes no extraction process, due to the compositional differences in the test articles. The effects reported on some serum biochemistry parameters may therefore be attributed to the concentrated fruit/leaf components following solvent extraction and/or any residual extraction solvents in the final product.

In addition to the published studies discussed above, EFSA reviewed a number of safety studies related to the dried fruits of *Synsepalum dulcificum* (*i.e.*, miracle fruit) as part of a novel food application (EFSA, 2021). The subject of the novel food application was dried miracle berries, which are fruits of *S. dulcificum* that have been pitted and dried by lyophilization, similar to the process used to produce the Miracle Fruit Powder as described herein. The composition of the dried miracle berries was approximately 4.4% moisture, 4.4% ash, 81% total carbohydrates, 5.1% total protein, and 2.6% total fat, which was generally similar to the miracle fruit powder described herein (see Section 2.4.2.1 for further details). The miraculin content of the dried miracle berries was reported to be 1.86%. The total polyphenol, oxalic acid, trypsin inhibitor, and sum of pyrrolizidine alkaloids content of the dried miracle berries was reported to be approximately 4.33 mg GAE/g, between 0.05 and 0.1%, 0.80 to 0.97 TIU/mg dry weight, and not detected to 7.2 µg/kg, respectively. It was noted that the oxalic acid content was below the levels in fonio and wheat bran, the trypsin inhibitor content was below the levels reported in chia seeds and soy beans. The safety of dried miracle berry was assessed using proprietary compositional and nutritional data, information on the allergenicity potential of the novel food, as well as a battery of safety studies, including genotoxicity, subchronic toxicity study, as well as data related to human exposure. A summary of the safety studies on dried miracle berries reviewed by EFSA is provided in Table 6.2-2 below. EFSA concluded that dried miracle berries do not have genotoxic potential based on the information on the ingredient, and the no-observed-adverse-effect-level (NOAEL) of dried miracle berries was concluded to be 2,000 mg/kg body weight/day. The reported NOAEL is approximately 7,900 times greater than the total population consumer-only mean intakes of Miracle Fruit Farm’s miracle fruit powder, *i.e.*, 253 µg/kg body weight/day.

Table 6.2-2 Summary of Genotoxicity and Toxicity Studies on Dried Miracle Berries (EFSA, 2021)

Study	Test System	Concentration/Dose	Outcome
Bacterial reverse mutation test (OECD TG 471)	<i>Salmonella</i> typhimurium TA98, TA100, TA102, TA1535, and TA1537	Up to 5,000 µg/plate (±59)	Equivocal
Bacterial reverse mutation test (OECD TG 471)	<i>S. typhimurium</i> TA98, TA100, TA102, TA1535, and TA1537	Up to 5,000 µg/plate (±59)	Negative
<i>In vivo</i> mammalian erythrocyte micronucleus test (OECD TG 474)	Wistar rats	2,000 mg/kg bw/day	Undetermined (due to uncertainty whether the test article reached target cells)
<i>In vitro</i> mammalian cell micronucleus test (OECD TG 487)	Mouse lymphoma L5178Y TK+/- 3.7.2C cells	Up to 1,000 µg/mL	Negative
Acute oral toxicity study by Up-and-Down Procedure (OECD TG 425)	Wistar rats	5,000 mg/kg bw/day	No adverse effects reported

Table 6.2-2 Summary of Genotoxicity and Toxicity Studies on Dried Miracle Berries (EFSA, 2021)

Study	Test System	Concentration/Dose	Outcome
90-day repeated dose oral toxicity study with a 14-day recovery period (OECD TG 408, limit test)	Wistar rats	2,000 mg/kg bw/day	NOAEL = 2,000 mg/kg bw/day

bw = body weight; NOAEL = no-observed-adverse-effect level; OECD = Organisation for Economic and Cooperative Development; TG = test guideline.

In addition to the animal toxicology studies, EFSA reviewed a number of human studies evaluating the taste altering properties of different products derived from *S. dulcificum* fruits (Capitanio *et al.*, 2011; Wong and Kern, 2011; Wilken and Satiroff, 2012; Igarashi *et al.*, 2013; Rodrigues *et al.*, 2016; Hudson *et al.*, 2018; Andrade *et al.*, 2019; Tafazoli *et al.*, 2019). EFSA noted that these studies reported no adverse effects regarding possible localized effects, such as irritation on the tongue and/or mouth, following consumption of up to 600 mg for period of 2 weeks (EFSA, 2021). It was also noted by EFSA that chronic consumption of dried miracle berries is not expected to pose any safety concern considering that the available sensory data indicate that the taste-altering effect of miraculin has a “rapid onset and disappearance with no desensitizing impact on the receptors,” and that taste cells have regenerative capabilities with an approximate life span of 8 to 22 days (EFSA, 2021). EFSA concluded that the NOAEL determined from the 90-day oral toxicity study supports a daily intake of 0.7 g/day, considering a margin of exposure of 200 to ensure safety of the dried miracle berries.

6.3 Compositional Analyses

The proximate analysis of miracle fruit powder has been compared with that of commonly consumed berries, such as blueberries, blackberries, and raspberries. As presented in Table 6.3-1, the proximate composition of miracle fruit powder is similar to other commonly consumed berries, with the exception of moisture content, considering that miracle fruit powder undergoes freeze-drying to obtain the powder form.

Table 6.3-1 A Proximate Analysis Comparison of Miracle Fruit Powder with Commonly Consumed Berries

Parameter	Miracle Fruit Powder ^a	Miracle Fruit Pulp ^a	Blueberries (USDA, 2020a) ^b	Blackberries (USDA, 2020b) ^b	Raspberries (USDA, 2020c) ^b
Carbohydrate (% dry basis)	~87	~87	~90	80	~86
Protein (% dry basis)	~7	~9	~4	~11	~8.5
Ash (% dry basis)	~4	~3.5	~1	~3	N/A
Fat (% dry basis)	~0.8	~0.8	~2	~4	5
Moisture (%)	~3.6	~85	~84	~88	~86

N/A = not available.

^a The values are the mean of the 9 batches in Table 2.4.2.1-1.

^b The “as is” values have been corrected for moisture content for ease of comparison.

6.4 Exposure to Polyphenols from Proposed Uses of Miracle Fruit Powder

Polyphenols are naturally occurring in various dietary sources, including fruits, vegetables, and beverages of plant origin, such as teas and can be divided into various classes according to their basic chemical structures. Miracle fruit powder is derived from miracle fruit berries without the use of any chemical solvents or processing aids through physical processing steps, including pulping, macerating, freeze-drying, and milling to form a powder. As such, miracle fruit powder is essentially the miracle fruit berry with water removed. In order to quantify and qualify how the polyphenol profile of miracle fruit powder compares to that of commonly consumed and recognized polyphenol-rich fruits and teas, and for the purposes of direct comparison, a detailed and comprehensive analysis of the polyphenol composition of miracle fruit pulp was first determined, using the analytical data reported in Table 2.5.1-1 for miracle fruit powder and adjusting for the average moisture content of 84.8%, as presented in Table 6.4-1.

Table 6.4-1 Determination of Polyphenol Composition of Miracle Fruit Pulp from Data on Miracle Fruit Powder

Polyphenol Class	Polyphenol Compound	Miracle Fruit Powder (dry basis) (mg/100 g)	Miracle Fruit Pulp (mg/100 g) ^a
Anthocyanidins	Cyanidin	48.5	7.37
	Delphinidin	0.58	0.09
Flavan-3-ols	(-)-Epicatechin	52.00	7.90
	(-)-Epigallocatechin 3-gallate	21.03	3.20
	(+)-Catechin	42.43	6.45
	(+)-Gallocatechin	13.70	2.08
	(+)-Gallocatechin gallate	84.70	12.87
Total Polyphenol (as GAE)		1,190.0^b	180.88

^a Total polyphenol in miracle fruit pulp calculated from powder data using average moisture content in pulp (84.8%).

^b Total polyphenol from Table 2.5.1-1.

The polyphenol content of miracle fruit pulp, as estimated based on the data on miracle fruit powder after adjustment for moisture, was then compared with commonly consumed berries, such as blackberry, blueberry, cranberry, strawberry, raspberry, as well as the polyphenol content of green tea and oolong tea using information obtained from the USDA database for flavonoid content in food v3.3. As shown in Table 6.4-2, the polyphenol content of miracle fruit pulp (~181 mg/100 g) is comparable or less than that of commonly consumed berries (155 to 569 mg/100 g). Specifically, the cyanidin level in miracle fruit pulp (7.37 mg/100 g) is comparable to the content in blueberries, but significantly less than the cyanidin levels in blackberries (up to 100 mg/100 g). The total catechins in miracle fruit pulp (~34 mg/100 g) is comparable to that of blackberries (~43 mg/100 g), brewed green tea (~43 mg/100 g), and brewed oolong tea (~50 mg/100 g), and lower than blueberries (~124 mg/100 g).

Table 6.4-2 Comparison of Polyphenols in Miracle Fruit Pulp and Commonly Consumed Berries

Polyphenol Class	Polyphenol Compound	Miracle Fruit Pulp (mg/100 g) ^c	Bilberry (raw) (mg/100 g)	Blackberry (raw) (mg/100 g)	Blueberry (wild, raw) (mg/100 g)	Blueberry (rabbiteye, raw) (mg/100 g)	Blueberry (cultivated, raw) (mg/100 g)	Cranberry (raw) (mg/100 g)	Strawberry (raw) (mg/100 g)	Raspberry (raw) (mg/100 g)	Green Tea (brewed)	Oolong Tea (brewed)
Anthocyanidins	Cyanidin ^a	7.37	85.26	99.95	19.35	9.60	8.46	29.88	1.68	45.77	NR	NR
	Delphinidin ^a	0.09	97.59	NR	37.59	23.41	35.43	7.66	0.31	1.32	NR	NR
	Malvidin ^a	ND ¹	39.22	NR	57.16	63.45	67.59	0.31	0.01	0.13	NR	NR
	Peonidin ^a	ND ¹	20.45	0.21	9.99	15.90	20.29	30.54	0.05	0.12	NR	NR
	Petunidin ^a	ND ¹	42.69	NR	23.52	36.25	31.53	NR	0.11	0.31	NR	NR
Flavonols	Quercetin ^a	ND ²	3.04	3.58	NR	14.42	7.67	16.64	0.11	0.93	1.69	1.30
Flavan-3-ols	(-)-Epicatechin ^a	7.90	NR	4.66	NR	25.66	0.62	4.37	0.42	NR	4.45	2.54
	(-)-Epicatechin 3-gallate ^a	ND ³	NR	NR	NR	NR	NR	NR	0.15	NR	5.11	6.33
	(-)-Epigallocatechin ^a	ND ³	NR	0.10	NR	NR	0.66	0.74	0.78	NR	13.34	6.10
	(-)-Epigallocatechin 3-gallate ^a	3.20	NR	0.68	NR	NR	NR	0.97	0.11	NR	19.97	34.48
	(+)-Catechin ^a	6.45	NR	37.06	NR	98.47	5.29	0.39	3.11	NR	NR	0.23
	(+)-Gallocatechin ^a	2.08	NR	NR	NR	NR	0.12	NR	0.03	NR	NR	NR
	(+)-Gallocatechin gallate ^a	12.87	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	Catechin gallate	1.24	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	Total catechin	33.75	NR	42.50	NR	124.13	6.69	5.47	4.60	0.00	42.87	49.68
Flavanones	Hesperetin ^a	ND ²	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	Naringenin ^a	ND ²	NR	NR	NR	NR	NR	NR	0.26	NR	NR	NR
Hydroxycinnamic acids	Caffeic acid ^b	ND ⁴	0.40	2.51	NR	1.07	NR	15.64	0.04	0.37	NR	NR
	Ferulic acid ^b	ND ⁴	NR	3.25	NR	4.43	NR	8.79	NR	NR	NR	NR
	p-Coumaric acid ^b	ND ⁴	2.40	1.24	NR	4.55	NR	25.38	1.98	1.17	NR	NR
	Sinapic acid ^b	ND ⁴	NR	NR	NR	NR	NR	21.18	NR	NR	NR	NR
Total polyphenol (Folin assay) ^b		180.88	525.00	569.43	471.55	549.98	223.41	315.00	289.20	154.65	NR	NR

HPLC = high-performance liquid chromatography; LOQ = limit of quantitation; ND = not detected; NM = not measured; NR = not reported; USDA = United States Department of Agriculture.

