Citric acid and salts

Handling/Processing

Identification of Petitioned Substance

Chemical Names:
Citric acid; calcium citrate; potassium citrate; sodium citrate.

Other Names:
Citric acid: 2-hydroxypropane-1,2,3-tricarboxylic acid; 3-carboxy-3-hydroxypentanedioic acid.
Calcium citrate: 2-hydroxy-1,2,3-propane-tricarboxylic acid calcium salt (2:3); 2-hydroxy-1,2,3-propanetricarboxylic acid.
Potassium citrate: potassium citrate tribasic; potassium citrate tribasic monohydrate; tripotassium citrate.
Sodium citrate: disodium hydrogen 2-hydroxypropane-1,2,3-tricarboxylate; sodium dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate; trisodium 2-hydroxypropane-1,2,3-tricarboxylate; trisodium citrate.

Trade Names:
There are no trade names for the pure chemicals.

CAS Numbers:
Citric acid:
77-92-9 (citric acid).
Calcium citrate:
813-94-5 (calcium citrate) (also is listed as 813-994-95 in 21 CFR 184.1195);
5785-44-4 (calcium citrate tetrahydrate).
Potassium citrate:
866-84-2 (potassium citrate);
6100-05-6 (potassium citrate tribasic monohydrate) (also is listed as 6100-905-96 in 21 CFR 184.1625).
Sodium citrate:
18996-35-5 (monosodium citrate);
144-33-2 (disodium citrate);
68-04-2 (trisodium citrate) (also is listed as 68-0904-092 in 21 CFR 184.1751);
6132-04-3 (trisodium citrate dihydrate);
6858-44-2 (trisodium citrate pentahydrate).

Other Codes:
E330 (citric acid);
E333 (calcium citrate);
E332 (potassium citrate);
E331 (sodium citrate).

Summary of Petitioned Use

This limited scope technical report provides updated technical information to the National Organic Standards Board (NOSB), for the support of the sunset reviews of citric acid listed at 7 CFR 205.605(a)(1); and calcium, potassium, and sodium citrate listed at §§ 205.605(b)(7), (25), and (31), respectively. This technical report focuses on the fermentation processes used to make these materials. Additionally, we describe the use of excluded methods related to the manufacture of these substances. Excluded methods are defined at § 205.2, as follows:

> A variety of methods used to genetically modify organisms or influence their growth and development by means that are not possible under natural conditions or processes and are not considered compatible with organic production. Such methods include cell fusion, microencapsulation and macroencapsulation, and recombinant DNA technology (including gene deletion, gene doubling, introducing a foreign gene, and changing the positions of genes when achieved by recombinant DNA technology). Such methods do not include the use of traditional breeding, conjugation, fermentation, hybridization, in vitro fertilization, or tissue culture.

Citric acid, calcium citrate, potassium citrate, and sodium citrate were all recommended for addition to the National List of Allowed and Prohibited substances (hereafter referred to as the "National List") in 1995 (NOSB, 2009). They were included on the National List with the first publication of the National Organic Program (NOP) Final Rule (65 FR 80547).

Focus Question Requested by the NOSB

Focus Question #1: What fermentation processes are used to produce these substances?
At present, submerged fermentation (SmF) using the fungus *Aspergillus niger* is the mainstream technology used to produce citric acid (CA) and CA salts globally (Y. Chen & Nielsen, 2016; Di Lorenzo et al., 2022; Tong et al., 2019, 2023; Wang et al., 2020; Zhang et al., 2020). About 80% of the world's CA is obtained by SmF. This method is preferred because of its lower initial investment and maintenance costs (Reena et al., 2022; Wang et al., 2020). Yeast SmF processes (*Candida guilliermondii, C. lipolytica, Yarrowia lipolytica*) using various carbon sources are also sporadically used today (Anastassiadis et al., 2008).

The main carbon source materials used for production of CA are plant materials in the form of starch carbohydrates isolated from plant materials or the plant material itself, such as potato, tapioca, maize, rice, or another grain (Tong et al., 2019). The primary substrate used in the *A. niger* CA industry is corn steep liquor (Xue et al., 2021). More than 90% of manufacturers in the U.S. rely on fermentation of corn-derived glucose or dextrose (Anastassiadis et al., 2008). Researchers have studied other feedstocks such as agro-industrial by-products (e.g., stalks, husks, industrial fluids, and so forth) as potential carbon sources for citric acid production (Tong et al., 2023), but these alternative substrates are only sporadically used today (Anastassiadis et al., 2008).[1]

As mentioned above, most manufacturers produce CA using submerged culture fermentation because of operation economics and performance (i.e. lower labor cost and higher yield of CA) (Anastassiadis et al., 2008; Behera et al., 2021). The other two batch fermentation processes used in the industry today are: Liquid surface culture and the Japanese Koji process, also known as solid-state fermentation.

