United States Department of Agriculture
Agricultural Marketing Service | National Organic Program
Document Cover Sheet
https://www.ams.usda.gov/rules-regulations/organic/national-list/petitioned

Document Type:

☐ **National List Petition or Petition Update**

A petition is a request to amend the USDA National Organic Program's National List of Allowed and Prohibited Substances (National List).

Any person may submit a petition to have a substance evaluated by the National Organic Standards Board (7 CFR 205.607(a)).

Guidelines for submitting a petition are available in the NOP Handbook as NOP 3011, National List Petition Guidelines.

Petitions are posted for the public on the NOP website for Petitioned Substances.

☒ **Technical Report**

A technical report is developed in response to a petition to amend the National List. Reports are also developed to assist in the review of substances that are already on the National List.

Technical reports are completed by third-party contractors and are available to the public on the NOP website for Petitioned Substances.

Contractor names and dates completed are available in the report.

L-Malic Acid
Handling/Processing

Identification of Petitioned Substance

Chemical Names:
L-malic acid
L(-)-malic acid
L-hydroxysuccinic acid
L-hydroxybutanedioic acid
L-1-hydroxy-1,2-ethanedicarboxylic acid
L-malate
(S)-hydroxybutanedioic acid
(S)-malate

Other Name:
Apple acid, Deoxytetraric acid, Methyl tartronic acid

Trade Names:
L-malic acid

CAS Numbers:
L-malic acid (97-67-6)

Other Codes:
L-malic acid EC 202-601-5

Summary of Petitioned Use

L-malic acid is currently listed at 7 CFR 205.605(a) along with other nonsynthetic, "nonagricultural (nonorganic) substances allowed as ingredients in or on processed products labeled 'organic' or 'made with organic (specified ingredients or food group(s)).'" L-malic acid is used as a flavor enhancer, flavoring agent, adjuvant, and pH control agent in a variety of foods (USDA, 2015a).

DL-malic acid was originally petitioned for inclusion on the National List of Allowed and Prohibited Substances ("the National List") in 2002; after review, the National Organic Standards Board (NOSB) recommended its inclusion at their May 2003 meeting. The addition of L-malic acid (CAS #97-67-6) to the National List was announced on September 11, 2006 (Federal Register Vol. 71, No. 175), and became effective September 12, 2006. L-malic acid underwent two sunset reviews at the Fall 2009 NOSB meeting and the April 2015 NOSB meeting (USDA, 2015a); the NOSB relisted the material in both cases. L-malic acid is again under review before its scheduled sunset date of September 2021.

The following additional technical report requests were identified by the NOSB Handling Subcommittee:
1. Describe how malic acid is produced via fermentation (in addition to any other methods of production) and how production via fermentation may affect its classification as either synthetic/nonsynthetic and agricultural/nonagricultural.
2. Is malic acid best classified as agricultural or non-agricultural, according to NOP Decision Tree NOP 5033-2?
3. Is malic acid best classified as nonsynthetic or synthetic according to NOP Decision Tree 5033-1?
4. Are nonsynthetic forms of malic acid available?

These questions are answered below in *Evaluation Question #1* and *Evaluation Question #2*.

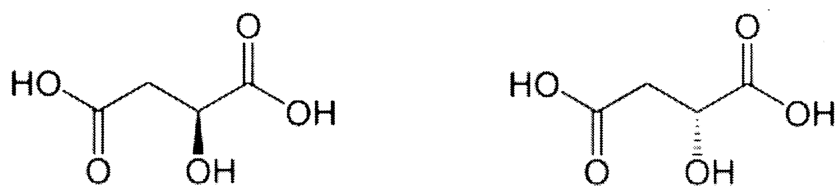
Characterization of Petitioned Substance

<u>Composition of the Substance:</u>
Malic acid is optically active and exists in D-, L-, and racemic DL-forms (mixtures of equal parts of both forms) (West 2017). Chemical names, CAS#'s, other names, and other codes for the D-malic and DL-malic

acid forms are included below. Use of the term "malic acid" in this technical report generally refers to the DL-malic acid form unless otherwise specified.

DL-malic acid is synthetic, and its synthesis is described in *Evaluation Question #1* below. D-malic acid does not occur naturally but can be obtained by separating DL-malic acid into its components. L-malic acid occurs naturally in many fruits, including apples and cherries (USDA 2002). It can be obtained by enzymatic synthesis or fermentation as described in *Evaluation Question* #2. The chemical structure of D- and L-malic acids are shown in Figure 1 below:

Figure 1. Optically Active Forms of Malic Acid (Wikipedia 2018; USDA 2002)



L-malic acid **D-malic acid**

The four-carbon dicarboxylic, succinic, malic, fumaric, and maleic acids are chemically related. Succinic acid is an aliphatic, saturated compound. Malic acid is the hydroxy- derivative of succinic acid. Fumaric acid is unsaturated succinic acid with a *trans*- double bond configuration. It is *trans*-1,2-ethylenedicarboxylic acid. Maleic acid is unsaturated succinic acid with a *cis*- double bond configuration. It is *cis*-1,2-ethylenedicarboxylic acid. In the naturally occurring Krebs or tricarboxylic acid cycle, succinic acid is oxidized to fumaric acid and then to malic acid (Goldberg et al. 2006; West 2017).

The chemical names and other identifiers for DL-malic and D-malic acid forms are as follows:

Chemical Names:
DL-malic acid
DL(±)-malic acid
DL-hydroxysuccinic acid
DL-hydroxybutanedioic acid
DL-1-hydroxy-1,2-ethanedicarboxylic acid

D-malic acid
D(+)-malic acid
D-hydroxysuccinic acid
D-hydroxybutanedioic acidD-1-hydroxy-1,2-ethanedicarboxylic acid
(R)-hydroxybutanedioic acid
(R)-malate

CAS Numbers:
DL-malic acid (6915-15-7)
D-malic acid (636-61-3)

Other Codes:
DL-malic acid INS 296, E 296, EC 230-022-8
D-malic acid EC 211-262-2

<u>Source or Origin of the Substance:</u>
In 1785, Scheele extracted L-malic acid from unripe apples. In addition to apples, L-malic acid is found naturally in fruits and vegetables including cherries, strawberries, papayas, pineapples, oranges, grapes, apricots, mangoes, plums, tomatoes, carrots, olives, peas, potatoes, and corn (NLM 2018). L-malic acid is also produced by enzymatic conversion of fumaric acid and by fermentation of glucose and other carbohydrates (Goldberg et al. 2006).

DL-malic acid is a synthetic produced from petroleum in a high-pressure, high-temperature, catalytic process. D-malic acid can be obtained from DL-malic acid by a process called chiral resolution, which is discussed in *Evaluation Question #1*.

Properties of the Substance:

Malic acid is a white, odorless, crystalline solid at room temperature and atmospheric pressure with a tart, acidic, lingering taste. Malic acid is optically active, and can form D-, L-, and racemic forms. The melting point of the D and L forms is near the boiling point of water (212°F/100°C), and the melting point of the racemic (i.e., DL-malic acid) form is about 266°F (130°C). Crystals are not volatile at room temperature but decompose when heated above 284°F (140°C).