^a USDA database for flavonoid content in food v3.3 (reported as aglycone, including glycosylated forms).

Table 6.4-2 Comparison of Polyphenols in Miracle Fruit Pulp and Commonly Consumed Berries

Polyphenol Class	Polyphenol Compound	Miracle Fruit Pulp (mg/100 g) ^c	Bilberry (raw) (mg/100 g)	Blackberry (raw) (mg/100 g)	Blueberry (wild, raw) (mg/100 g)	Blueberry (rabbiteye, raw) (mg/100 g)	Blueberry (cultivated, raw) (mg/100 g)	Cranberry (raw) (mg/100 g)	Strawberry (raw) (mg/100 g)	Raspberry (raw) (mg/100 g)	Green Tea (brewed)	Oolong Tea (brewed)

^b Phenol Explorer v3.6 (reported as aglycone, HPLC measurement after hydrolysis).

^c Powder data converted using the average of moisture measured in the pulp.

ND¹: LOQ is 0.25 mg/100 g; ND²: LOQ is 124 mg/100 g; ND³: LOQ is 10.3 mg/100 g; ND⁴: LOQ is 3.45 mg/100 g.

Based on the proposed food uses of miracle fruit powder, the highest 90th percentile intake of miracle fruit powder in the U.S. population is estimated to be in female teenagers at 48.7 mg/day (see Table 3.2.2-1). Taking into account the highest 90th percentile intakes of miracle fruit powder (48.7 mg/day), the maximum daily intakes of total polyphenols (1,190 mg/100 g as GAE; see Table 6.4-1) from the proposed uses of miracle fruit powder is calculated to be 0.58 mg/day as GAE in female teenagers. In comparison, the highest 90th percentile intake of total polyphenols from the proposed uses of miracle fruit powder is lower than the intakes of total polyphenols from other berry fruits, such as blackberry (435 mg GAE/100 g), blueberry (348 mg GAE/100 g), or strawberry (83.9 mg GAE/100 g) (Heinonen *et al.*, 1998; León-González *et al.*, 2013), based on a serving size of 100 g (USDA, 2022). Therefore, it is anticipated that dietary exposure to total polyphenols from the proposed food uses of miracle fruit powder in water-based beverages would not substantially contribute to the total polyphenol exposure in the U.S. population.

6.5 Exposure to Antinutrients from Proposed Uses of Miracle Fruit Powder

Several production batches of miracle fruit powder have been analyzed for chemical, microbiological, and environmental contaminants originating from the manufacturing process or cultivation practices (see Section 2.5). The results of this analysis demonstrate that the final ingredient is absent of contaminants (*e.g.*, heavy metals, pesticides, and mycotoxins) and microbiological hazards, and the levels are in compliance with the established product specifications.

Analysis of 3 production batches of miracle fruit powder demonstrate the presence of low levels of oxalic acid (ranging between 1,170 to 1,350 ppm; see Section 2.5.2) and does not contain any detectable levels of phytic acid or trypsin inhibitors. The oxalic acid content of miracle fruit powder was compared against the levels naturally occurring in several commonly consumed fruits. On an absolute basis, the proposed food uses of miracle fruit powder would provide approximately 0.03 mg/day (mean) or 0.06 mg/day (90th percentile) of oxalic acid in the highest consumer-only intake population group (*i.e.*, female teenagers). In comparison, 1 serving of black raspberries or concord grapes would provide approximately 82.5 or 37.5 mg/day of oxalic acid, which is appreciably greater than the amount of oxalic acid contributed by miracle fruit powder (USDA, 2020c,d).

6.6 Publicly Available Safety Data on Miraculin

The search of the scientific literature identified 1 publication related to the *in vitro* digestibility and safety of the glycoprotein miraculin (*i.e.*, allergenicity, toxigenicity) using *in silico* tools (Tafazoli *et al.*, 2019, 2020). The results of this study are discussed in further detail in the sections that follow. The resulting miraculin exposure based on the proposed food uses of miracle fruit powder was estimated to be in the range of 0.02 to 0.05 mg/person/day at the mean and 90th percentile, respectively, for the total population.

6.6.1 Metabolic Data on Miraculin

6.6.1.1 *In Silico* Prediction of Digestibility of Miraculin

The digestibility of the 191 amino acid sequence of miraculin (*i.e.*, without the signal peptide) was predicted using the PeptideCutter tool maintained by ExPASy. PeptideCutter predicts possible cleavage sites within a peptide sequence by proteases under various gastric conditions. The *in silico* digestibility of the glycoprotein was predicted with pepsin (pH >2.0). PeptideCutter predicted 48 cleavage sites along the miraculin peptide sequence, with the resulting lengths of the peptide digest ranging between 1 and 19 amino acids (see Table 6.6.1.1-1).

Table 6.6.1.1-1 Results of *In Silico* Pepsin Digestion (pH 2.0) of Miracle Fruit Protein (Miraculin) Using PeptideCutter

Position of Cleavage Site	Enzyme (pH)	Resulting Peptide Sequence	Peptide Length (amino acids)	Peptide Mass (Da)
8	Pepsin (pH>2)	DSAPNPVL	8	811.890
14	Pepsin (pH>2)	DIDGK	6	675.693
15	Pepsin (pH>2)	L	1	131.175
20	Pepsin (pH>2)	RTGTN	5	547.569
21	Pepsin (pH>2)	Y	1	181.191
22	Pepsin (pH>2)	Y	1	181.191
27	Pepsin (pH>2)	IVPVL	5	539.716
33	Pepsin (pH>2)	RDHGGG	6	597.588
34	Pepsin (pH>2)	L	1	131.175
44	Pepsin (pH>2)	TVSATTPNGT	10	947.998
45	Pepsin (pH>2)	F	1	165.192
63	Pepsin (pH>2)	VCPPRVVQTRKEVDHDRP	18	2131.441
65	Pepsin (pH>2)	LA	2	202.253
67	Pepsin (pH>2)	FF	2	312.368
81	Pepsin (pH>2)	PENPKEDVVRVSTD	14	1584.703
82	Pepsin (pH>2)	L	1	131.175
85	Pepsin (pH>2)	NIN	3	359.382
86	Pepsin (pH>2)	F	1	165.192
88	Pepsin (pH>2)	SA	2	176.172
94	Pepsin (pH>2)	FMPCRW	6	839.041
99	Pepsin (pH>2)	TSSTV	5	493.514
100	Pepsin (pH>2)	W	1	204.228
102	Pepsin (pH>2)	RL	2	287.362
104	Pepsin (pH>2)	DK	2	261.278
105	Pepsin (pH>2)	Y	1	181.191
111	Pepsin (pH>2)	DESTGQ	6	635.585
112	Pepsin (pH>2)	Y	1	181.191
113	Pepsin (pH>2)	F	1	165.192
130	Pepsin (pH>2)	VTIGGVKGNPGPETISS	17	1612.800
131	Pepsin (pH>2)	W	1	204.228
132	Pepsin (pH>2)	F	1	165.192
136	Pepsin (pH>2)	KIEE	4	517.580
137	Pepsin (pH>2)	F	1	165.192
141	Pepsin (pH>2)	CGSG	4	322.336
142	Pepsin (pH>2)	F	1	165.192
143	Pepsin (pH>2)	Y	1	181.191
144	Pepsin (pH>2)	K	1	146.189
145	Pepsin (pH>2)	L	1	131.175
164	Pepsin (pH>2)	VFCPTVCGSCKVKCGDVGI	19	1915.329
165	Pepsin (pH>2)	Y	1	181.191
176	Pepsin (pH>2)	IDQKGRRLAL	11	1325.580
180	Pepsin (pH>2)	SDKP	4	445.473

Table 6.6.1.1-1 Results of *In Silico* Pepsin Digestion (pH 2.0) of Miracle Fruit Protein (Miraculin) Using PeptideCutter

Position of Cleavage Site	Enzyme (pH)	Resulting Peptide Sequence	Peptide Length (amino acids)	Peptide Mass (Da)
182	Pepsin (pH>2)	FA	2	236.271
183	Pepsin (pH>2)	F	1	165.192
184	Pepsin (pH>2)	E	1	147.131
185	Pepsin (pH>2)	F	1	165.192
190	Pepsin (pH>2)	NKTVY	5	623.707
191	end of sequence	F	1	165.192

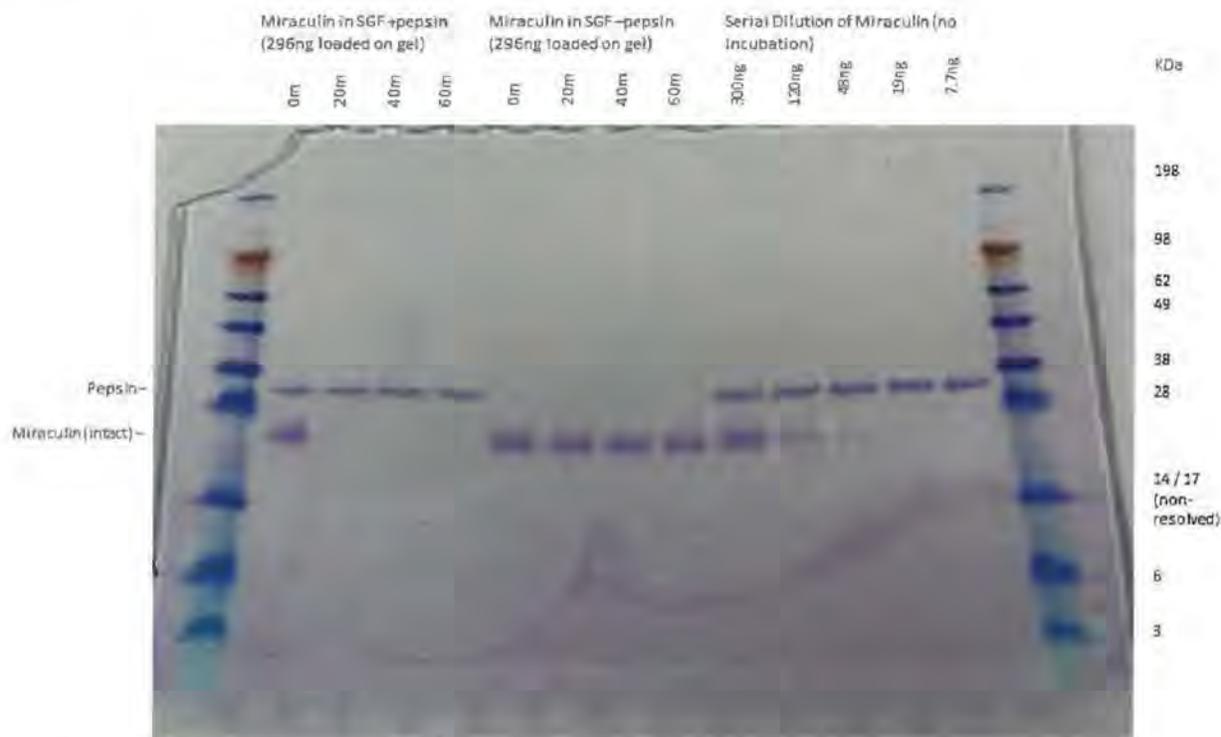
6.6.1.2 *In Vitro* Pepsin Digestibility of Miraculin

The protein digestibility of miraculin was reported using an *in vitro* simulated gastric fluid (SGF) model (Tafazoli *et al.*, 2019). Miraculin (0.08 mL; 2 mg/mL or 0.1 mg/mL final concentration in reaction) was added to a preincubation mixture consisting of SGF (10 U/μg pepsin) and incubated for up to 60 minutes. After 0, 20, 40, and 60 minutes of incubation, sample mixtures were quenched with sodium bicarbonate, tricine buffer solution, and a reducing agent, and heated at 85°C for 10 minutes. The digestibility of the protein was evaluated by gel electrophoresis after heating. The results of digestion are presented in Figure 6.6.1.2-1 and demonstrate that miraculin was completely digested within 20 minutes.