In general, all the industrial fermentation processes have three phases: media preparation and inoculation, fermentation, and recovery of the CA or CA salts (Behera et al., 2021; Sweta V. Lende et al., 2021).

The three fermentation methods mentioned above, together with techniques used for the recovery of CA and CA salts, are described within the *Evaluation Question #1* of the 2015 *Citric Acid and Salts* technical report (USDA, 2015). The information describing the manufacturing processes of CA found in the 2015 technical report is still accurate and represents the current state of CA production today.

Focus Question #2: Which products are manufactured using organisms developed by "excluded methods" in Appendix A? Which products are manufactured using organisms developed through allowed methods, including (but not limited to) those listed as "Methods Allowed" in Appendix A?
Based on available information, the majority of CA manufacturers use wild type fungal strains, as well as those that are products of classical induced mutagenesis (i.e., mutagenesis caused by exposure to UV light, chemicals, irradiation, or other stress-causing activities) (Pacher & Puchta, 2017). The use of organisms developed using excluded methods (i.e., genetic engineering) appears to remain in an experimental phase. However, unraveling the microbial origin of each one of the CA and CA salts in the market requires information that is often not publicly available.

We were able to locate specific information on a few CA producing strains from international culture collections. It is unclear to us how representative of the CA industry these strains are. Most of the specimens available in these collections have a wild-type origin (Deutsche Sammlung von Mikroorganismen und Zellkulturen-DSMZ, personal communication, August 2023). Attributes (including the origin) of industrial CA-producing strains are often proprietary information held by the manufacturers. Keeping these challenges in mind, the following section provides a summary of the CA producing microorganisms and the origin of those strains whenever we were able to obtain this information.

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[1] *Throughout this report the terms substrate, feedstock, and media are used interchangeably. These terms refer to the material from which a cultured microbe obtains its nutrients. Typically, these include a carbon source, a nitrogen source, and in some cases electrolytes (salts) and other nutrients in a liquid or solid medium, such as agar. In some cases, they may also contain materials which inhibit the growth of other organisms, such as antibiotics.*

127 *Industrial CA-producing strains origin*
128 Cairns et al. (2018) provide a comprehensive list of citric acid manufacturers. Using this list, we searched
129 through company websites and publicly available documents in order to identify the various species of
130 fungi used to produce CA and, when possible, the excluded methods status. In some cases, patents
131 provided more detailed strain information. When identities were not available from digital platforms, we
132 reached out personally to manufacturers. In most cases, the documentation found through the digital
133 search and individual inquiries was not sufficient for us to identify specific strains used by each company.
134
135 China is the world's largest producer of citric acid (CA) with a total production about 2.02 million metric
136 tons, or approximately 75% of the total world production in 2018 (Tong et al., 2023). Companies including
137 Tate & Lyle, ADM, Cargill, and Jungbunzlauer, account for the remaining 25%(Tong et al., 2023).
138
139 The multinational companies mentioned above mostly use submerged fermentation (SmF) using *A. niger*
140 (see Table 1). However, the specific strains within this species were not disclosed. In some instances, we
141 were able to identify patents in which more specific information was disclosed. For example, an Adcuram
142 patent describes the genes useful for the industrial production of CA, and the specific methods (genetically
143 engineered plasmids) used to transform several microbes species in order to increase CA production
144 (Bauweleers & Robert, 2014).[2] Despite this patent, it is unclear if Adcuram is currently utilizing strains
145 derived from these processes to produce their commercial CA at an industrial level. A second patent by
146 Dai & Baker (2015) also describes inactivation and increased expression of genes utilizing genetic
147 engineering techniques.
148
149 **Table 1. CA (Multi)national manufacturers**

Company	Headquarter	Method	Strain	Origin	Media	Citation
COFCO	China	SmF	*Aspergillus niger*	Unknown	Corn pretreated with amylases enzymes	(夏令和 et al., 2013)
		SmF	*Candida mycoderma or Aspergillus wentii*	Unknown	Sugar liquid from corn	(唐宏泉, 2016)
Cargill	USA	SmF	*Unknown*	Unknown	Dextrose carbohydrate	(Cargill, 2023)
Jungbunz-lauer	Switzerland	SmF	*Aspergillus niger*	Strict non-GMO policy	Glucose syrup from corn	(Jungbunzlauer, 2023)
Weifang Ensign Industry Co., Ltd.	China	SmF	*Aspergillus niger*	Unknown	Carbohydrates from corn	(Cairns et al., 2018), (Ma et al., 2019)
RZBC	Shandong / China	Unknown	*Aspergillus niger* (CGMCC 10142)	Unknown	Unknown	(Xue et al., 2021)
Adcuram (Citribel)	Germany	LsF	*Aspergillus niger*	Unknown, Patent describing GE available	Sugar molasses	(Bauweleers & Robert, 2014; Citribel, 2022, 2023)
ADM	USA	SmF	Unknown	Non-GM Microbe	Fermentable carbohydrates from corn and molasses	Personal communication with ADM, 2023