The anhydrous crystals are stable in air under normal conditions but pick up moisture in high humidity. DL-malic acid is readily soluble in water (55.8 g/100g water) at room temperature (68°F/20°C), whereas the water solubility of L-malic acid is somewhat less (36.3 g/100g water). Solubility increases with temperature. DL- and L-malic acid are more soluble in polar solvents such as methanol and water than in non-polar solvents such as benzene. More dissolves in water than in octanol, and the log of the octanol/water partition coefficient for DL-malic acid is -1.26.

Malic acid forms acidic solutions when dissolved in water. It is a relatively strong dibasic acid, with a pKa1 of 3.46 at 77°F (25°C); thus, it is extensively ionized at pH 3.5. It forms salts easily, many of which are water soluble (Fiume 2001; Bartek 2018a; Baker and Grant 2016). The properties of DL-, D-, and L-malic acid are summarized in Table 1 below:

Table 1. Properties of DL-malic acid, D-malic acid, and L-malic acid

Substance	Property	Value	Reference
DL-, D-, or L-malic acid	Molecular weight	134.09 g/mol	Bartek 2018a
	Molecular formula	$C_4H_6O_5$	Bartek 2018a
	Taste	Smooth, tart, acidic	Bartek 2018a
DL-malic acid	Melting point	267.8–269.6°F (131–132°C)	FCC 1981, Fiume 2001
	Density at 68°F (20°C)	1.601 g/cm^3	Bartek 2018a
	Log Kow (Octanol/water partition coefficient)	-1.26	Bartek 2018a
	pKa1 at 77°F (25°C), zero ionic strength	3.46	Bartek 2018a
	pKa2 at 77°F (25°C), zero ionic strength	5.10	Bartek 2018a
	Heat of solution	-4.9 kcal/mol	Bartek 2018a
	Vapor pressure	<0.1 hPa (<0.1mm Hg) at 68°F/20°C	Baker and Grant 2016
	Boiling point	302°F/150°C (decomposes)	Fiume 2001
	Solubility (g/100g solvent at 68°F/20°C)	Water 55.8, methanol 82.70, ethanol 45.53, acetone 17.75, ether 0.84, nearly insoluble in benzene	Baker and Grant 2016
D-malic acid	Melting point	213.8°F/101°C	Fiume 2001
L-malic acid	Melting point	212°F/100°C	Fiume 2001
	Optical rotation	$[alpha]_D$ = -2.3° (8.5 g/100 ml water)	Fiume 2001
	Solubility (g/100g solvent at 68°F/20°C)	Water 36.3, methanol 197.22; ethanol 86.60; acetone 60.66; ether 2.7	Santa Cruz Biotechnology 2018

D- or L-malic acid	Boiling point	284°F/140°C (decomposes)	Fiume 2001

Specific Uses of the Substance:

DL-malic and L-malic acid are both FDA-approved food additives for use as a pH control agent, flavor enhancer, flavoring agent, and adjuvant (U.S. FDA 2018). DL-malic acid is a 50:50 (w/w) mixture of D-malic acid and L-malic acid. It is not approved for baby food because infants cannot quickly metabolize the D-isomer, which can lead to acidosis (Baker and Grant 2016).

According to the 2002 malic acid petition, "malic acid is used in dry mix beverages, carbonated beverages, bakery products, fruit juices, candies, gelatins, desserts, frozen specialties, and tea as a flavor enhancer and food acidulant, and that malic acid "provides greater tartness and better taste retention than other major food acids" (USDA 2002). Malic acid is also used to acidify milk and cream and is found in French dressing, mayonnaise, and salad dressing (Fiume 2001). In non-food uses, malic acid is added to hundreds of cosmetics such as shampoos, hair sprays, hair tonics, wave sets, nail polish, face creams, skin conditioners, and moisturizers (Fiume 2001). It is also used in pharmaceuticals, paints, metal cleaning, electroplating, soaps, and as a chelating agent (USDA 2002).

Approved Legal Uses of the Substance:

USDA Food Safety and Inspection Service (FSIS)

Malic acid is listed in the "Table of Safe and Suitable Ingredients" as a flavoring agent for fish. (USDA FSIS 2018).

U.S. Environmental Protection Agency (EPA)

DL-malic acid is listed as a "FIFRA 25(b)" active ingredient for minimum-risk pesticides for non-food uses (U.S. EPA 2018a). DL-malic acid is also listed for use as an inert ingredient for use in minimum-risk pesticides for non-food use only (U.S. EPA 2016). DL-malic acid is tracked by the EPA Substance Registry Services (SRS) with tracking number 145912. It is listed by the Toxic Substances Control Act (TSCA) as a chemical in commerce. The EPA Inert Finder lists DL-malic acid as approved for use as an inert ingredient for non-food uses (U.S. EPA 2018c). It is tracked as an organic hazardous air pollutant (U.S. EPA 2018b). There are no EPA-registered pesticides with malic acid as an active ingredient (Baker and Grant 2016).

U.S. Food and Drug Administration (FDA) GRAS Food Additive

21 CFR Section 184.1069 refers to both DL-malic and L-malic acid as Generally Recognized as Safe (GRAS) approved food additives, which are used as pH control agent, flavor enhancer, flavoring agent, and adjuvant in all food types except for baby food. Maximum good manufacturing practice (GMP) levels are 3.4 percent for non-alcoholic beverages; 3.0 percent for chewing gum; 0.8 percent for gelatins, puddings, and fillings; 6.9 percent for hard candy; 2.6 percent for jams and jellies; 3.5 percent for processed fruits and fruit juices; 3.0 percent for soft candy; and 0.7 percent for all other food categories (21 CFR Section 184.1069; U.S. FDA 2018).

Action of the Substance:

Malic acid is a pH control agent, a flavor enhancer, and a flavoring agent. According to Bartek Ingredients, malic acid has a "smooth, persistent sourness" and can be blended with other organic acids, sugars, sweeteners, and flavors. Its extended sourness masks the aftertaste of artificial sweeteners such as aspartame in diet soda and stimulates the flow of saliva when used in toothpastes and mouth washes (Bartek 2018c). In sports drinks, consumer preference for the taste of malic acid is stable over time (Kinnear et al. 2011).

Malic acid also intensifies and extends the impact of flavors, allowing producers to reduce the amount of added flavoring. For example, adding malic acid to jams, jellies, and fruit preparations results in a more natural flavor profile. Fruit fillings in bakery products also have a stronger and more natural flavor with malic acid added, and the buffering action helps with the gelling texture (Bartek 2018c). In soft candy, malic acid extends the fruit flavor profile and helps with gelling and product clarity (Bartek 2018c). According to the 2002 malic acid petition, it boosts the savory flavors of cheese and pepper in snack foods, and "enhances fruit flavors, improves pH stability, and masks the aftertaste of some salts" in non-carbonated drinks. Malic acid is also used to reduce the pH of bottled tea from neutral to pH 4.6 (USDA 2002).

Combinations of the Substance:

Malic acid is often used in combination with other organic acids such as fumaric, citric, and tartaric acids in candy, soft drinks, and baking goods. Malic and fumaric acid are used together in non-carbonated beverages, powdered beverage mixes, chewing gum, gummy bears, jams, gelatin desserts and mixes, corn tortillas, herbal drinks, and nutri-bars (Bartek 2018b; Jarrett 2012). In candy and chewing gum, citric acid is added for initial sourness, with malic and fumaric acid added to extend the sour taste (Bartek 2018c). In jams and jellies, optimal taste is achieved through a combination of citric (40 percent), malic (35 percent), and tartaric acid (25 percent) (Kesava et al. 2016).