The effect of pepsin concentration on the digestion of miraculin was investigated in a second experiment in which miraculin was added to SGF containing 5.45 U/μg or 10 U/μg of pepsin, and incubated for 0, 1, 5, or 10 minutes. Miraculin was fully digested within 1 minute, indicating that the protein is rapidly metabolized, and the digestion kinetics are pepsin-dependent.

These results indicate that, following ingestion, miraculin would be completely and rapidly digested in the gastrointestinal tract.

Figure 6.6.1.2-1 Results of Digestion of Miraculin in Simulated Gastric Fluid at 37°C



The *in vitro* digestibility of miraculin (purity 85 to 90%) was investigated using the methods described by Thomas *et al.* (2004). The glycoprotein and digestion control proteins (pepsin sensitive² or resistant³ proteins) were dissolved at 3.67 mg/mL in phosphate buffer saline (pH 7.0) or deionized water. For each protein, a sample tube containing 1.52 mL of SGF (1,600 U pepsin) was pre-heated to 37°C for 10 minutes prior to the addition of 0.08 mL of the glycoprotein or digestion control proteins. The test concentrations were 3.67 mg/mL or 2.00 mg/mL, providing either 5.45 U pepsin/μg protein or 10 U pepsin/μg protein, respectively. The samples were mixed by vortexing and placed in a 37°C water bath for up to 4 hours. At each timepoint, 100 μL of each sample was removed and quenched by sodium bicarbonate, buffer solution, and reducing agent. At time point 0, samples were quenched prior to the addition of test proteins. Quenched samples were heated for 10 minutes at 85°C and stored at -20°C. Samples were thawed and loaded onto tricine gels to run for 80 minutes at 125V. Serial dilutions of miraculin were prepared identical to time 0 samples. Gels were stained with Coomassie Blue or silver staining.

² Bovine serum albumin and peroxidase from horseradish served as pepsin-sensitive control proteins.

³ Albumin from chicken egg white served as the pepsin-resistant control protein.

Miraculin was demonstrated to be completely digested within 20 minutes in the presence of SGF containing pepsin at a concentration of 10 U/μg protein (see Figure 6.6.1.2-1). No peptide fragments were detected. In a subsequent study with shorter digestion times (*i.e.*, 0, 1, 5, and 10 minutes), as well as a lower pepsin concentration (5.45 U/μg protein), similar results were reported (see Figure 6.6.1.2-2). The miraculin protein was rapidly digested within 1 minute. The digestion control proteins were digested as expected, and pepsin was reported to be intact throughout the incubation period. Miraculin was stable and intact at all timepoints in SGF without pepsin, suggesting that digestion of the glycoprotein is pepsin dependent. Fragments of the miraculin protein were not detected, indicating that digestion with pepsin is rapid and extensive.

Figure 6.6.1.2-2 Results of Digestion of Miraculin and Digestion Control Proteins at Different Concentrations of Pepsin (5.45 and 10 U/μg) for 0, 1, 5, and 10 Minutes (Coomassie Blue Staining)

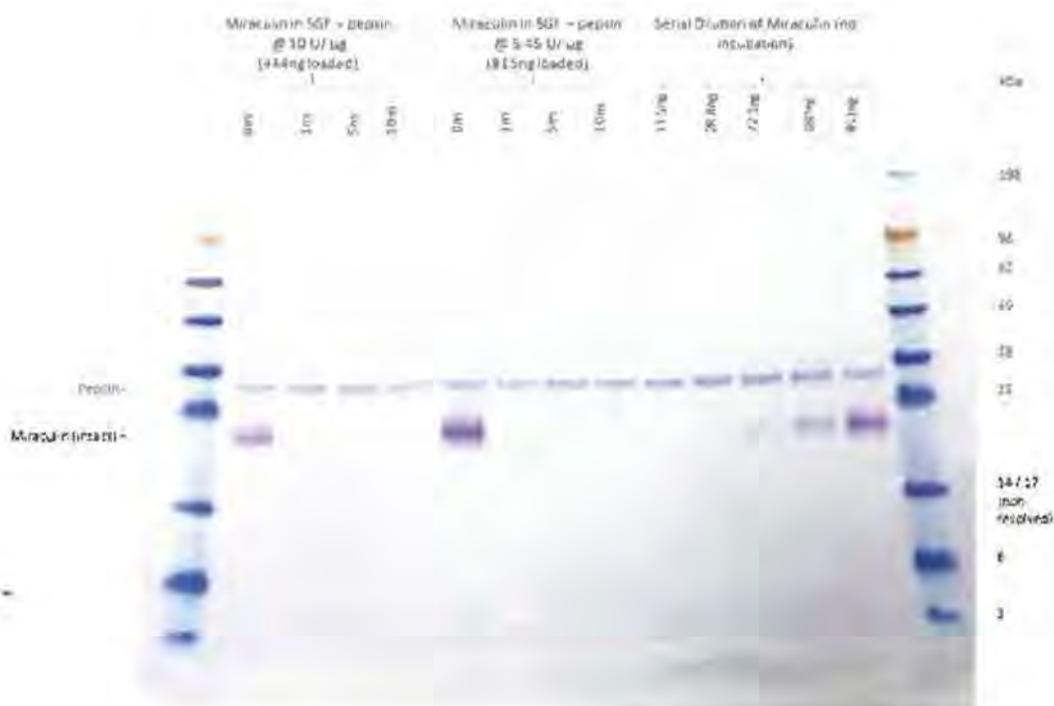
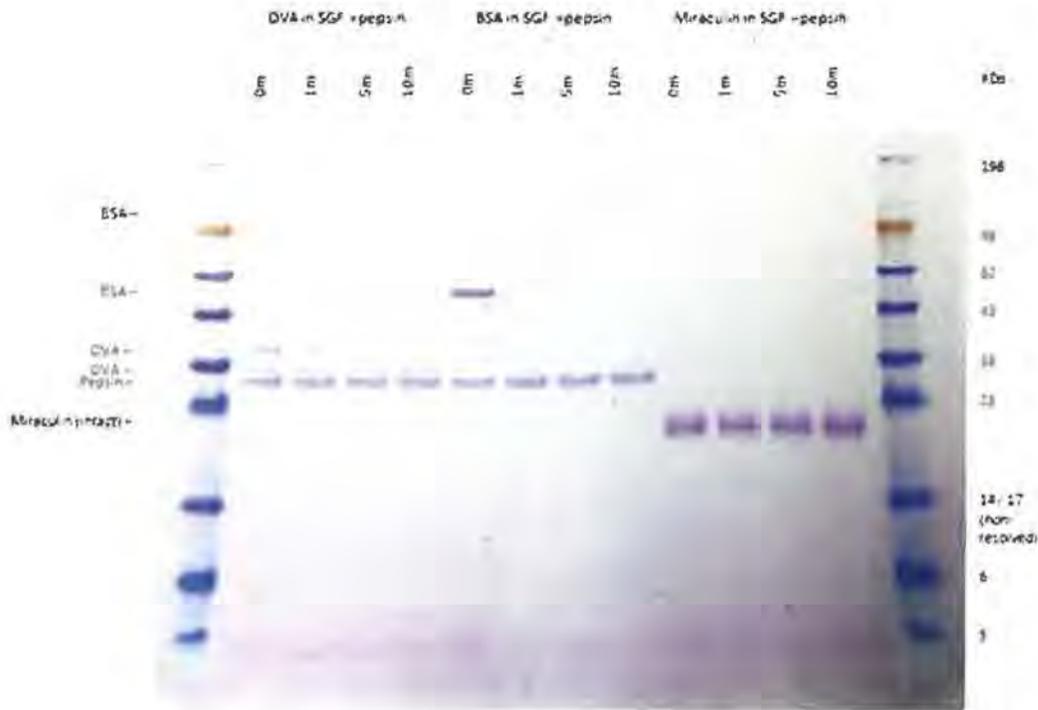
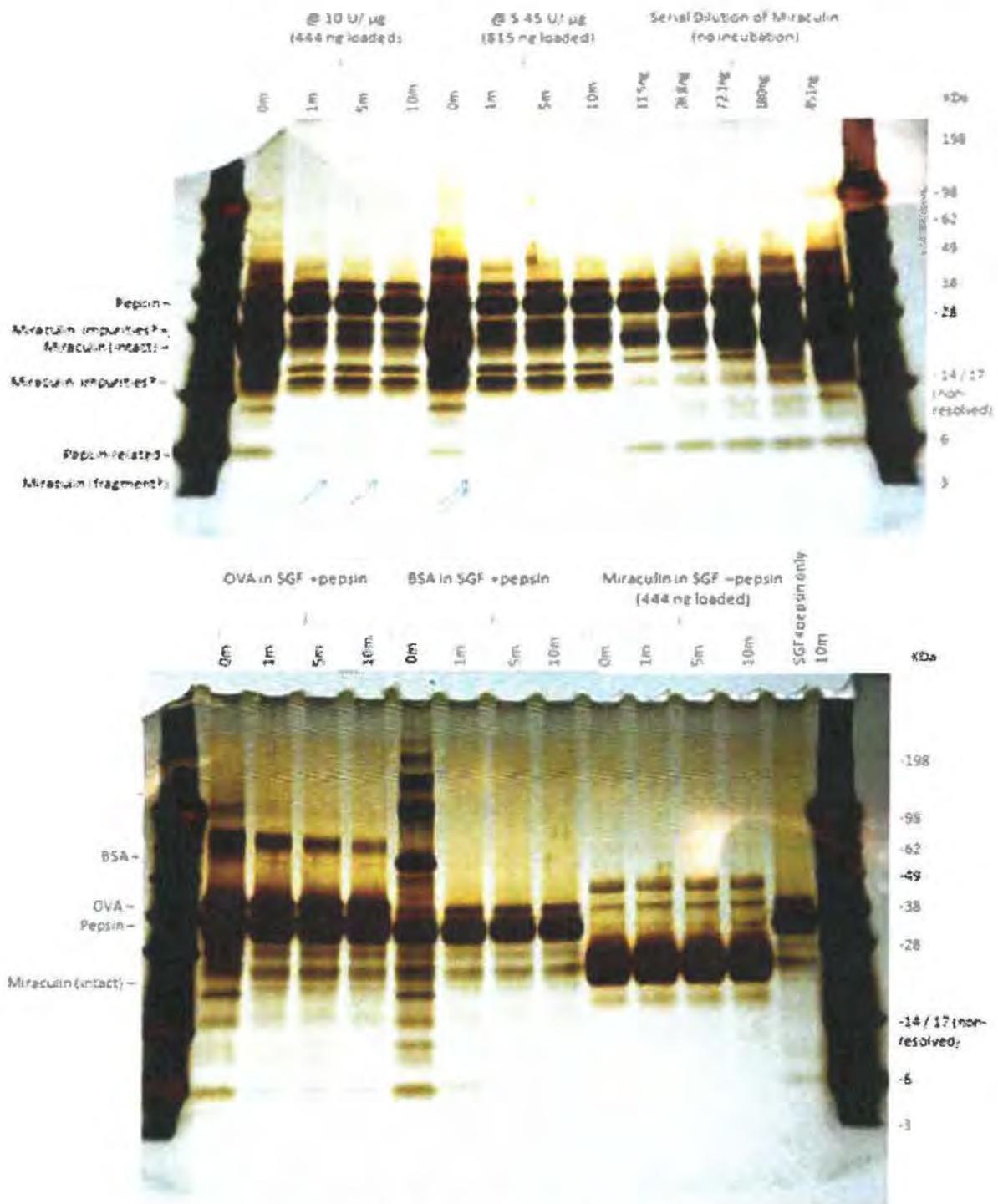


Figure 6.6.1.2-2 Results of Digestion of Miraculin and Digestion Control Proteins at Different Concentrations of Pepsin (5.45 and 10 U/ μ g) for 0, 1, 5, and 10 Minutes (Coomassie Blue Staining)



Due to the low sensitivity of Coomassie Blue staining and in order to enhance detection of lower abundance peptides, the above experiment was repeated using the more sensitive Silver Staining approach. Following digestion by pepsin for 0, 1, 5, and 10 minutes, the results of the Silver Staining corroborated the previous findings that miraculin was rapidly digested at both 5.45 and 10 U pepsin/ μ g protein. Minor peptide fragments were reported at approximately 4 kDa at both concentrations within 10 minutes of digestion. The other bands were reported to be pepsin or miraculin impurities, as similar bands were observed at time point 0 and serial dilutions of miraculin, which were not digested (see Figure 6.6.1.2-3).