150 SmF= Submerged Fermentation, LsF= Liquid Surface Fermentation
151

[2] *Methods involved introducing bioengineered genetic material into an organism (Rivera et al., 2014). In fungi these techniques are divided into two types: biological and physical. Biological methods are based on Agrobacterium tumefaciens-mediated transformation and protoplast transformation using various cell wall-degrading enzymes. The production of protoplasts remains the most common method for preparation of cells for transformation. Technologies based on physical genetic transformation methods, such as electroporation, biolistics, agitation with glass beads, vacuum infiltration and shock waves contributed significantly towards improving the capacities and have enabled the design of genetically manipulated strains of different fungi (Rivera et al., 2014).*

152 Most of the CA produced commercially comes from wild type strains of *A. niger*, or selected varieties
153 which have been optimized through classical mutagenesis and screening techniques to select the hyper-
154 producing mutant strains (Anastassiadis et al., 2008). [3, 4]
155
156 *Genetic engineering of A. niger in the context of research*
157 Until recently, the main strategy for strain improvement was through chemical or physical mutagenesis
158 followed by screening (Di Lorenzo et al., 2022). These protocols, although time consuming, successfully
159 allowed the improvement of CA yields (Di Lorenzo et al., 2022). For instance, a combination of UV
160 exposure, ethyl methane sulfonate (EMS) and acridine orange treatment to *A. niger* UMIP 2564 resulted in a
161 3.2-fold increase in CA product yield (Lotfy et al., 2007). In another study, Adeoye et al. (2015) reported a
162 45.97-fold increase in CA production by *A. niger*, FUO 2 strain, subjected to UV radiation and cultivated on
163 cassava peel substrate (Di Lorenzo et al., 2022).
164
165 Researchers are exploring using genetic engineering (excluded methods) to redesign and optimize *A. niger*
166 (Tong et al., 2019). A major goal of researchers using biotechnology is to generate designer strains and cell
167 factory with higher yield and efficiency[5]. The release of the first *A. niger* genome data in 2007 paved the
168 way to genetic engineering approaches that targeted (Di Lorenzo et al., 2022):
169 - modifying carbon source utilization and uptake
170 - enhancing CA secretion and biosynthesis pathways
171 - modifying mycelial morphology of the fungus
172 - modifying regulation of the respiratory pathway
173
174 **Table 2. Genetic engineering outcomes for enhancing CA production in a variety of *A. niger* strains**
175 **(Modified from Tong et al. and Zhang et al. (2023; 2020)). It is not clear if any of these strains are**
176 **currently being used to produce CA at an industrial level.**

Strain	Original strain	Engineered change
TNA 101ΔagdA	CGMCC10142	Carbon utilization
OG1	CGMCC10142	Carbon utilization
50-2-12	NW129/ NW131	Enhancing citric acid biosynthesis pathway
55-14	NW129/ NW131	Enhancing citric acid biosynthesis pathway
acl1-acl2	ATCC1015	Enhancing citric acid biosynthesis pathway
Δacl	AB4.1	Enhancing citric acid biosynthesis pathway
Frds (V)-FumRs	N402	Enhancing citric acid biosynthesis pathway
NW185	NW131	Removal of by-product formation
Δ1–3	ATCC11414	Reducing feedback inhibition
TE23	A158	Reducing feedback inhibition
Brsa-25-3	ATCC11414	Engineering Mn2+ response and morphology
chsC-3	CBS513.88	Engineering Mn2+ response and morphology
CGMCC10142-72	CGMCC10142	Regulating the respiratory chain
CGMCC10142-102	CGMCC10142	Regulating the respiratory chain
CGMCC10142-3-4	CGMCC10142	Regulating the respiratory chain
CGMCC10142-4-10	CGMCC10142	Regulating the respiratory chain
several pyrG deficient mutants	WT-D and D353	inhibition of uridine/pyrimidine synthesis

177
178 To date, the principal engineering strategies in the CA industry focus on the improvement of the central
179 metabolic fluxes and the respiratory energy efficiency of *A. niger* (Xue et al., 2021). For example, Xu et al.

[3] *Naturally occurring.*
[4] *Methods where mutations are randomly induced through physical or chemical factors (Pacher & Puchta, 2017).*
[5] *A cell factory is an artificially designed microbial metabolism system (H. Chen & Wang, 2017)*

(2021) genetically modified the industrial CA-production *A. niger* CGMCC 10142 so that it overexpressed glucose transporter genes. This led to an increase in sugar utilization and an increase in the production of CA (Xue et al., 2021). However, genetic engineering strategies are limited because the majority of genes with potential industrial applications to elevate CA production remain hypothetical and have not been identified in the laboratory (Zhang et al., 2020).