For fruit-flavored products, Bartek Ingredients recommends that for fruits where the predominant acid is citric acid, up to 10 percent malic acid can be used as a percentage of total acid. For acid combinations in apricot, peach, and pear flavors, two-thirds of the total should be malic acid and one-third should be citric acid. For flavors with astringency, such as grape or cherry, there should be at least 90 percent fumaric acid and less than 10 percent malic acid. Apple and watermelon flavors should use 100 percent malic acid. For prolonged taste sensations, malic acid should be used as the acidulant in hard candy (Sortwell and Woo 1996).

Most of these acid combinations are allowed in organic processing. The L-malic acid form is currently listed at 7 CFR 205.605(a) as a nonsynthetic, "nonagricultural (nonorganic) substances allowed as ingredients in or on processed products labeled 'organic' or 'made with organic (specified ingredients or food group(s)).'" Combinations of L-malic acid and citric acid are allowed, as citric acid is used in organic processing and is one of the allowed nonsynthetics at 7 CFR 205.605(a). Combinations of L-malic, citric, and tartaric acid (one of the allowed nonsynthetics at 7 CFR 205.605(a)) are also allowed, though the tartaric acid must be made from grape wine. Because fumaric acid is not listed at 7 CFR 205.605(a), commercial combinations of malic acid with fumaric acid would not be allowed in organic processing.

Status

Historic Use:

A petition by Honest Tea of Bethesda, Maryland, to add DL-malic acid to the National List as a "direct food additive and pH adjuster" for organic processing was submitted to the NOSB on November 1, 2002 (USDA 2002). A technical advisory panel (TAP) review was subsequently written in April 2003 (USDA 2003), which concluded that DL-malic acid was synthetic and "should not be allowed on the national list because a nonsynthetic viable alternative is available." However, the authors believed that L-malic acid produced by fermentation was nonsynthetic and could be added to the National List. Their conclusion was based on the status of citric acid: citric acid produced by "microbial fermentation of carbohydrate substances," is nonsynthetic, and is allowed in NOP Final Rule at 205.605(a) for the processing of organic products. The TAP review also identified viable alternatives such as vinegar or citric acid but concluded that L-malic acid may provide some unique properties in handling and processing that those alternatives cannot. The TAP review did not indicate any unacceptable risks to human health or the environment from producing or using L-malic acid (USDA 2003; USDA 2009).

The NOSB reviewed L-malic acid at their May 13–14, 2003 meeting in Austin, Texas and recommended adding L-malic acid to the National List (USDA 2015a). The addition of L-malic acid (CAS #97-67-6) to the National List was announced on September 11, 2006 (Federal Register Vol. 71, No. 175) and became effective on September 12, 2006. On November 5, 2009, NOSB voted 13 to 0 to relist L-malic acid (USDA 2009) after finding that L-malic acid was being used by large producers in the "wine, juice, and bottled tea sectors" (USDA 2015a). A Sunset Renewal Notice was published in the *Federal Register* on August 3, 2011 (Fed Reg 2011).

At the NOSB meeting in La Jolla, California from April 27–30, 2015, the NOSB voted to relist L-malic acid on the National List at 205.605(a) (USDA 2015b), which the USDA National Organic Program (NOP) announced in the *Federal Register* on February 23, 2016. The renewal was effective September 12, 2016 (Fed Reg 2016; USDA 2017).

L-malic acid is currently listed at 7 CFR 205.605(a) along with other nonsynthetic, "nonagricultural (nonorganic) substances allowed as ingredients in or on processed products labeled 'organic' or 'made with organic (specified ingredients or food group(s)).'"

Organic Foods Production Act, USDA Final Rule:
Malic acid is not listed in the Organic Foods Production Act of 1990 (OFPA). L-malic acid is now listed as an allowed nonsynthetic at 7 CFR 205.605(a).

International
Canada, Canadian General Standards Board – CAN/CGSB-32.311-2015, Organic Production Systems Permitted Substances List
http://www.inspection.gc.ca/food/organic-products/standards/eng/1300368619837/1300368673172

In Table 6.3, "Ingredients classified as food additives," "Malic acid" is listed as a food additive with no restrictions (Canada 2018).

CODEX Alimentarius Commission – Guidelines for the Production, Processing, Labelling and Marketing of Organically Produced Foods (GL 32-1999)
http://www.fao.org/docrep/005/Y2772E/Y2772E00.HTM

In Table 3 of "Annex 2: "Permitted substances for production of organic foods," "Malic acid" with INS 296 is a permitted food additive listed without conditions (Codex 2001).

European Economic Community (EEC) Council Regulation – EC No. 834/2007 and 889/2008
https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32007R0834
http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32008R0889

Malic acid is not specifically mentioned in EC No. 834/2007. In EC No. 889/2008, Annex 8, "Certain productions and substances for use in organic processed foods," "Malic acid" with E number 296 is allowed as a food additive (EU 2007; EU 2008).

Japan Agricultural Standard (JAS) for Organic Production
http://www.maff.go.jp/e/jas/specific/criteria_o.html

On page 4, "Attached Table 1, Food Additives," DL-malic acid INS 296, is an approved food additive with the annotation, "Limited to be used for processed foods of plant origin"(JAS 2012).

IFOAM – Organics International
http://www.ifoam.bio/en/ifoam-norms

L-malic acid assigned INS 296 is listed on page 79 in Appendix 4, "Table 1: List of approved additives and processing aids for post-harvest handling." L-malic acid is listed both as a food additive and post-harvest handling aid without restrictions (IFOAM 2014).

Evaluation Questions for Substances to be used in Organic Handling

Evaluation Question #1: Describe the most prevalent processes used to manufacture or formulate the petitioned substance. Further, describe any chemical change that may occur during manufacture or

formulation of the petitioned substance when this substance is extracted from naturally occurring plant, animal, or mineral sources (7 U.S.C. § 6502 (21)).

Production of L-malic Acid

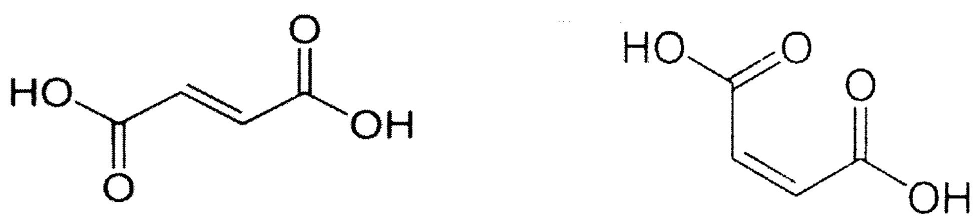
The naturally occurring form of malic acid is L-malic acid, which is found in apples and many other foods. It is not economical to extract L-malic acid from natural foodstuffs such as apple juice. Industrial quantities of L-malic acid are made using biological processes (West 2017), with the major industrial process to produce L-malic acid being a two-step procedure: 1) production of fumaric acid either synthetically from petroleum or by fermentation of carbohydrates and 2) enzymatic conversion of fumaric acid to L-malic acid by immobilized microbes producing the enzyme fumarase (Engel et al. 2008; Chi et al. 2016a; Chibata et al. 1983). See *Evaluation Question #2* for more information on the production process.

L-malic acid can also be produced by microbes in a one-step fermentation processes fueled by glucose or other carbohydrates. Reaction conditions are adjusted to cause overproduction of L-malic acid, which is an essential product of microbe metabolism (West 2017; Zou et al. 2013; Khan et al. 2014). See "One Step Fermentation Method to Produce Malic Acid" in *Appendix A*.