Figure 6.6.1.2-3 *In Vitro* Digestion of Miraculin and Digestion Control Proteins at Different Concentrations of Pepsin (5.45 and 10 U/ μ g) for 0, 1, 5, and 10 Minutes (Silver Staining)



Arrows denote minor potential miraculin digestion fragments.

6.6.1.3 Proteolytic Fate of Miraculin

Using the same pepsin digestion method as described in Section 6.2.1, Tafazoli *et al.* (2019) reported the proteolytic fate of the miraculin protein following pepsin digestion. The digested peptides of miraculin were evaluated using LC-MS/MS. Miraculin was added to SGF containing 5.45 U/ μ g pepsin and incubated for up to 10 minutes at 37°C. Digest samples were collected after 0, 0.5, 1, and 10 minutes. Tafazoli *et al.* (2019) reported that miraculin was increasingly digested with a longer digestion time (*i.e.*, number of unique peptides increased with longer digestion periods). After 0 minutes of digestion, the authors reported 5 unique peptides while the number of unique peptides after 0.5, 1, and 10 minutes of digestion were 33, 54, and 61, respectively. The number of unique peptides after 10 minutes of pepsin digestion encompassed approximately 75% of the amino acid sequence. The authors reported that the only peptides that were not identified after 10 minutes of digestion were peptides with cysteine residues (*i.e.*, disulfide bonds) that may be resistant to digestion. The authors further evaluated the unique peptides from miraculin digestion for their allergenic potential (see Section 6.3 for further details). The authors concluded that the *in vitro* digestibility studies suggest that miraculin, following ingestion, would be rapidly and completely metabolized into small peptides, and ultimately its amino acid components. This was further supported by the results of the Silver Staining method over a longer digestion period of up to 240 minutes, demonstrating that in the *in vitro* pepsin digestibility study, miraculin was rapidly digested to fragments that had a molecular weight below the detection limit of the gel electrophoresis method (*i.e.*, 2 to 5 kDa), suggesting the complete digestion of the protein.

6.6.2 Bioinformatics Assessment of Miraculin

The amino acid sequence of miraculin (UniProt Accession No. P13087) was subject to bioinformatic analyses to predict the potential allergenicity of miraculin and to determine whether the candidate protein shares any structural homology to known toxins in the absence of sufficient *in vivo* toxicological data. Structural homology between the candidate proteins and known allergens or toxins may suggest the candidate protein has allergenic or toxic potential. Interpretation of the sequence alignment data involved an evaluation of the percent identity (*i.e.*, quantification of amino acid alignment between the queried protein and a known allergen or toxin) and the expectation value (E-value) and query cover. Based upon the information from these bioinformatics searches, additional information and experimental approaches can be directed, as might be necessary, to reach conclusions on the allergenic or toxic potential of a candidate protein. The results of these searches are summarized in the sections that follow.

6.6.2.1 Allergenicity of Miraculin

The potential allergenicity of miraculin (UniProt Accession No. P13087) was investigated using bioinformatics searches on the AllergenOnline Database⁴ (version 21; updated 14 February 2021) (FARRP, 2021) and Allermatch Database⁵ (updated 04 July 2019) to determine whether the amino acid sequence of miraculin shares similarity to known allergens. The databases contain a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of assessing the potential allergenicity of novel proteins. In addition, the Allermatch database was constructed using the allergenic proteins from the COMPARE⁶, UniProt⁷, and WHO/IUIS⁸ allergen databases. Sequence homology

⁴ <http://www.allergenonline.org/>.

⁵ <http://www.allermatch.org/>.

⁶ <https://comparedatabase.org/>.

⁷ <https://www.uniprot.org/docs/allergen>.

⁸ <http://www.allergen.org/>.

searches of the full-length amino acid sequence and 80-amino acid sliding window alignment were conducted using FASTA. The results are summarized in Table 6.6.2.1-1 below.

In the full-length amino acid sequence search, an identity cut-off value of 50% was used as allergic cross-reactivity may occur at matches greater than 50% (Aalberse, 2000). However, it is noted that cross-reactivity is rare at 50%, and, in fact, allergic cross-reactivity may require greater than 70% identity over the full-length sequence (Aalberse, 2000). Nevertheless, in the full-length amino acid sequence search using the AllergenOnline and Allermatch databases, no hits greater than 50% identity were identified, suggesting the unlikely potential for cross-reactivity to putative allergens.

In the 80-amino acid sliding window alignment search, segments of 80-amino acids (1–80, 2–81, 3–82, etc.) derived from each full-length amino acid sequence were searched in accordance with the methodology described by the FAO/WHO (2001) and Codex Alimentarius (2003, 2009). Significant homology was defined as an identity match of greater than 35% in accordance with the FAO/WHO (2001) and Codex Alimentarius (2003, 2009) criteria. Immunoglobulin E (IgE) cross-reactivity to putative allergens may be considered a possibility at matches greater than 35% identity. A number of sequences with identity matches ranging from 36 to 39% with known allergens from commonly consumed agricultural products, *Solanum tuberosum* (potato) and *Glycine max* (soybean), were identified using AllergenOnline (see Table 6.6.2.1-1). The clinical significance of low identity matches (35 to 40% over 80 amino acid windows) is questionable; the recommended criterion of >35% identity over 80 amino acid windows is quite conservative and other factors should be considered when the percent identity is low (Goodman, 2006).

Table 6.6.2.1-1 Search Results of AllergenOnline (Version 21) and Allermatch

Sequence Identifier	Source	Description	80 mer		Full Length		
			% Identity	# Hits (>35%)	Length	E-value	% Identity
AllergenOnline							
994779	<i>Solanum tuberosum</i>	Proteinase inhibitor	39.30	23/141	227	4.0x10 ⁻¹²	28.60
124148	<i>Solanum tuberosum</i>	Aspartic protease inhibitor 11	35.80	5/141	194	1.6x10 ⁻¹¹	29.9
256429	<i>Glycine max</i>	Kunitz trypsin inhibitor KTI	37.54	12/141	215	1.8x10 ⁻¹¹	31.6
18770	<i>Glycine max</i>	Trypsin inhibitor subtype A	37.5	12/141	215	1.8x10 ⁻¹¹	31.6
256635	<i>Glycine max</i>	Kunitz trypsin inhibitor KTI1	37.54	14/141	212	1.6x10 ⁻⁶	33.5
18772	<i>Glycine max</i>	Trypsin inhibitor subtype B	37.5	12/141	215	4.5x10 ⁻⁸	31.6
256636	<i>Glycine max</i>	Kunitz trypsin inhibitor KTI2	37.54	8/141	213	3.2x10 ⁻⁵	32.4
Allermatch							
P25273	<i>Glycine max</i>	Kunitz-type trypsin inhibitor KTI2	37.52	8	187	-	33.7
Q39899	<i>Glycine max</i>	Kunitz trypsin inhibitor	35.04	2	189	-	32.8
Q39898	<i>Glycine max</i>	Kunitz trypsin inhibitor	35.04	2	189	-	32.8
P01070	<i>Glycine max</i>	Trypsin inhibitor A	35.04	2	189	-	32.8
P25272	<i>Glycine max</i>	Kunitz-type trypsin inhibitor KTI1	36.23	7	186	-	34.9

The potential for cross-reactivity between miraculin and these potato and soybean trypsin inhibitors is low based on the lack of significant full-length identity and the rather low (35 to 39%) identities over sliding 80-mer windows between the potato and soybean trypsin inhibitors and miraculin. Also, although these trypsin inhibitors are recognized as known allergens, neither the potato proteinase inhibitors nor the soy Kunitz trypsin inhibitor are considered as important food allergens from a clinical context (Taylor *et al.*, 2015).

While the soybean Kunitz trypsin inhibitor is one of the known allergens from soy, this protein is very infrequently identified as an allergen in investigations conducted with soy-allergic patients. The soybean Kunitz trypsin inhibitor (SKTI) consists of 181 amino acids and represents 4 to 7% of the total extractable protein in soy. SKTI is a tightly packed protein with 2 disulfide bonds between Cys39-Cys86 and Cys138-Cys145, both of which are critical for trypsin inhibition and resistance to denaturation (Sessa and Ghantous, 1987). SKTI is not glycosylated, and trypsin inhibition is achieved through reversible binding of SKTI to the trypsin enzyme (Kunitz, 1947; Friedman and Brandon, 2001; Barać *et al.*, 2004; Mikić *et al.*, 2009). SKTI is an inhalation allergen associated with occupational exposure to flour dust in bakers (Baur *et al.*, 1996; Quirce *et al.*, 2006). As an allergen, SKTI primarily affects bakers exposed to large amounts of inhaled soy flour. Individuals with SKTI-induced baker's asthma have serum IgE specific for binding to SKTI, positive skin prick test to extracts of SKTI, and reactions during a specific inhalation challenge with purified SKTI (Baur *et al.*, 1996; Quirce *et al.*, 2006). The incidence of inhaled SKTI related allergic reactions is very low. Ingestion of SKTI has only been confirmed to cause an allergic reaction in 1 individual, although symptoms were severe in this case (Moroz and Yang, 1980). This patient's sensitization to SKTI may not have occurred through the ingestion of soybeans because the patient worked in a biochemical laboratory that used SKTI in experiments. Although this report in 1980 was one of the first identifications of a soybean allergen, no other cases of food-related soybean allergies have been attributable to SKTI in the intervening years. Instead, the major soy allergens have been identified as Gly m 5 (conglycinin), Gly m 6 (glycinin), Gly m 4 (a starvation associated message protein cross-reactive to the major birch tree pollen allergen, Bet v 1), and perhaps Gly m 8 (a 2S albumin) (Kattan and Sampson, 2015; Taylor *et al.*, 2015).

The major allergen in potatoes is Sol t 1, a 43 kDa protein known as patatin (Seppälä *et al.*, 1999; Astwood *et al.*, 2000; Majamaa *et al.*, 2001). Patatin is the main storage protein of the potato tuber. Potatoes also contain several proteinase inhibitors that have been identified as allergens by Seppälä *et al.*, 2000, 2001. The initial study investigated 12 patients sensitized to raw potato. On subsequent investigation, only 7 of these 12 subjects reacted to oral challenges with cooked potato (Seppälä *et al.*, 2001); the other patients had positive reactions when their skin was rubbed with raw potato. Seven of these 12 potato-allergic patients showed IgE binding to proteins in the 20kD region and 3 of 12 to proteins in the 16kD and 18kD regions after electrophoretic separation of potato proteins (Seppälä *et al.*, 2000). N-Terminal sequencing of the purified proteins showed that all belonged to the family of Kunitz-type trypsin inhibitors (Seppälä *et al.*, 2000). Subsequently, Seppälä *et al.* (2001) described the 20kD protein as Sol t 2, the 18kD protein as Sol t 3, and the 16 kD protein as Sol t 4. Sol t 2 was further identified as a cathepsin D proteinase inhibitor with an IgE-binding N-glycan region that was identical to the major grass allergen, Lol p 11 (Seppälä *et al.*, 2001). In an enzyme-linked immunosorbent assay (ELISA), 43 to 67% of 39 allergic children showed specific IgE binding to the new allergens, Sol t 2–4 (Seppälä *et al.*, 2001). However, oral challenges were not conducted on the additional 27 potato-sensitized subjects to demonstrate that they would actually react upon consumption of potato. While Seppälä *et al.* (2001) characterized Sol t 2-4 as major potato allergens, the allergenicity of these proteins has not subsequently been corroborated by other clinical investigators. The importance of these proteinase inhibitors from potato as allergens remains uncertain. Together with the low identity of these proteins with miraculin, the likelihood of cross-reactivity between miraculin and potato seems remote.

The allergenic profile of the glycoprotein miraculin was investigated using AlgPred, which utilizes a support-vector machine (SVM) analysis. The *in silico* search with AlgPred identified the following on the miraculin protein:

1. Non-allergen-based on algorithms for IgE epitopes, Motif Alignment and Search Tool (MAST), and allergen representative peptides (ARP); and
2. Allergen based on SVM analysis of the amino acid composition and dipeptide composition.