Production of DL-malic acid

Butane, butene, or benzene from petroleum are the starting materials for synthesis of DL-malic acid. In one method, benzene is catalytically oxidized at high temperature (752–842°F/400–450°C) to produce maleic anhydride. Vapors of hot benzene are passed over a catalyst of vanadium pentoxide on alumina. Maleic anhydride yields are 50–60 percent of theoretical. Maleic anhydride is then hydrated with hot steam to become DL-malic acid. This process produces a product of high purity, with fumaric acid and maleic acid as impurities. These compounds are closely related geometric isomers (see Figure 2 below); fumaric acid is *trans*-1,2-ethylenedicarboxylic acid and maleic acid is *cis*-1,2-ethylenedicarboxylic acid (Skinner and Tieszen 1961).

Figure 2: Precursors of Malic Acid (USDA 2002; Skinner and Tiezen 1961)



Fumaric Acid **Maleic Acid**

In another process, a mixture of butenes at about 1 percent concentration in air is passed over a catalyst of phosphomolybdate on silica gel. Optimum yields of about 27 percent maleic acid are obtained at 842°F/450°C (Skinner and Tieszen 1961). Maleic acid's carboxylic acid groups are on the same side of the double bond and react with each other in this process, losing water and forming maleic anhydride that is then hydrated with steam to form DL-malic acid (USDA 2002). The production of DL-malic acid is a synthetic process according to NOP Guidance 5033-1; the maleic acid undergoes a chemical change that is not the result of a naturally occurring biological process (USDA 2016b). Note this is similar to the method of production for synthetic fumaric acid used as precursor for industrial L-malic production (see *Evaluation Question* #2).

Production of D-malic acid and L-malic acid

Research quantities of D-malic acid and L-malic acid can be obtained by chemically separating the racemic DL-malic acid into its components in a process called chiral resolution. Most commonly, chiral resolution involves a racemate that is reacted with an optically active reagent to form D- derivatives and L-derivatives that have different solubility properties. The derivatives, called diasteroisomers, can be separated by fractional crystallization. Pure D- and pure L- can be regenerated from the diasteroisomers by yet another chemical reaction. For example, DL-malic acid has been resolved into its optically active forms using (+)-cinchonine and (-)-cinchonine (Streitwieser and Heathcock 1976; Bathori et al. 2015). Chiral resolution is an expensive process that is not used to make large commercial quantities. D or L-malic acid produced by chiral resolution is synthetic according to NOP Guidance 5033-1 because the isomers are isolated by chemical processes (USDA 2016b; West 2017).

<u>Evaluation Question #2:</u> Discuss whether the petitioned substance is formulated or manufactured by a chemical process, or created by naturally occurring biological processes (7 U.S.C. § 6502 (21)). Discuss whether the petitioned substance is derived from an agricultural source.

Commercial quantities of L-malic acid are produced in part by biological processes and are usually produced in a two-step synthesis. In the first step, fumaric acid is produced; in the second step, the fumaric acid is converted to L-malic acid using immobilized microbes that secrete fumarase (Chibata et al. 1983). There are two options for obtaining the fumaric acid in the first step in this process; more detailed information on the two-step process can be found in *Appendix A*.

- Option one: the fumaric acid precursor is obtained through the fermentation of carbohydrates (i.e., *Rhizopus* spp.)
- Option two: the fumaric acid precursor is obtained as a synthetic product from maleic acid of petroleum origin

Commercial quantities of nonsynthetic L-malic acid may also be produced using a one-step fermentation process through biological methods such as microbial fermentation using *Aureobasidium pullulans* and *Penicillium vitacola*, though the major commercial source of L-malic acid is enzymatic conversion of synthetic fumaric acid to L-malic acid by immobilized microbes (Chibata et al. 1983; Chi et al. 2016a; Dai et al. 2018). If the malic acid produced by this method is synthetic, most if not all, of the L-malic acid on the market is therefore synthetic (Goldberg et al. 2006; Chibata et al. 1983; Engel et al. 2008; Chi et al. 2016a; Dai et al. 2018). More information on this production process can be found in *Appendix A*.

L-malic acid can also be made from ethanol and biodiesel production waste. Thin stillage is a byproduct of corn fermentation in the production of ethanol from which *Aspergillus niger* ATCC 9142 can produce L-malic acid (West 2017). Another L-malic acid production process is the fermentation of crude glycerol obtained from production of biodiesel. Non-engineered *Ustilago trichophora* can be used for high yield production. The pH is maintained at 6.5 by addition of NaOH. CaCO3 is then added in a batch process. Production rate is 0.74g/liter/hour reaching a concentration of 195g/liter of L-malic acid (Liu et al. 2018; Zambanini et al. 2017). *A. niger* MTCC 281 can also produce L-malic acid from crude glycerol (Iyyappan et al. 2018ab).

Status of L-malic Acid from Nonsynthetic Fumaric Acid Production

According to NOP 5033-1, the enzyme conversion of nonsynthetic fumaric acid to L-malic acid is nonsynthetic. L-malic acid is produced by a naturally occurring biological process, an enzyme secreted by immobilized cells of microbes (Chibata et al. 1983; Takata et al. 1980; West 2017). The fumaric acid starting material is also produced by a naturally occurring biological process, fermentation or enzymatic conversion. The enzyme fumarase, fumaric acid, and L-malic acid are all components of the natural Krebs cycle; the conversion of fumarate to L-malic acid by fumarase is one step of the cycle. L-malic acid is extracted from the effluent solution by a combination of acid, base, and physical extraction, and when derived from nonsynthetic fumaric acid, it meets all the requirements of item 4.6 on NOP Guidance 5033 to be considered nonsynthetic (USDA 2016a):

1) L-malic acid has not been converted into a different substance
2) L-malic acid occurs in nature
3) Any synthetic materials used to isolate or extract L-malic acid have been removed by the purification process.

Similarly, citric acid has been classified as nonsynthetic and, like L-malic acid, is produced from fermentation followed by extraction. Nonsynthetic citric acid is produced through *A. niger* fermentation of molasses, sucrose, starch, and other substrates. To isolate citric acid, lime is added first to the fermentation product to produce the insoluble salt tricalcium citrate tetrahydrate, which is then filtered off from the fermentation solution. The isolated salt is treated with sulfuric acid (an acid-base reaction) to produce soluble citric acid and insoluble calcium sulfate. The calcium sulfate is filtered off and the solution is concentrated until the citric acid crystallizes. Alternatively, removing the citric acid from the fermentation medium is done through solvent extraction (Soccol et al. 2006; USDA 2015c). With this method, synthetic materials used to separate, isolate, or extract the substance are removed from the final substance and the citric acid is not transformed into a different substance via chemical change, nor is it altered into a form that does not occur in nature.

Status of L-malic Acid from Synthetic Fumaric Acid Production

Whether the L-malic acid produced by enzymatic conversion of synthetic fumaric acid is synthetic or nonsynthetic depends on the interpretation of NOP Guidance 5033-1 and what is considered the "natural source" from which L-malic acid is produced (USDA 2016b). If synthetic fumaric acid, as a microbial substrate, is considered the source, then L-malic acid is synthetic. If, however, the microbial product (i.e., column effluent) the L-malic acid is extracted from is considered the source, L-malic acid could be considered nonsynthetic. As previously discussed, the second phase of this production method—conversion of fumaric acid to L-malic acid through an enzymatic process, and extraction of L-malic acid from a preparation of immobilized microbial cells—uses nonsynthetic methods (naturally occurring biological processes and extraction).