The results are summarized in Table 6.5.2.1-2. AlgPred was used to evaluate the allergenicity of soy leghemoglobin, and it was concluded that the reliability of this tool was questionable due to a reported high false positive rate (GRN 737 – U.S. FDA, 2018). Furthermore, it was concluded that allergenicity assessments using established databases, such as AllergenOnline, was “*more than adequate to demonstrate that [...] have little or no allergenic potential*” (GRN 737 – U.S. FDA, 2018). Considering the multitude of results from established methodologies that have been successfully used in the assessment of allergenicity potential of proteinaceous compounds (FAO/WHO, 2001; Codex Alimentarius, 2009; EFSA, 2017), it can be concluded that miraculin has a low risk of allergenicity.

The allergenicity potential of miraculin was also evaluated using AllerTOP (version 2.0), which is based on auto cross covariance transformation of protein sequences into uniform equal-length vectors (Wold *et al.*, 1993). AllerTOP predicted the miraculin sequence to be a “probable non-allergen,” with the nearest protein to be *beta*-galactosidase (Accession No. P48980), a non-allergen.

Table 6.6.2.1-2 Assessment of the Allergenicity Potential of Miraculin Using AlgPred

Algorithm	Result	Sensitivity (True Allergen)	Specificity (True Non-Allergen)	Error Rate (False Allergen)	Analysis Type
IgE Epitopes	Non-allergen	10.84%	98.25%	1.75%	Sequence motif
Motif Alignment and Search Tool (MAST)	Non-allergen	22.05%	86.68%	13.32%	Sequence motif
Allergen Representative Peptides (ARP)	Non-allergen	66.56%	97.97%	2.03%	Sequence motif
Support Vector Machine (SVM) Amino Acid Composition	Allergen	84.21%	56.07%	43.93%	Amino acid composition
Support Vector Machine (SVM) Dipeptide Composition	Allergen	84.83%	61.09%	38.91%	Amino acid composition

As part of the novel food application for dried miracle berries, EFSA evaluated the allergenicity potential of dried miracle berries (EFSA, 2021). The total protein fraction of dried miracle berries was between 5 and 6%, of which, the glycoprotein miraculin comprised 15 to 40% of this amount (*i.e.*, between 0.75 to 2.4% miraculin in the final product). In the EFSA opinion, it was noted that no allergic reactions have been reported in the scientific literature following consumption of miracle fruits/berries. EFSA (2021) reported the results of an *in silico* search of miraculin (UnitProt No. P13087) against the National Center for Biotechnology Information (NCBI) amino acid database, and matches greater than 50% identity were identified to belonging to proteins from peach, sesame, and bitter lemon (Kunitz trypsin inhibitor 2) and miraculin precursor from tomato. In addition, the sequential identity between miraculin and widely known protein allergens from other plants was evaluated by EFSA, and the results indicated sequence identities of 83% with latex, 50% with peach, 53% with soy, and 80% with peanuts, with low query coverage across all matches (EFSA, 2021). It was concluded that there are “*no significant homology between miraculin and*

widely known pan-allergens" using the Basic Local Alignment Search Tool (BLAST) maintained by the NCBI (EFSA, 2021). The EFSA Panel concluded that these *in silico* findings are preliminary and do not allow to draw definitive conclusions on cross-reactivity. The results of Tafazoli *et al.* (2019, 2020) were also reviewed and discussed by EFSA, as well as a preliminary ELISA screening of the novel food (Dried Miracle Berries) with the major food allergens, in which positive results for peanut allergens were identified. It is also noted that the details of the *in silico* searches performed by the applicant of Dried Miracle Berries, as well as the methodology of the ELISA test, are scarce within the EFSA scientific opinion, however, it appears that the findings were subsequently published in the scientific literature by Menéndez-Rey *et al.* (2022). The authors employed an *in silico* allergenicity prediction model based on the protein sequence homology and predicted the protein structure using *in silico* methods to investigate the cross-reactivity potential of miraculin (UniProt Accession No. C5NU63) to known peanut allergens. In addition, the authors performed an ELISA assay using blood sera from peanut-allergic patients to investigate the cross-reactivity potential of proteins from the miracle fruit berry (*S. dulcificum*), including miraculin. Serum samples were obtained from 4 patients allergic to peanuts and 3 patients that were non-allergic to peanuts. The authors considered peanut allergy to be an immediate adverse reaction suggestive of IgE-mediated allergy, together with demonstration of a peanut-specific IgE level higher than 3.5 kU/L, as determined by ImmunoCAP™. The miracle berry extract was obtained by solid-liquid protein extraction of a lyophilized miracle berry powder (*i.e.*, the novel food reviewed by EFSA). The authors reported no significant responses in the miracle berry protein extract compared to the negative control, bovine serum albumin, suggesting that the miracle berry fruit does not contain any protein that may present a concern for cross-reactivity to known peanut allergens.

A BLAST search against the NCBI database with the results limited to proteins originating from *Arachis hypogaea* (peanut) did not identify any significant sequence homology to known peanut allergens. The highest identity match (50.52%) was to miraculin from peanuts; the query coverage was 85% and E-value of 10^{-53} suggest this match to be significant. It is noted that peanuts are one of the most well characterized foods with respect to allergenic proteins; miraculin from peanuts has not been identified as a major allergenic protein. A search of the AllergenOnline database confirm that miraculin from peanuts is not an allergenic protein. Likewise, the search results using internationally recognized guidelines for *in silico* allergenicity assessments revealed sequence homology matches to proteins from soybean and potatoes, with no matches to proteins from peanuts (see Table 6.6.2.1-1). Therefore, based on the totality of the evidence discussed herein, and on the basis of the results from various *in silico* assessments, it can be concluded that miracle fruit powder has low risk for allergenicity or cross-reactivity with any major protein allergens, including peanuts.

6.6.2.2 Allergenicity Assessment of Protein Digests of Miraculin

The 61 identified peptides were evaluated for allergenicity potential using a similar approach as described above (*i.e.*, full-length amino acid sequence and 80-amino acid sliding window). The use of these approaches on short peptides (<25 amino acids) are not commonly performed as part of allergenicity assessments and are not known to be predictive. The full-length search of each peptide digest revealed a number of matches with known allergens, with identity scores ranging from 36 to 67% and similarity scores ranging from 60 to 100%. The corresponding E-values ranged from 0.00067 to 0.95 with an amino acid overlap of 8 to 25. With short peptides (<25 amino acids), the likelihood of partial matches would be predicted to be higher than for the corresponding full-length protein. An appropriate criterion for potential significance is not known but should be lower than the <50% full-length identity used for intact proteins recommended by Aalberse (2000). The lack of predictability of sequence identity matches for short peptides was evaluated by Hileman *et al.* (2002) for peptides up to 8 amino acids in length, and it was determined that 100% identity over such short peptides was not a predictive criterion. Therefore, considering the high

E-values and an identity match of less than 67% over a short amino acid coverage (<25), it is unlikely that these peptide digests would pose any allergenic risk. The 80-amino acid sliding window searches with each peptide digest did not reveal any significant structural identity with any known allergens. The authors concluded that the results of the *in silico* searches with the peptide digests do not suggest that miraculin will pose a risk of cross-reactivity with known allergens (Tafazoli *et al.*, 2019, 2020).

In order to enhance detection of lower abundance peptide fragments that were detected within 10 minutes of digestion in an *in vitro* digestibility model with SGF, a follow-up experiment was conducted using the more sensitive Silver Staining method over a longer digestion period of 240 minutes. The results of the Silver Staining demonstrated complete and rapid digestion of miraculin within 20 minutes to fragments that had a molecular weight below the detection limit of gel electrophoresis method (*i.e.*, 2 to 5 kDa). The weight of the available evidence and the nature of the potential matches to trypsin inhibitors from soy and potato, including *in silico* results from the allergenicity assessment, suggest that miraculin is unlikely to have potential for allergenicity. These conclusions are further corroborated with the fact that exposures to miraculin *via* miracle berry and miracle fruit in commercial products in the U.S. have not been reported to be associated with any allergenic reactions.

6.6.2.3 Toxicogenicity of Miraculin

A sequence alignment query was conducted for the miraculin amino acid sequence against downloaded protein sequences obtained from a curated database of animal venom proteins and toxins maintained in the UniProtKB/Swiss-Prot Tox-Prot⁹ database. The amino acid sequences were compared using BLAST. The BLAST search results are summarized in Table 6.6.2.3-1 below. Several matches to animal toxins/venoms were identified, with sequence identities ranging from 25 to 54% and corresponding E-values of 0.61 to 9.3 with generally low query coverage (<25%). While there are no formal guidelines established for what constitutes a significant sequence similarity between an introduced protein and protein toxins (Hammond *et al.*, 2013), considering the low query coverage and high E-values/scores (Pearson, 2000; Bushey *et al.*, 2014) identified for the alignments between miraculin and the animal toxins/venoms, as presented in Table 6.6.2.3-1, the results of the full-length sequence alignment search of miraculin suggest that it does not share homology or structural similarity to any animal venom protein, toxins, virulence factors or harbors any toxic potential.

Table 6.6.2.3-1 Search Results of UniProtKB/Swiss-Prot Tox-Prot with Miraculin

Organism	Description	Sequence Length	Query Cover	E-value	% Identity
<i>Dabola siamensis</i>	Snaclec 5	148	19%	0.61	29%
<i>Dabola siamensis</i>	Snaclec 3	148	19%	0.58	29%
<i>Stichodactyla helianthus</i>	Kappa-stichotoxin-She3a	35	5%	1.8	54%
<i>Lychas mucronatus</i>	Lipolysis-activating peptide 1-alpha chain	83	25%	1.9	25%
<i>Conus leopardus</i>	Alpha-conotoxin-like Lp1.6a	61	9%	6.1	50%
<i>Apis cerana cerana</i>	Mast cell degranulating peptide	50	6%	6.6	53%
<i>Dendroaspis angusticeps</i>	Muscarinic toxin 1	66	23%	7.6	33%
<i>Tityus obscurus</i>	Potassium channel toxin alpha-KTx 13.1	23	8%	7.7	44%
<i>Stichodactyla haddoni</i>	Kappa-stichotoxin-Shd5a	74	14%	8.2	32%
<i>Heterodactyla hemprichii</i>	Kappa-thalatoxin-Hhe2a	75	14%	8.4	32%

⁹ The UniProtKB/Swiss-Prot Tox-Prot database is available at: <http://www.uniprot.org/uniprot/?query=taxonomy%3A%22Metazoa%22+AND+%28keyword%3Atoxin+OR+annotation%3A%28type%3A%22tissue+specificity%22+AND+venom%29%29+AND+reviewed%3Ayes&sort=score>.

Table 6.6.2.3-1 Search Results of UniProtKB/Swiss-Prot Tox-Prot with Miraculin

Organism	Description	Sequence Length	Query Cover	E-value	% Identity
<i>Thalassianthus oster</i>	Kappa-thalatoxin-Tas2a	75	5%	8.5	54%
<i>Dendraaspis angusticeps</i>	Muscarinic toxin 4	66	16%	8.6	42%
<i>Ethmostigmus rubripes</i>	U-scoloptoxin(16)-Er4a	130	20%	8.9	37%
<i>Stichodactyla gigantea</i>	Kappa-stichotoxin-Sgt4a	74	14%	9.3	32%

6.7 Biochemical Mechanisms of Miraculin Interaction with Sweet Taste Receptor

Miracle Fruit Farm conducted several human sensory panel experiments at miracle fruit powder concentrations of either 50 or 80 ppm that are representative of the levels that are proposed for use within water-based beverages (see Section 2.7). Consistent with the findings of Kurihara and Beidler (1969), Miracle Fruit Farm demonstrated that consumption of a miracle fruit powder-containing beverage does not interfere with taste qualities such as sweet, bitter, and sour following subsequent consumption of an acid solution. There were no detectable taste-modifying effects of citric acid solutions consumed post ingestion of miracle fruit powder at either 50 ppm or 80 ppm (approximately 3 to 4 nM) in a model soft drink formulation. This is consistent with the findings from Kurihara and Beidler (1969) reporting that a concentration of 20 nM of miraculin resulted in the disappearance of taste-modifying effects after 20 minutes. As such, a lower concentration, similar to what is being used in miracle fruit powder-containing beverages, would be expected to result in even a shorter latency of the effect. The results of the experiments conducted by Miracle Fruit Farm further confirmed that repeated stimulation of an 80-ppm miracle fruit powder model soft drink does not have any taste-modifying effects on other taste qualities such as sweet, bitter, and sour, when citric acid solutions are consumed post ingestion of miracle fruit powder solutions. The repeated exposure analysis further supports that repeated administration of an 80-ppm miracle fruit powder containing mock beverage has no additive effect at the receptor level when consumed close together. The results of the human sensory panel studies conducted by Miracle Fruit Farm further demonstrated that the receptor binding of miraculin in miracle fruit powder at a typical concentration of 80 ppm in a mock beverage does not impact the sweetness perception of other sweeteners at a neutral pH over time. While these results corroborate the human sensory study findings of Lipatova *et al.* (2016), they are contradictory to the findings of Koizumi *et al.* (2011) who reported that miraculin protein, when incubated with the sweet taste receptor at neutral pH, reduced the sweet taste response to various types of sweeteners, including carbohydrate, protein, and “artificial” sweeteners. Therefore, it is likely that there were some limitations in the *in vitro* study design of Koizumi *et al.* (2011).