If L-malic acid is considered to be extracted from a natural source, then it is non-synthetic according to NOP Guidance 5033-1. Question 2 of NOP Guidance 5033-1 asks whether the substance has undergone a chemical change making it chemically or structurally different than how it occurs in the source material (USDA 2016b). L-malic acid pre- and post-extraction is not chemically or structurally different and could therefore be considered nonsynthetic. If extracted, Question 2b asks whether the substance meets all the criteria described at 4.6 of NOP 5033-1 at the end of the extraction process (USDA 2016b). This is true in the case of L-malic acid, where acid, base, physical and/or solvent extraction methods have been used. At the end of the extraction process:

1) L-malic acid that is isolated has not been transformed into a different substance
2) L-malic acid has not been altered into a form that does not occur in nature
3) Any synthetic materials used to separate, isolate, or extract L-malic acid have been removed by the purification process

Identifying the culture broth (i.e. extracellular metabolic product) as the "natural source" as opposed to the beginning growth substrate used is a commonly applied interpretation of the 5033-1 Decision Tree. For example, citric, lactic, and gibberellic acid are all nonsynthetic substances extracted from the fermentation medium produced by the growth of various microorganisms. In this interpretation, the culture broth produced by a microorganism is considered biological matter and a natural source material.

It is worth noting that the starting material or the type of growth medium has not consistently been used to categorize whether a fermentation process or extraction is synthetic or nonsynthetic. For instance, the *Aspergillus* production of citric acid by fermentation uses synthetic mineral salts and synthetic reagents, but the overall process is considered nonsynthetic (USDA 2015c). NOSB has recognized that nonsynthetic microorganisms used in handling have synthetic ancillary substances in the formulation but described leftover substrate as natural substances (USDA 2015c, USDA 2015d).

Status of L-malic Acid from One-Step Fermentation Process

L-malic acid produced by microbial fermentation of glucose from the natural source *A. pullulans* is nonsynthetic according to NOP 5033-1 (USDA 2016b; West 2017). Glucose fuels the fermentation, and L-malic acid is enzymatically produced from fumarate. The chemical change from fumarate to L-malic acid is a naturally occurring biological process. L-malic acid produced by *A. pullulans* fermentation was nonsynthetically converted into PMA during the fermentation. The polymer was physically extracted from the reaction. Acid is added to a solution of the polymer, converting it back to L-malic acid.

The L-malic acid produced using *Penicillium* fermentation is produced by a biological process and is nonsynthetic. The *Penicillium* produces L-malic acid, which is converted into calcium malate by reaction with calcium carbonate. Calcium malate is extracted from the medium by a combination of physical extraction, acid-base extraction, and solvent extraction. Following NOP Guidance 5033-1, at the end of the process, L-malic acid has not been transformed into a different substance, L-malic acid does occur in nature, and all synthetic materials have been removed (USDA 2016b).

Agricultural Status

According to NOP Guidance 5033-2 "Decision tree for classification of agricultural and non-agricultural materials for organic livestock production or handling" (USDA 2016c), L-malic acid is non-agricultural:

1) It is not a mineral or bacterial substance
2) It is not a micro-organism or enzyme
3) It is not a crop or livestock product and it is not derived from crops or livestock

<u>Evaluation Question #3:</u> If the substance is a synthetic substance, provide a list of nonsynthetic or natural source(s) of the petitioned substance (7 CFR 205.600(b)(1)).

DL-malic acid is a synthetic substance. L-malic acid occurs naturally in many fruits, and the natural form is nonsynthetic. It is possible, but not economical, to extract it from apple juice or other sources. There are no naturally sourced versions of L-malic acid from plant sources. Nonsynthetic L-malic acid can be manufactured by enzymatic conversion of fumaric acid (produced by fermentation), or by fermentation of carbohydrates (West 2017; USDA 2002; Chibata et al. 1983; Zou et al. 2013; Khan et al. 2014).

<u>Evaluation Question #4:</u> Specify whether the petitioned substance is categorized as generally recognized as safe (GRAS) when used according to FDA's good manufacturing practices (7 CFR 205.600(b)(5)). If not categorized as GRAS, describe the regulatory status.

Malic acid (both DL and L-malic acid) is an FDA GRAS food additive approved for use as a pH control agent, flavor enhancer, flavoring agent, and adjuvant. Malic acid in 21 CFR Section 184.1069 refers to both DL-malic acid (CAS 617-48-1) and L-malic acid (CAS 97-67-6). It is used in food, except for baby food. As mentioned in *Approved Legal Uses of the Substance* above, maximum GMP levels are 3.4 percent for non-alcoholic beverages; 3.0 percent for chewing gum; 0.8 percent for gelatins, puddings and fillings; 6.9 percent for hard candy; 2.6 percent for jams and jellies; 3.5 percent for processed fruits and fruit juices; 3.0 percent for soft candy; and 0.7 percent for all other food categories (21 CFR Section 184.1069) (U.S. FDA 2018).

<u>Evaluation Question #5:</u> Describe whether the primary technical function or purpose of the petitioned substance is a preservative. If so, provide a detailed description of its mechanism as a preservative (7 CFR 205.600(b)(4)).

Malic acid (both DL-malic acid and L-malic acid) is a GRAS food additive permitted for use by the FDA as an acidulant, flavor enhancer, flavoring agent and adjuvant; these are the primary technical functions of malic acid (U.S. FDA 2018; USDA 2002). However, acidifying food can incidentally lead to destruction of microbes. For instance, malic acid incorporated into thin films of soy protein was more effective against *Listeria* sp., *Escherichia coli* O157:H7, and *Salmonella* sp. than incorporating citric, lactic, or tartaric acid (Eswaranandam et al. 2004; Baker and Grant 2016). Malic acid added to apple, pear, and melon juices inhibited the growth of *Listeria* sp., *E. coli* O157:H7, and *Salmonella* sp. (Baker and Grant 2016). In soft drinks, organic acids such as malic acid are able to lower the internal pH of microbes, causing denaturation of enzymes (Azeredo et al. 2016).

<u>Evaluation Question #6:</u> Describe whether the petitioned substance will be used primarily to recreate or improve flavors, colors, textures, or nutritive values lost in processing (except when required by law) and how the substance recreates or improves any of these food/feed characteristics (7 CFR 205.600(b)(4)).

Malic acid is not used primarily to replace flavor, colors, textures or nutritive values lost in processing. Its value is improving and enhancing the existing flavors. See *Action of the Substance* above for additional information on malic acid's flavor impact.

<u>Evaluation Question #7</u>: Describe any effect or potential effect on the nutritional quality of the food or feed when the petitioned substance is used (7 CFR 205.600(b)(3)).

The addition of malic acid to soft drinks, tea, candies, and other approved foods adds a small caloric content; 100 grams of malic acid contains 239 Kcal (Bartek 2018d), while typical human consumption of L-malic acid is about 1.5–3.0 grams per day (USDA 2002).

The Toxnet database of the National Library of Medicine has no citations about L-malic acid interfering with vitamin metabolism. However, animal experiments cited there show that L-malic acid may help with the absorption of aluminum (NLM 2018). Small studies with human subjects suggest that L-malic acid may help with the absorption of magnesium (Russell et al. 1995), or with the absorption of creatinine (Tyka et al. 2015).

<u>Evaluation Question #8:</u> List any reported residues of heavy metals or other contaminants in excess of FDA tolerances that are present or have been reported in the petitioned substance (7 CFR 205.600(b)(5)).

Any malic acid used as a food additive must meet FDA standards listed in the *Food Chemicals Codex*. Malic acid must be not less than 99 percent pure—it can contain no more than 1 percent fumaric acid, and no more than 0.05 percent maleic acid. There can be no more than 0.002 percent heavy metals, not more than 3 ppm arsenic, and not more than 10 ppm lead (FCC 1981).

The Toxnet database of the National Library of Medicine showed no instance of poisoning with malic acid contaminated with heavy metals on October 4, 2018 (NLM 2018).

<u>Evaluation Question #9:</u> Discuss and summarize findings on whether the manufacture and use of the petitioned substance may be harmful to the environment or biodiversity (7 U.S.C. § 6517 (c) (1) (A) (i) and 7 U.S.C. § 6517 (c) (2) (A) (i)).

Manufacturing DL-malic acid from petroleum leaves a number of industrial waste products, including spent catalysts that must be buried in landfills. It is a high-energy process using fossil fuels that produce carbon dioxide when burned. The carbon dioxide released could contribute to global warming (Skinner and Tieszen 1961).

The manufacture of L-malic acid by fermentation is much more benign to the environment. Waste products such as spent cells and fermentation media can be composted. Processing chemicals include low toxicity acids and bases; while some of these can be recycled, they may end up in industrial landfills (West 2017; Dai et al. 2018).

L-malic acid is found extensively throughout the environment in rotting fruit in agricultural or garden applications. Because it is soluble in water, L-malic acid eventually leaches out into the soil, where it is degraded by microbes. The soil half-life is 112 hours, and the water half-life is 55.9 hours. If any of the manufactured material is spilled into the environment, about 38 percent ends up in water, 1 percent ends up in sediments, 62 percent ends up in soil, and about 1 percent ends up in air. Malic acid has high mobility in soil, and the half-life of vapor-phase malic acid is about 2 days. Traces of malic acid have been found in rainwater, and melted snow in Japan contains about 0.49–6.76 µg/liter (Baker and Grant 2016; NLM 2018).

The total worldwide production of all forms of malic acid is about 40,000 metric tons (88 million pounds) a year, though this is not enough to satisfy the global demand of 200,000 metric tons (nearly 441 million pounds) (Liu et al. 2018). The total U.S. consumption in 1998 was 18 million pounds (USDA 2002). For comparison, the annual U.S. apple production is about 11 billion pounds (Seetin 2018). Manufactured malic acid is not deliberately released into the environment, and the amounts released incidentally into the environment through manufacturing processes and spills are likely to be small compared to the amounts already found in nature. The impacts of the manufactured material on beneficial insects, diversity, and other important aspects of environmental quality are negligible compared to natural exposures from rotting vegetation (Baker and Grant 2016).

Any DL- or L-malic acid ingested as a food additive is quickly oxidized and released into the atmosphere as carbon dioxide. When rats were given 2.5 mg/kg of C14 radioactive DL- or L- malic acid by gavage (oral) or by intraperitoneal injection as part of a study, about 88–91.6 percent of the dose was excreted as carbon dioxide into the air, about 3 percent was eliminated in urine, and less than 1.4 percent was eliminated in feces. Human metabolism should be similar because much of the metabolism is through the Krebs cycle. Malic acid is not appreciably eliminated in urine or feces, and thus should not be an important environmental contaminant when used as a food additive (NLM 2018; Fiume 2001).

__Evaluation Question #10:__ Describe and summarize any reported effects upon human health from use of the petitioned substance (7 U.S.C. § 6517(c)(1)(A)(i), 7 U.S.C. § 6517(c)(2)(A)(i)) and 7 U.S.C. § 6518(m)(4)).

L-malic acid occurs naturally in apples, cherries, and many other fruits. The average intake from natural sources is 1.5–3.0 grams a day (Hartmann and Hillig 1934). DL-malic acid is an approved GRAS food additive, though it has caused acidosis in infants because the D-isomer is metabolized more slowly than the L-isomer (USDA 2002). The D- isomer is also more toxic to rats than the L-isomer; whereas intraperitoneal administration of 1,000 mg/kg L-malic acid was not lethal to rats, the same dose of D-malic acid killed them within 20 minutes (Fiume 2001).

Animal tests show that malic acid has low acute toxicity. The oral median lethal dose (LD50) in rats is 3,500 mg/kg and 5,000 mg/kg in rabbits. A chronic feeding study in rats showed only weight gain effects. Malic acid did not cause reproductive toxicity in mice, rats, or rabbits. A range of standard tests showed no mutagenic effects. Beagles treated with up to 50,000 mg/kg of DL-malic acid for 104 weeks showed no adverse effects. Mice receiving 85.8 mg/kg day of malic acid showed increased levels of aluminum in brain and bone after one month (NLM 2018; Fiume 2001).

Malic acid is an eye and skin irritant. The consumption of acidic soft drinks can lead to erosion of tooth enamel, and can cause tooth decay. Patients with atopic dermatitis can develop allergic sensitivity to malic acid in the diet (NLM 2018; Fiume 2001).

__Evaluation Question #11:__ Describe any alternative practices that would make the use of the petitioned substance unnecessary (7 U.S.C. § 6518(m)(6)).

Malic acid food additives are only needed in processed foods; thus, if processed foods were discontinued, a malic acid food additive would not be necessary. Alternative food additives to malic acid are discussed in *Evaluation Question #12*.

__Evaluation Question #12:__ Describe all natural (nonsynthetic) substances or products which may be used in place of a petitioned substance (7 U.S.C. § 6517(c)(1)(A)(ii)). Provide a list of allowed substances that may be used in place of the petitioned substance (7 U.S.C. § 6518(m)(6)).

L-malic acid is used as a pH adjuster and a flavor enhancer. If L-malic acid is not used as a food additive, other simple organic acids might be substituted as acidulants. The 2003 TAP review identified citric, fumaric, lactic, and tartaric acids as possible alternatives, but fumaric acid is not a permitted nonsynthetic additive at 205.605(a) (USDA 2003). The only simple organic acids listed at 205.605(a) are citric, L-malic, lactic, and tartaric. Ascorbic acid is a listed synthetic at 205.605(b) that might be appropriate for some uses. Alginic acid is also listed at 205.605(b), but it would not be an appropriate substitute for malic acid as a pH adjuster because it has different properties.

These are all possible substitutions, but in practice they may not be effective alternatives. For example, authors of the 2003 TAP review found that citric acid might be an alternative acidulant for green teas, but that malic acid was better for black teas (USDA 2003).

Other acids might be appropriate as pH adjusters, but according to major formulators, the taste profile in sodas, non-carbonated drinks, and other uses would not be the same if malic acid were eliminated (Bartek 2018c). Authors of the 2003 TAP review concluded that "although the petition is not for improving taste, this seems to be the main reason [malic acid] is preferred over other acids" (USDA 2003).

Evaluation Information #13: Provide a list of organic agricultural products that could be alternatives for the petitioned substance (7 CFR 205.600(b)(1)).