Overall, the human sensory experiments conducted by Miracle Fruit Farm can corroborate those low levels of miraculin protein in miracle fruit powder-containing water-based beverages that will have little to no taste-modifying effects on foods and beverages that are subsequently consumed.

6.8 Conclusions

Miracle fruit powder is compositionally similar to the fruit with the only major difference being the removal of the moisture content. Miracle fruit has a long history of consumption dating back centuries and was introduced into the U.S. over 100 years ago by the USDA. Currently, several commercially available supplement products marketed in the U.S. contain miracle fruit/miracle berry, and derivatives thereof. The recommended dosage of these products is greater than the expected daily consumption of miracle fruit powder from use within water-based beverages. Overall, the maximum daily exposure to miracle fruit powder from use in water-based beverages, at levels between 3 to 4 nM (50 to 80 ppm), resulted in a maximum exposure of up to 48.7 mg/day, which is less than the currently recommended intakes as a supplement. The highest 90th percentile intake of miracle fruit powder of 48.7 mg/day would equate to the consumption of approximately 40% of a single miracle fruit. Taking into account the highest miraculin content of 0.32%, the highest mean and 90th percentile consumer-only intakes of miraculin were determined to be 72.64 µg/day and 155.84 µg/day, respectively, in female teenagers. The long history of safe consumption of miracle fruit in the U.S. for over 100 years, in conjunction with detailed compositional analysis and sensory data, as well as the published studies demonstrating the safety of miracle fruit protein, miraculin, confirms the safe use of miracle fruit powder in water-based beverages.

The 90th percentile intake of miracle fruit powder is also significantly lower than the 700 mg per day level considered safe by EFSA following the safety assessment of dried miracle berries, which is compositionally similar to miracle fruit powder described herein. Apart from the overall low exposure levels under the conditions of intended use, the safety of miracle fruit powder is also supported through detailed compositional analysis of the fruit including both macro ingredient components, and by demonstrating that the daily consumption of phytochemicals from miracle fruit is below the occurrence from consuming common fruits and teas. Therefore, the proposed use of miracle fruit powder within water-based beverages would not substantially contribute to the daily phytochemical exposure levels. Likewise, the daily exposure to antinutrients from the proposed uses of miracle fruit powder is significantly lower than from a serving of commonly consumed fruits.

Published human sensory studies and *in vitro* receptor binding studies clearly show low concentrations of miraculin in water-based beverages at levels of less than 4 nM have no impact on the taste profile of food during or following miraculin consumption. These findings were corroborated in 3 human sensory panel experiments, which reported that miracle fruit powder at the proposed use levels within water-based beverages (3 to 4 nM) had no impact on taste qualities such as sweet, bitter, and sour following either single or repeat stimulation. Similarly, miracle fruit powder did not impact the sweetness perception of other sweeteners at neutral pH over time. In addition, several published animal efficacy studies on miracle plant-based products report no significant adverse outcomes, albeit limited toxicological endpoints were assessed.

Regarding the safety of the miracle fruit protein, miraculin, both an *in vitro* digestibility and *in silico* bioinformation analyses indicated that the miraculin protein does not represent a risk of allergy or toxicity to humans and has a low cross-reactivity with other known allergens. This was further supported through proteomic analysis, which demonstrated that the miraculin glycoprotein was fully and readily digested by pepsin.

Based on the above data and information presented herein, Miracle Fruit Farm has concluded that the intended uses of miracle fruit powder in water-based beverage products, as described in Section 1.3, is GRAS based on scientific procedures. General recognition of Miracle Fruit Farms' GRAS conclusion is supported by the unanimous consensus rendered by an independent panel of experts, qualified by experience and scientific training, to evaluate the use of miracle fruit powder, as described herein is GRAS. Therefore, Miracle Fruit Farms' miracle fruit powder may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*. The GRAS Panel Consensus Statement is provided in Appendix B.

PART 7 § 170.255 LIST OF SUPPORTING DATA AND INFORMATION

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APPENDIX A
List of Tested Alkaloids

List of Tested Alkaloids and their Reporting Limits

Compound	Reporting Limit
Echimidine	1 µg/kg
Echimidine N-oxide	1 µg/kg
Erucifoline	1 µg/kg
Erucifoline N-oxide	1 µg/kg
Europine	1 µg/kg
Europine N-oxide	1 µg/kg
Heliotrine	1 µg/kg
Heliotrine N-oxide	1 µg/kg
Intermedine	1 µg/kg
Intermedine-N-oxide/Indicine-N-oxide	1 µg/kg
Jacobine	1 µg/kg
Jacobine N-oxide	1 µg/kg
Lasiocarpine	1 µg/kg
Lasiocarpine N-oxide	1 µg/kg
Lycopsamine/Indicine	1 µg/kg
Lycopsamine N-oxide	1 µg/kg
Monocrotaline	1 µg/kg
Monocrotaline N-oxide	1 µg/kg
Retrorsine	1 µg/kg
Retrorsine N-oxide	1 µg/kg
Senecionine	1 µg/kg
Senecionine N-oxide	1 µg/kg
Seneciophylline	1 µg/kg
Seneciophylline N-oxide	1 µg/kg
Senecivernine	1 µg/kg
Senecivernine N-oxide	1 µg/kg
Senkirkine	1 µg/kg
Trichodesmine	1 µg/kg
Sum of all positive pyrrolizidinalkaloids	-
Anisodamine	2 µg/kg
Atropine	1 µg/kg
Atropine-NO _x	2 µg/kg
Homatropine	2 µg/kg
Littorin	2 µg/kg
Norscopolamine	2 µg/kg
Scopolamine	1 µg/kg
Scopolamine-N-oxide	1 µg/kg
Sum of Atropin/Scopolamin	-
Sum of Atropin/Scopolamin and their N-oxides	-
Sum of all positive tropanalkaloids	-

APPENDIX B
GRAS Panel Consensus Statement

GRAS Panel Statement Concerning the Generally Recognized as Safe (GRAS) Status of the Proposed Use of Miracle Fruit Powder in Water Based Beverages

03 May 2023

INTRODUCTION

At the request of Miracle Fruit Farm, LLC. (Miracle Fruit Farm), an Expert Panel (the “GRAS Panel”) of independent scientists, qualified by their scientific training and relevant national and international experience in the safety evaluation of food ingredients, conducted a critical and comprehensive assessment of data and information pertinent to the safety of miracle fruit powder to determine whether the intended uses of miracle fruit powder as an ingredient in water-based beverages would be Generally Recognized as Safe (GRAS) based on scientific procedures. The GRAS Panel consisted of the below-signed qualified scientific experts: Professor Emeritus George C. Fahey, Jr., Ph.D. (University of Illinois); Professor Emeritus I. Glenn Sipes, Ph.D., Fellow AAAS and ATS (University of Arizona College of Medicine), and Emeritus Professor Stephen L. Taylor, Ph.D. (University of Nebraska).

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of publicly available scientific data and information compiled from the literature and summarized in a dossier titled “*GRAS Notice for Miracle Fruit Powder*” (dated 6 March 2023), which included an evaluation of available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended uses of miracle fruit powder. This dossier was prepared in part from a comprehensive search of the scientific literature performed at the request of Miracle Fruit Farm through March 2023 and included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, and the safety of miracle fruit powder and its active glycoprotein component, miraculin.

Following its independent and collective critical evaluation, and on the basis of scientific procedures, the GRAS Panel unanimously concluded that Miracle Fruit Farm’s miracle fruit powder, meeting food-grade specifications and manufactured in accordance with current Good Manufacturing Practice (cGMP), is GRAS for use as an ingredient in water-based beverages. A summary of the information critically evaluated by the GRAS Panel is presented below.

COMPOSITION, MANUFACTURING, AND SPECIFICATIONS

Miracle fruit powder is obtained from de-seeded miracle fruit berries. Miracle fruit, which is also referred to as “miraculous berry”, “sweet berry”, or “miracle berry”, is a small (2 to 3 cm) bright red fruit from *Synsepalum dulcificum*, an evergreen bush or tree that is native to tropical West Africa, but also grown in Florida. Miracle fruit powder is intended for use as an ingredient for addition to water-based beverages for its ability to impart sweetness by modifying taste from sour to sweet due to the active glycoprotein, miraculin.

Miraculin, present within the thin-layered pulp of the miracle fruit berries, is a single polypeptide with 191 amino acid residues that was isolated in 1968 by researchers at Florida State University (Theerasilp and

Kurihara, 1988). Miraculin imparts a taste-modifying effect when consumed by binding to the sweet receptors of the tongue, turning sour tastes into sweet (Morris, 1976). The protein has 2 glycosylated sites (Asn-42 and Asn-186) crosslinked by disulfide bonds with a molecular weight of 28 kDa (Theerasilp and Kurihara, 1988; Theerasilp *et al.*, 1989). The amino acid sequence of miraculin is publicly available on the UniProt/SwissProt database under Accession No. P13087.

Miracle fruit powder is manufactured consistent with cGMP, and the production process complies with the principles of Hazard Analysis and Critical Control Points (HACCP). The finished ingredient is produced by pulping, maceration, and freeze-drying the de-seeded-miracle fruit berries and does not involve the use of any solvent or chemical processing aid.

Miracle Fruit Farm has established food-grade specifications for miracle fruit powder which include organoleptic and compositional parameters, including heavy metals and microbiological contaminants. All analytical methods are internationally recognized or have been developed internally and validated. The GRAS Panel reviewed the results from 3 to 9 non-consecutive batches of miracle fruit powder for physical, chemical and microbiological parameters and concluded that the manufacturing process produces a consistent product that conforms to the established specifications.

Miracle fruit powder has been fully characterized and is primarily comprised of carbohydrates (~87% on dry basis), protein (~6.6% on dry basis), ash (~3.9% on dry basis), and moisture (~3.6%), with a minimum miraculin content of ~0.05% on dry basis. Detailed analyses of antinutrients including oxalic acid, phytic acid, and trypsin inhibitors demonstrated that the levels of these antinutrients in miracle fruit powder are comparable with those occurring in other commonly consumed fruits or they are present at low or non-detectable levels and therefore, they are not associated with any safety concerns. A detailed characterization of the polyphenol profile of miracle fruit powder has also been performed, which demonstrated that the polyphenol content of miracle fruit powder is primarily comprised of anthocyanidins and glycosides, anthocyanosides, and catechins and is comparable with commonly consumed berries, such as blackberry, blueberry, cranberry, strawberry, raspberry, as well as green and oolong teas. Taking into account the polyphenol content of miracle fruit powder, the anticipated dietary exposure to total polyphenols from the proposed food uses of miracle fruit powder in water-based beverages would not substantially contribute to the total polyphenol exposure in the U.S. population.

The GRAS Panel also reviewed the results of a 52-week shelf-life stability study. Samples from 3 non-consecutive lots of miracle fruit powder were stored at 25±2°C and 60% relative humidity in metalized barrier pouches for 52 weeks. The miraculin and moisture contents of each sample were measured at 0, 2, 4, 8, 14, 26, and 52 weeks. The results indicate that miracle fruit powder is stable for up to 52 weeks when stored at ambient temperature and humidity.