Acidic juices (e.g., cranberry, lemon, lime, grape, apple, orange, grapefruit, pomegranate, and blueberry juices) naturally contain some of the organic acids discussed as food additives, including citric, malic, and tartaric acids. Other more exotic juices that might be used include tamarind, *Tamarindus indica*; kokum, *Garcinia indica*; and Indian gooseberry, *Embilica officinalis* (D'Souza et al. 2018). In some formulations, adding these juices might adjust pH, but the taste would have to be compatible with the product (USDA 2002; USDA 2003).

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Appendix A
Two-Step and One-Step Production Processes for L-malic Acid

Two-Step Production Process

Step One, Option One: Production of Fumaric Acid by Fermentation with Rhizopus Species

In the fermentation method, fumaric acid is produced in stirred batch bioreactors using *Rhizopus oryzae* or *Rhizopus arrhizus*. The glucose fermentation medium is fortified with mineral salts and a nitrogen source such as ammonium sulfate. After the solution is sterilized, the microbe is introduced. Sodium carbonate is then added to neutralize the fumaric acid, maintain pH, and provide a source of carbon dioxide for carboxylation of reaction intermediates (Engel et al. 2008). Fermentation continues until the glucose is mostly depleted. Some of the glucose is used for cell growth, while the rest is used to produce fumaric acid and other substances. The fermentation is run under reduced nitrogen conditions to force overproduction of fumaric acid; other acids such as malic, lactic, acetic, and succinic acid are also formed (Engel et al. 2008; West 2017). When fermentation is complete, cells and other solids are filtered off to leave a solution containing sodium fumarate. The solution is then acidified with sulfuric acid to pH 1. Fumaric acid precipitates from the acidic solution and is isolated by filtration. Impurities remain in solution (Engel et al. 2008).

Though this fermentation method was used widely in the early 20th century, it is uncommon today. For instance, in 1940, Dupont produced about 4,000 tons of fumaric acid per year through fermentation with *Rhizopus* spp. (Goldberg et al. 2006). In 2008, the entire production of fumaric acid was from petrochemicals due to favorable oil prices (i.e., more than $61/barrel). Non-synthetic sources of the precursor will probably not be available until petroleum prices increase significantly (Engel et al. 2008).

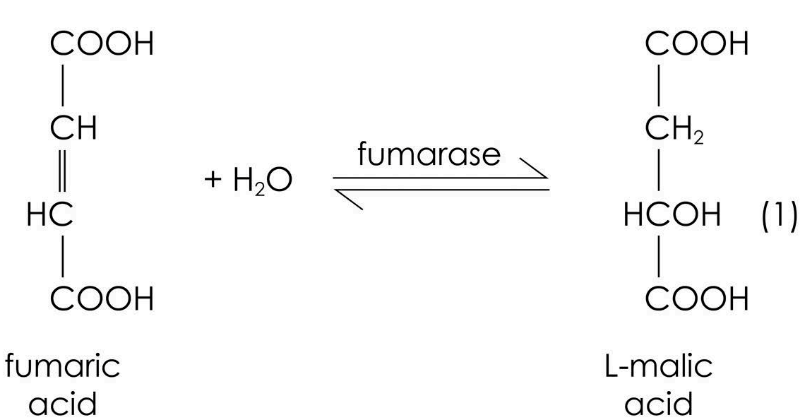
Step One, Option Two: Synthesis of Fumaric Acid from Petroleum Products

Most industrially produced fumaric acid is sourced from petroleum. In the first step of this production process, maleic anhydride is synthesized by catalytic oxidation of benzene or mixtures of butane and butene. The maleic anhydride is then hydrated to maleic acid, which is catalytically or thermally isomerized to fumaric acid in almost quantitative yield (Engel et al. 2008). The synthetic fumaric acid is then converted to L-malic acid by the enzyme process described below.

Step Two: Enzymatic Production of L-malic Acid from Nonsynthetic or Synthetic Fumaric Acid

In the second step of the two-step industrial process, fumaric acid (either synthetic or nonsynthetic, based on which option from above was chosen) is enzymatically converted to L-malic acid by fumarase (see Figure 3 below). This reaction is reversible, but is faster in the forward direction of fumaric acid to malate. Equilibrium is at about 82 percent L-malic acid (Yamamoto et al. 1977).

Figure 3: Enzymatic conversion of fumaric acid to L-malic acid (Chibata et al. 1983)



Solutions of fumaric acid salts such as sodium fumarate at pH 7 are passed through columns containing immobilized cells. Fumarase released by the cells convert fumaric acid into L-malic acid. When 450 liters per hour of 1 molar pH 7 sodium fumarate are passed through a 1000-liter *B. flavum* MA-3 column, about 30 tons of high-purity L-malic acid are produced each month. Yields are about 70 percent, and unreacted fumaric acid is recovered by recycling (Chibata et al. 1983; Takata et al. 1980). About 20 percent of the L-malic acid produced is converted back to fumaric acid by fumarase in the reversible reaction (Yamamoto et al. 1977). Consistent with the requirement in 5033.1, any traces of fumaric acid remaining in the final product have "no technical or functional effect in the final product." A typical analysis of L-malic acid produced this way has a maximum of 0.07 percent fumaric acid (Prescribed for Life 2018a).

To isolate L-malic acid, the effluent from the column is collected and concentrated by water evaporation. The addition of hydrochloric acid causes fumaric acid to crystallize, and the crystals are removed by filtration. Calcium hydroxide is added to the acidic solution until the pH is between 6 and 7. The calcium malate produced is filtered off and dried. Crystals of calcium malate are dissolved in water, then sulfuric acid is added and the calcium sulfate precipitate is filtered off. The solution is passed through Amberlite IR-120 (H+)-type and Amberlite IR-120 (OH^-)-type columns to remove remaining impurities. The solution is concentrated in a vacuum and crystallized from isopropyl alcohol. Yields are about 70 percent L-malate (Yamamoto et al. 1976; Chibata et al. 1983; Takata et al. 1980).

Beginning in 1974, L-malic acid was produced commercially by enzymatic conversion of fumaric acid with immobilized microbial cells producing fumarase. Initially, *Brevibacterium ammoniagenses* MA-2 cells immobilized by polyacrylamide were used (Yamamoto et al. 1977; Chibata et al. 1983). Later, cells of *B. flavum* MA-3 immobilized by kappa-carrageneen in 1000-liter columns were used for industrial production of L-malic acid in a flow process. Cell preparation treatments with bile extracts reduced the production of contaminants such as succinic acid (Tosa et al. 1979; Takata et al. 1980; Chibata et al. 1983).

One-Step Fermentation Method to Produce L-malic acid

L-malic acid can also be produced in a one-step fermentation process. In 1924, Dakin showed that glucose and other sugars could be converted by yeast fermentation into L-malic acid (Dakin 1924). Several other one-step fermentation methods for L-malic acid have been developed, but most of these are still in the research stage and are hard to scale up for industrial production.

L-malic acid production by fermentation of carbohydrates has been extensively studied (Chi et al. 2016a). There is an oxidative pathway, a glyoxylic acid pathway, and a reductive pathway; these are nonsynthetic pathways because they involve natural processes that are part of normal microbial metabolism (Chi et al. 2016a; West 2017; Dai et al. 2018).