TECHNICAL EFFECT

Miracle fruit powder will be marketed in powdered form and added to beverage products for its ability to impart sweetness by modifying taste from sour to sweet due to the active glycoprotein, miraculin. The sweetness profile of miracle fruit was evaluated by 6 trained panelists (Tafazoli *et al.*, 2019). A baseline sweetness intensity was established with lemonade juice with a sweetness intensity of 7 Brix. Following establishment of a baseline sweetness intensity, each panelist consumed 0.08 g of miracle fruit powder and was instructed to hold the powder in the mouth for 1 minute before swallowing. Each panelist then consumed 60 mL of the original lemonade juice every 5 minutes for 30 minutes, and the sweetness of each cup was recorded. The results indicate that miracle fruit significantly increased the perceived sweetness of lemonade juice, and the perceived sweetness of the juice returned to baseline levels in all subjects after 30

minutes. These results indicate the taste-modifying effect of miraculin is rapid with no lasting desensitization effect. It is noted that this sensory study was conducted with a miracle fruit powder amount (0.08 g) that is expected to be much greater than the amounts proposed for use in water-based beverages (50 ppm) and would reflect a “worst-case” effect scenario. In another published study evaluating the taste-modifying effect of miraculin, the maximum relative sweetening effect was achieved within 3 minutes of consumption, and rapidly declined after 30 minutes. These effects were concentration-dependent (Kurihara and Beidler, 1969). While the results of human sensory studies confirmed the increase of sweetness and corresponding decrease of sourness of sour tastants after pre-exposure with miraculin, there is striking evidence that miraculin has no effect on bitter, salty, and sweet stimuli, rendering it very unlikely that miraculin acts on the biological targets responsible for the detection of these taste qualities. Nevertheless, an intense increase of sweetness after exposure to miraculin combined with a lack of a sourness effect can cause a diversion of the overall flavor from the expected traditional flavor in complex food products. Overall, the results of human sensory studies indicate that miraculin does not impact the overall taste profile and does not interfere with other taste qualities such as bitter and salty, and has little or no impact on the taste profile of foods consumed with or following miraculin consumption. Those human sensory studies conducted by Kurihara and Beidler (1969) indicated that the taste modifying effect of miraculin dissipates quickly at low concentrations of 20 nM and knowing the correlation between concentration and affinity at the receptor, it may be assumed that the residence time of miraculin will be even shorter at concentrations of between 0.4 and 4 nM that are aligned with the proposed uses of miracle fruit powder in the water-based beverages.

FUNCTIONALITY

Miracle fruit powder is intended for use in water-based beverage products for its sweetening and taste-modifying properties due to the active glycoprotein, miraculin. Products to which miracle fruit powder will be added will not carry any structure/function or health claims and will be marketed similar to conventional products that do not contain this ingredient. The only difference between the products containing Miracle Fruit Farm’s miracle fruit powder and the conventional products will be in the ingredient list. Therefore, no increases or changes in the consumption pattern of food products containing miracle fruit powder is expected compared to conventional food products that do not contain the ingredient.

While addition of miracle fruit powder to water-based beverages is expected to change their sweetness profile, this ingredient will not have an impact on the pH of the beverage to which it is added. Considering that the miracle fruit powder-containing product will be marketed in a similar manner as any other conventional water-based beverage, there will be no risk to consumers that suffer from conditions such as acid-reflux or other digestive disorders; therefore, consumers who may experience digestive disorders such as acid-reflux would be expected to self-regulate products that contain Miracle Fruit Farm’s miracle fruit powder in the same manner as any other acidic products and refrain from consuming them.

INTENDED USE AND ESTIMATED EXPOSURE

Miracle fruit powder is intended to be added as an ingredient to water-based beverages at a use level of 50 ppm (0.005%). These uses fall under the “Beverages and Beverage Bases” food category as defined under 21 CFR §170.3 (U.S. FDA, 2022). Miracle fruit powder is not intended for use in infant formula or infant food products or bottled water, and the proposed food category does not include food uses that are subject to the oversight by the U.S. Department of Agriculture (USDA) and the USDA Food Safety & Inspection Service (FSIS).

The GRAS Panel reviewed data related to the estimated dietary exposure to miracle fruit powder based on an assessment of its anticipated intake as an ingredient under the intended conditions of use and use levels using the information from the 2015-2016 cycle of the National Health and Nutrition Examination Survey (NHANES).

Among the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of miracle fruit powder were determined to be 16.5 and 36.0 mg/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest statistically reliable mean consumer-only intakes of miracle fruit powder on an absolute basis at 22.1 mg/person/day. Female adults had the lowest statistically reliable mean consumer-only intakes of 14.9 mg/person/day (see Table 3.2.2-1). The 90th percentile value was not considered to be statistically reliable in any of the age groups, as the number of consumers within the individual population groups were all below 80. Similarly, the mean intakes of miracle fruit powder by individual populations ages ≤19 years were not statistically reliable based on low sample size (n<30). While the values using NHANES were not statistically reliable, an alternative serving-size approach for estimating exposure likewise determined that heavy consumers (consuming 2 bottles of the 360 mL water-based beverages per day) would result in a similar intake of intake of 36 mg/day.

The GRAS Panel also reviewed estimated exposure to the active glycoprotein, miraculin, from the proposed food uses of miracle fruit powder. Corresponding intakes of miraculin were calculated based on the highest miraculin content of 0.32% across 9 tested batches. In the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of miraculin were determined to be approximately 52.8 and 115.2 µg/person/day, respectively. In the female teenagers, the highest miraculin exposure was approximately 72.6 µg/day (mean) and 155.8 µg/day (90th percentile).

SAFETY NARRATIVE

Miracle fruit powder is obtained by mechanical processing steps (*i.e.*, pulping, maceration, freeze-drying, milling) from miracle fruit as the starting material. Analytical composition data demonstrated that there are no compositional differences between the miracle fruit powder and the starting material, aside from the moisture content considering that the miracle fruit powder undergoes freeze-drying to obtain the powder form. Mean values from the analysis of 6 production batches of miracle fruit powder demonstrate that the ingredient is primarily composed of carbohydrates (~88% on dry basis), protein (~6% on dry basis), ash (~4% on dry basis), and moisture (~3.5%).

Miracle Fruit Farm's conclusion on the GRAS status of miracle fruit powder under its conditions of intended use, as an ingredient in various beverage products, as reviewed by the GRAS Panel is based on scientific procedures supported by the following:

1. Long history of consumption of miracle fruit globally;
2. The low intakes of miracle fruit powder under the intended conditions of use that equate to consuming approximately only 40% of a single miracle fruit;
3. Publicly available data related to the safety of miracle fruit, miracle fruit powder, and miraculin;
4. Compositional data on miracle fruit powder and pulp, including individual polyphenols, and comparison with other commonly consumed fruits;

5. Exposure to antinutrients from proposed uses of miracle fruit powder; and
6. Sensory studies on Miracle Fruit Powder demonstrating that the sensory profile of beverages consumed after consumption of the ingredient is not adversely impacted.

Miracle fruit powder is compositionally similar to the fruit with the only major difference being the removal of the moisture content. Miracle fruit has a long history of consumption dating back centuries and was introduced into the U.S. over 100 years ago by the USDA. Currently, several commercially available supplement products marketed in the U.S. contain miracle fruit/miracle berry, and derivatives thereof. The recommended dosage of these products is greater than the expected daily consumption of miracle fruit powder from use within water-based beverages. Overall, the maximum daily exposure to miracle fruit powder from use in water-based beverages, at levels between 3 to 4 nM (50 to 80 ppm) is estimated at a maximum exposure of 48.7 mg/day, which is less than the currently recommended intakes as a supplement. The highest 90th percentile intake of miracle fruit powder of 48.7 mg/day would equate to the consumption of approximately 40% of a single miracle fruit. Taking into account the highest miraculin content of 0.32%, the highest mean and 90th percentile consumer-only intakes of miraculin were determined to be 72.6 µg/day and 155.8 µg/day, respectively, in female teenagers. The long history of safe consumption of miracle fruit in the U.S. for over 100 years, in conjunction with detailed compositional analysis and sensory data, as well as the published studies demonstrating the safety of miracle fruit protein, miraculin, confirms the safe use of miracle fruit powder in water-based beverages.

The 90th percentile intake of miracle fruit powder is also significantly lower than the 700 mg per day level considered safe by EFSA following the safety assessment of Dried Miracle Berries, which is compositionally similar to miracle fruit powder described herein. Apart from the overall low exposure levels under the conditions of intended use, the safety of miracle fruit powder is also supported through detailed compositional analysis of the fruit including both macro ingredient components, and by demonstrating that the daily consumption of phytochemicals from miracle fruit is below the occurrence from consuming common fruits and teas. Therefore, the proposed use of miracle fruit powder within water-based beverages would not substantially contribute to the daily phytochemical exposure levels. Likewise, the daily exposure to antinutrients from the proposed uses of miracle fruit powder is significantly lower than from a serving of commonly consumed fruits.

Published human sensory studies and *in vitro* receptor binding studies clearly show that low concentrations of miraculin in water-based beverages, at levels of less than 4 nM, have no impact on the taste profile of food during or following miraculin consumption. These findings were corroborated in 3 human sensory panel experiments, which reported that miracle fruit powder at the proposed use levels within water-based beverages (3 to 4 nM) had no lasting impact on taste qualities such as sweet, bitter, and sour following either single or repeat stimulation. Similarly, miracle fruit powder did not impact the sweetness perception of other sweeteners at neutral pH over time. In addition, several published animal efficacy studies on miracle plant-based products report no significant adverse outcome albeit there were limited toxicological endpoints assessed.

Regarding the safety of the miracle fruit protein, miraculin, results of both *in vitro* digestibility and *in silico* bioinformatics analyses indicated that the miraculin protein does not represent a risk of allergy or toxicity to humans and has a low cross-reactivity with other known allergens. This was further supported through proteomic analysis which demonstrated that the miraculin glycoprotein was fully and readily digested by pepsin.

Based on the above data and information presented herein, Miracle Fruit Farm has concluded that the intended uses of miracle fruit powder in water-based beverage products is GRAS based on scientific

procedures. General recognition of Miracle Fruit Farms' GRAS conclusion is supported by the unanimous consensus rendered by an independent panel of experts, qualified by experience and scientific training, to evaluate the use of miracle fruit powder to be GRAS. Miracle Fruit Farms' miracle fruit powder; therefore, may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*.

CONCLUSION

We, the members of the GRAS Panel, have, independently and collectively, critically evaluated the data and information summarized above, and unanimously conclude that the proposed uses of Miracle Fruit Farm's miracle fruit powder are safe.

We further unanimously conclude that the proposed uses of Miracle Fruit Farm's miracle fruit powder, meeting appropriate food-grade specifications and produced in accordance with current Good Manufacturing Practice (cGMP), are Generally Recognized as Safe (GRAS) under conditions of intended use based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

[Redacted Signature]

George C. Fahey, Jr. Ph.D.
Professor Emeritus
University of Illinois

May 4, 2023

Date

[Redacted Signature]

I. Glenn Sipes, Ph.D.
Fellow AAAS and ATS
Professor Emeritus
University of Arizona College of Medicine

May 3, 2023

Date

[Redacted Signature]

Steve L. Taylor, Ph.D.
Professor Emeritus
University of Nebraska

May 3, 2023

Date

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7 November 2023

Renata Kolanos, Ph.D.
Regulatory Review Scientist
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration

Dear Dr. Kolanos,

Re: GRAS Notice No. GRN 001144

Thank you for your questions and comments on the GRAS Notice No. GRN 1144 for use of Miracle Fruit Powder as a taste modifier in water-based beverages. We have reviewed FDA's questions and comments and provided information to address each question below. We hope these sufficiently address the Agency's questions and remain at your disposal if additional information or clarification is needed.

Question 1: The notice describes the intended use for miracle fruit powder as an ingredient at up to 0.005% in water-based beverages. Please elaborate on the types of beverages that are included in the category of water-based beverages. For example, what types of beverages from the National Health and Nutrition Examination Survey (NHANES) food consumption dataset were included in the notifier's dietary exposure assessment for the intended use of miracle fruit powder?