- The **oxidative** pathway utilizes the Krebs or tricarboxylic acid cycle. Glucose and other carbohydrates are oxidized into organic acids, producing acetyl CoA and adenosine triphosphate (ATP). As part of this cycle, L-fumarate is converted into L-malate by the enzyme fumarase (Chi et al. 2016a; West 2017; Dai et al. 2018). Several wild-type organisms—including *Aspergillus flavus* ATCC 13697 (Battat et al. 1991), *A. oryzae* DSM 1863 (Ochsenreiter et al. 2014), *A. niger* ATCC 9142 (Knuf et al. 2013), *Penicillium vitacola* 152 (Khan et al. 2014), and *A. pullulans* ZX-10 (Zou et al. 2013; Zhang et al. 2011)—have been used to produce L-malic acid from glucose. Genetically engineered microbes have also been used in this process (West 2017; Dai et al. 2018; Chi et al. 2016a).
- The **glyoxylic acid** pathway enzymatically converts oxaloacetate to citrate, then isocitrate, then glyoxylate, then malate (Chi et al. 2016a; West 2017; Dai et al. 2018).
- The **reductive** pathway carboxylates pyruvate or phosphoenolpyruvate to oxaloacetate, which is enzymatically converted to malate. Most microbes employed in the production of L-malic acid use the reductive pathway (Chi et al 2016a; West 2017; Dai et al. 2018).

<u>Extraction of L-malic Acid from One-Step Fermentation</u>

L-malic acid is isolated by physical and acidic extraction from fermentation solution (Zou et al. 2013). The fermentation solution is centrifuged to remove solids, then is passed over an Amberlite IRA-900 ion exchange column to which PMA sticks. A solution of 1.2 M sodium chloride elutes PMA from the column, which is chemically identical to PMA in the reaction solution. The isolated polymer is dissolved in 1 M sulfuric acid, which converts the polymer into L-malic acid with no detectable impurities (Zou et al. 2013).

There are several problems with one-step production of L-malic acid by fermentation. For one, *A. flavus* cannot be used to produce GRAS L-malic acid because of possible aflatoxin contamination (West 2017); however, *A. niger* or *A. oryzae* can produce L-malic acid without aflatoxins (West 2017; Chi et al. 2016a). Another issue is the simultaneous production of other acids, such as succinic, fumaric, or acetic acid, though reaction conditions can be adjusted to maximize production of L-malic acid. There are also no commercial one-step production methods in the United States that involve fermentation. A commercial one-step fermentation of glucose to produce L-malic acid did not exist in 2006 (Goldberg et al. 2006); if one exists now, it is probably not in the United States. Fermentation expert Professor Thomas West of Texas A&M is "not aware of any U.S. company producing L-malic acid by a one-step fermentation process." (West 2018). Chinese and Indian researchers, on the other hand, have been actively working on fermentation processes, and developed an L-malic acid production method in 2013 that uses agricultural wastes to lower production costs (Wang et al. 2013; Zou et al. 2013; Khan et al. 2014).

Product inhibition is also a major hindrance. High concentrations of L-malic acid must accumulate to make the process economical, but as L-malic acid accumulates, it starts to poison the fermentation microbes, limiting possible concentrations (Zou et al. 2013; Chi et al. 2016a; Goldberg et al. 2006). To optimize fermentation, bases such as sodium hydroxide have to be added, which convert L-malic acid into a salt (Dai et al. 2018). Another limitation is the time the fermentation takes. Some fermentations of L-malic acid take more than 300 hours to complete (Khan et al. 2014; Zou et al. 2013). Two of the fastest one-step fermentation methods use *Penicillium vitacola* 152 (which completes in 96 hours) and *Aureobasidium pullulans* (which completes in 144 hours). These examples are discussed below:

Fermentation with *Penicillium vitacola* 152

The fermentation process using *P. vitacola* produces higher concentrations and higher yields of L-malic acid faster than using other microbes. In a 10-liter fermentation, 168 g/liter of calcium malate was produced in 96 hours, with a yield of 1.28 grams from each gram of glucose. The fermentation medium consisted of mineral salts, 140 g/liter of glucose, 40 g/liter calcium carbonate, and 0.5 percent (v/v) corn steep liquor. The calcium carbonate was added to provide a carboxylation source and to keep the pH near 6.5, converting the L-malic acid produced into calcium malate (Khan et al. 2014).

To isolate and purify calcium malate, microbial cells and insolubles were removed from the fermentation medium by centrifugation. The soluble supernate was then treated with methanol to precipitate polysaccharides. The polysaccharides were removed by centrifugation, and cold methanol (39.2°F/4°C) was added to the supernate, precipitating calcium malate. The calcium malate crystals were dried at 140°F/60°C; recrystallization from methanol led to pure calcium malate (Wang et al. 2013). To obtain L-malic acid, calcium malate can be dissolved in water. The addition of sulfuric acid causes calcium sulfate to precipitate, leaving L-malic acid in solution, which can be isolated by evaporation of water (Khan et al. 2014).

Aureobasidium pullulans ZX-10 Fermentation

The wild type yeast *A. pullulans* ZX-10 was obtained from the standard strain NRRL-Y2311-1 by serial culture without genetic modification (Zou et al. 2013). *A. pullulans* ZX-10 converts glucose into L-malic acid, which polymerizes under the conditions of the fermentation into polymalic acid (PMA). PMA is a water-soluble polyester produced by reaction of one of the carboxylic acid groups in an L-malic acid monomer with the alcohol group of another L-malic acid monomer. Monomers are added stepwise in this fashion to produce the polymer (Zou et al. 2013). The polymerization is not well understood, but it is uniquely directed by the microbe, as other microbes that produce L-malic acid do not initiate polymerization (Battat et al. 1991; Zhang et al. 2011; Khan et al. 2014; Knuf et al. 2013; Chi et al. 2016b). This water-soluble polymer is isolated by column chromatography on Amberlite IRA-900, then hydrolyzed to L-malic acid with sulfuric acid.

The fermentation medium for *A. pullulans* contains glucose (60–150g/liter), mineral salts, and calcium carbonate (30 g/liter) to maintain the fermentation near pH 6 and provide a source of carboxylation. This reaction uses the reductive metabolic pathway to produce L-malic acid. As described above, pyruvate is carboxylated to oxaloacetate, which is reduced to L-malic acid. The maximum yield in this pathway is 2 moles L-malic acid from 1 mole of glucose (Zou et al. 2013). In stirred tank bioreactors, the free cells produce the PMA equivalent of 87.6 g/liter L-malic acid. When cells are immobilized in a fibrous-bed bioreactor, fed batch production gives PMA equivalent to 144.2 g/liter of L-malic acid. Reaction proceeds at 0.74 g/liter/hr, and overall yield of L-malic acid is 0.55g per gram of glucose (Zou et al. 2013).

The final product from this fermentation process has no detectable contaminants, and the process can be scaled up for industrial production. The microbe can also produce polymalic acid and L-malic acid from agricultural wastes. Therefore, this process is one of the most efficient because product inhibition is not a problem, there are few side products, and isolation of near 100 percent pure L-malic acid is possible without recrystallization (Zou et al. 2013).

In other fermentation schemes, the L-malic acid produced poisons the microbes, limiting the concentrations of malic acid that can be produced. Also, fermentation of glucose to L-malic acid produces other acids such as succinic acid, and separating the desired product from the side reactions can be difficult. In contrast, the PMA produced in the *A. pullulans* process does not inhibit cell growth or production of the desired product. Reduced nitrogen concentrations encourage PMA synthesis. The reaction continues until the glucose added is completely consumed (Zou et al. 2013; Wang et al. 2016).