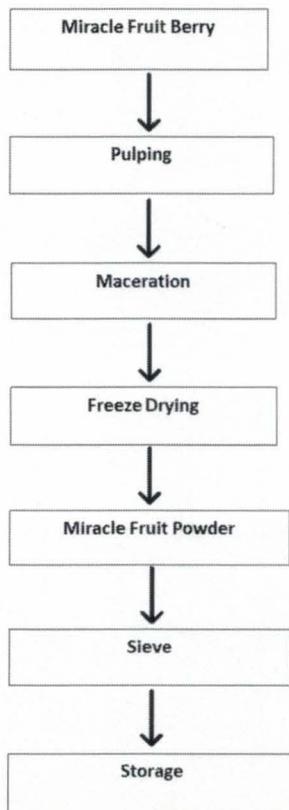
Response 1

The types of beverages from the National Health and Nutrition Examination Survey (NHANES) food consumption dataset were reserved to (1) sweetened or flavored bottled water (such as vitamin water) as well as (2) "niche" water-based beverages, such as coconut water, aloe vera drink, and sugar cane beverage. It excludes other beverage products that could be interpreted as water-based beverages, such as soft drinks and sports/energy drinks.

Question 2: The manufacturing process described on p. 7 of the notice for miracle fruit powder includes a freeze-drying step that also includes a quality control step with an analysis of the reduction in water activity. However, the flowchart depicted in Figure 2.2-1 (p. 7) indicates that the freeze-drying step is "optional." Please clarify whether the freeze-drying step is considered optional and if other methods of drying are used.

Response 2

Currently, the manufacturing process of miracle fruit powder only employs freeze-drying. Originally, the pulp of the miracle fruit was intended for use in the finished food product in addition to the freeze-dried powder. However, use of the fruit pulp is no longer under consideration by Miracle Fruit Farm. The revised schematic of the manufacturing process is provided below. The sieving step has been included to ensure there will be no transfer of the skin granules from the fruit into the final powder before storage.



Question 3: *Specifications are provided for miracle fruit powder that include a visual appearance parameter of “red/red-brown to pink powder.” The results of the analyses of three batches presented in Table 2.4.1-1 (p. 10) all describe the result for appearance as a “pink granular powder with black specks.” Please confirm that the notifier considers these observations to be in compliance with the listed specification.*

Response 3

The black specs were associated with the seed skin granules in the first generation of the miracle fruit powder. The process has since been improved and a sieving step has been included that will prevent the transfer of skin granules into the final freeze-dried powder prior to the storage, and the batches of miracle fruit powder that have since been tested, do not contain black specs and conform to the product specification with the final product being a pink to reddish colour.

Question 4: *Specifications include a limit for total fatty acids ($\geq 0.4\%$), and the notifier cites the following methods of analysis: AOAC 996.06 and AOCS Ce 2-66/Ce2b-11. AOAC method 996.06 is described as the determination of total fat that is calculated as the sum of individual fatty acids expressed as triglyceride equivalents. Please clarify if this specification is intended to be for total fat or total fatty acids.*

Response 4

For clarity the specification parameter in Table 2.4.2.1-1 is revised as follow: “Total fatty acids expressed as triglyceride equivalents (dry basis)”. Accordingly, the data for lot nos. 2019-09-0060, 2019—11-0060 and 2019-13-0060 in Table 2.4.2.1-1 are corrected to be expressed as the sum of individual fatty acids as triglyceride

equivalents on dry basis. The corrected values are presented in Table 2.4.2.1-1 below in red text. The calculated carbohydrate values are also corrected accordingly (please refer to the response to Question 6). The data for the other 6 lots in the table are already expressed as the sum of individual fatty acids as triglyceride equivalents.

Table 2.4.2.1-1 Proximate Analysis of 9 Non-Consecutive Batches of Miracle Fruit Powder

Specification Parameter	Specification Limit	Manufacturing Lot No.								
		2019-09-0060*	2019-11-0060*	2019-13-0060*	KVS20200506 AD Powder 2020-19-001A	KVS20200506 06AE_Powder 2020-19-002B	KVS20200506 AF_Powder 2020-19-003C	20220608-A	20220608-B	20220608-C
Carbohydrates (dry basis)	≥80%	90.8	90.7	90.8	86.2	85.4	86.9	88.4	88.9	88.9
Total dietary fiber (dry basis)	≥5.5%	13.0	13.6	13.3	10.9	11.8	10.2	16.0	16.4	16.3
Total fatty acids expressed as triglyceride equivalents (dry basis)	≥0.4%	0.705	0.706	0.706	0.788	0.872	0.811	0.89	0.86	0.91
Protein (dry basis)	≥4.5%	5.16	5.23	5.14	7.56	8.34	7.22	7.01	6.98	6.92
Ash (dry basis)	<6%	3.38	3.38	3.40	5.45	5.41	5.11	3.66	3.27	3.22
Moisture	<6%	1.82	1.87	1.78	5.63	5.91	4.53	3.37	4.25	3.08
Miraculin (dry basis)	≥0.048%	0.055	0.052	0.054	0.28	0.24	0.32	0.12	0.14	0.13

*Values for Lots 2019-09-0060, 2019—11-0060, and 2019-13-0060 are corrected for moisture, and also corrected for fatty acid as triglyceride equivalents. All values are presented on a dry basis.

Question 5: Specifications include a limit for cadmium of <0.2 mg/kg and a limit of <0.1 mg/kg for lead, arsenic, and mercury. The results of the analyses of three batches of miracle fruit powder demonstrate that the levels of these heavy metals are below the specified limits. We note that specifications help to ensure that the ingredient is being manufactured in accordance with good manufacturing practices. In addition, FDA’s recent “Closer to Zero” initiative focuses on reducing dietary exposure to heavy metals from food. We request that specifications for heavy metals be as low as possible and consistent with the methods used and the results obtained from the batch analyses. Please consider reducing the specification for cadmium to a level comparable to the other heavy metals.

Response 5

For consistency with other heavy metals, the specification limit for cadmium in Tables 2.3-1 and 2.4.2.2-1 has been changed to <0.1 mg/kg.

Question 6: A specification is provided for carbohydrates with the method listed as determination by calculation. Please confirm how this parameter is calculated. For example, the footnote to Table 2.4.2.1-2 notes that total carbohydrates is determined as the sum of mono- and di-saccharides, starch (including maltodextrin), total dietary fiber, and total organic acids. Calculation of carbohydrate content by difference

in some cases (e.g., food labeling) is determined by subtraction of the sum of the protein, total fat, moisture, and ash from the total weight of the food. We note that the sum of carbohydrates, total fatty acids, protein, ash, and moisture for the nine batches presented in Table 2.4.2.1-1 in some cases exceed 100% and are in the range of 98.34 to 105.932%. Please indicate the reason for the composition exceeding 100%.

Response 6

The carbohydrate values, as presented in Table 2.4.2.1-1 below, are determined on dry basis through calculation using the following formula:

$$\% \text{ Carbohydrate} = 100 - (\% \text{ total fatty acids as triglycerides} + \% \text{ Ash} + \% \text{ Protein})$$

Please note that moisture was not included in this calculation, as the values are already corrected for moisture. For clarity and consistency, we have also revised the carbohydrate values for lot nos. 20220608-A, 20220608-B, and 20220608-C based on the above formula and the revised values are presented below in red text. Taken into account this calculation, the sum of carbohydrates, total fatty acids, proteins, and ash, on dry basis, for 9 non-consecutive batches of miracle fruit powder are in the range of 99.95 % to 100.05%.

Table 2.4.2.1-1 Proximate Analysis of 9 Non-Consecutive Batches of Miracle Fruit Powder

Specification Parameter	Specification Limit	Manufacturing Lot No.								
		2019-09-0060*	2019-11-0060*	2019-13-0060*	KVS20200506AD_Powder 2020-19-001A	KVS20200506AE_Powder 2020-19-002B	KVS20200506AF_Powder 2020-19-003C	20220608-A	20220608-B	20220608-C
Carbohydrates (dry basis)	≥80%	90.8	90.7	90.8	86.2	85.4	86.9	88.4	88.9	88.9
Total dietary fiber (dry basis)	≥5.5%	13.0	13.6	13.3	10.9	11.8	10.2	16.0	16.4	16.3
Total fatty acids expressed as triglyceride equivalents (dry basis)	≥0.4%	0.705	0.706	0.706	0.788	0.872	0.811	0.89	0.86	0.91
Protein (dry basis)	≥4.5%	5.16	5.23	5.14	7.56	8.34	7.22	7.01	6.98	6.92
Ash (dry basis)	<6%	3.38	3.38	3.40	5.45	5.41	5.11	3.66	3.27	3.22
Moisture	<6%	1.82	1.87	1.78	5.63	5.91	4.53	3.37	4.25	3.08
Miraculin (dry basis)	≥0.048%	0.055	0.052	0.054	0.28	0.24	0.32	0.12	0.14	0.13

*Values for Lots 2019-09-0060, 2019—11-0060, and 2019-13-0060 are corrected for moisture, and also corrected for fatty acid as triglyceride equivalents. All values are presented on a dry basis.

Please note that we have removed the sum of Total Carbohydrates in Table 2.4.1.1-2 below. As discussed earlier, the total sum of carbohydrates was obtained using a calculation method, whereas the carbohydrate profile and organic acid content of lot nos. 20220608-A, 20220608-A and 20220608-C, as presented in Table 2.4.1.1-2 below, were analytically measured.

Table 2.4.2.1-2 Carbohydrate Profile and Organic Acid Content of 3 Non-Consecutive Batches of Miracle Fruit

Parameter	20220608-A	20220608-B	20220608-C
Mono-/di-saccharide (% dry basis)	51.70	51.30	51.40
Glucose (% dry basis)	21.40	20.90	20.30
Fructose (% dry basis)	23.80	23.00	22.40
Sucrose (% dry basis)	6.50	7.40	8.70
Starch incl. maltodextrin (% dry basis)	0.70	0.60	0.60
Total dietary fiber (% dry basis)	16.00	16.40	16.30
Insoluble IDF (% dry basis)	10.50	11.30	11.20
Soluble SDFP+LMWSDF (% dry basis)	5.40	5.11	5.19
Total organic acid (% dry basis)	15.51	17.76	15.91
Citric acid	12.8	13.8	13
Pyruvic acid	0.0058	0.0057	0.0049
Malic acid	1.83	2.29	2.05
Quinic acid	0.875	0.888	0.851
Succinic acid	ND ^{2a}	0.737	ND ^{2a}
Glycolic acid	ND ^{3a}	0.044	ND ^{4a}

LMWSDF = low molecular weight soluble dietary fiber; LOQ = limit of quantitation; ND = not detected; SDFP = soluble dietary fiber, which precipitates in the presence of 76% ethanol.

^a Sum of mono-/di-saccharide, starch incl. maltodextrin, total dietary fiber, and total organic acid.

ND¹: LOQ is 0.4950%; ND²: LOQ is 0.4840%; ND³: LOQ is 0.0289%; ND⁴: LOQ is 0.0320%.

Question 7: For the administrative record, please specify whether “Salmonella” that appears in Table 2.3-1 (p. 8) refers to Salmonella serovars.

Response 7

Analysis for *Salmonella* was conducted based on FDA BAM method, which includes screening for all serovars within the genus. In case of a presumptive positive, additional testing would be performed to determine the serovars. However, this was not required for the lots tested, as they were all negative as presented in Table 2.4.3-1.

Question 8: Estimates of dietary exposure to miracle fruit powder are provided in the notice that includes summaries in Tables 3.2.2-1 (p. 32) and 3.2.2-2 (p. 33). We note small discrepancies between the number of reported consumers in these tables, specifically, female teenagers (n=13 vs. n=12), female adults (n=52 vs. n=51), male adults (n=46 vs. n=45), and total population (n=156 vs. n=153). Please address this discrepancy between the number of consumers observed.

Response 8

Table 3.2.2-1 presents the exposure estimates on an absolute basis while Table 3.2.2-2 presents the estimates on a body weight basis. The discrepancy in the number of consumers indicates that body weight data is unavailable for 3 individuals and therefore the body weight values could not be calculated. This is a fairly common occurrence in surveys for reasons such as measurement or reporting errors, leading to this information not being collected or recorded.

Yours sincerely,



Erik Tietig
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