Genetic engineering of other fungi, in the context of research

While most of the commercial CA production comes from *A. niger*, there are other microorganisms that have been genetically modified to produce CA as an exercise in basic research. For example, researchers have modified the yeast *Yarrowia lipolytica* to produce CA using inulin as the primary substrate (Reena et al., 2022). To achieve this, a gene from another yeast, *Kluyveromyces marxianus,* was transferred to *Y. lipolytica* to increase the hydrolysis of inulin (Reena et al., 2022). The resulting *Y. lipolytica* produced high levels of CA (Reena et al., 2022).

Intergeneric protoplast fusion

We found few studies related to intergeneric protoplast fusion of microbes to improve CA production. To the best of our knowledge, this technique has been used mostly in research and experimental settings rather than widespread commercial applications. However, due to the proprietary nature of commercial CA production, it is not possible to form a definitive conclusion.

Kirimura et al. (1990) carried out intergeneric protoplast fusion between *A. niger* (producing CA) and *Trichoderma viride* (producing cellulases) and have succeeded in obtaining two types of intergeneric fusants[6] (El-bondkly, 2006). El-bondkly et al. (2006) focused on producing *A. niger/ Trichoderma spp.* hybrids that could potentially ferment agricultural waste with large cellulosic materials. Wild type *A. niger* strains are not able to degrade cellulose. The strains obtained through protoplast fusion with *T. reesei, T. harzianum* and *T. viride* possessed enzymes required for cellulose degradation, and some of them were able to produce up to 200% more CA than the parental *A. niger* CA-producer strain when consuming a fermentation medium based on ground rice straw (El-bondkly, 2006). The experiments published on the above-mentioned study were not done at an industrial scale, but performed in a small-scale laboratory setting where flasks were incubated with constant shaking.

Microbial strain catalogs and strain origin

Collections of microbial strains (or cultures) exist worldwide, and their catalogs are often accessible via the internet, their primary function is to gather, maintain, and distribute strains which have unique properties and are of practical value (Sievers, 2013). These collections are a resource from which microbial strains can be obtained for experimentation but also a source of informative documents associated those strains (Sievers, 2013). From such documents the origin of the strain can be elucidated.

Strains are often identified with codes, which are assigned by the organization that maintains the collection. A single strain can exist in multiple collections and may be identified by different codes depending on the microbial collection from where it is stored and retrieved.

Many collections do not explicitly include the origin (i.e., wild-type, product of classical mutagenesis or product of excluded methods/genetic engineering) of its strains; however, some do. For example, the DSMZ-German Collection of Microorganisms and Cell Cultures provides information on whether strains were developed using genetic engineering (DSMZ, 2023). Most of the strains they preserve are "wild-types." Another catalog, the Japan Collection of Microorganisms (JCM), also identifies the origin of their collected strains (JMC, 2023). From a search performed on August 3rd of 2023, we found 43 strains of *A. niger* available in this collection. Of the 43 strains, three of them are explicitly marketed as citric acid producers:

- *A. niger* 22282
- *A. niger* 22344
- *A. niger* 22437

[6] *A fusion of two different species of fungus.*

233
234 None of these CA producing strains are genetically modified (JMC, 2023).
235
236 The Global Catalogue of Microorganisms (GCM) is a virtual catalog consisting of multiple collections from
237 around the world (GMC, 2023). This catalog includes advanced search options. Utilizing the "application
238 section," we identified 26 microbial strains considered useful in CA production:

239 - *Aspergillus awamori* (2 *strains)*
240 - *Aspergillus carbonarius (1 strain)*
241 - *Aspergillus niger (18 strains)*
242 - *Candida albicans (1 strain)*
243 - *Metschnikowia pulcherrima (1 strain)*
244 - *Yarrowia lipolytica (3 strain)*

245
246 Where possible, we further identified the origin of the 18 *A. niger* strains considered important for CA
247 production (see Table 3). We were not able to identify if some of the strains were or were not produced
248 with excluded methods, in those cases we assigned "Unknown" on the "Origin" column (Table 1).
249
250 **Table 3. GMC CA-producing *A. niger* strains**

Strain number	Other code names/Literature	Origin (Isolated from)
NBRC 111403		Soil
GFCC16905	Tiegh., Annls Sci. Nat., Bot., ser. 5 8: 240 (1867)	Arachis hypogea kernel
GFCC16907		Unknown
GFCC16909		Unknown
GFCC16912		Unknown
GFCC16913		Unknown
GFCC16914		Unknown
GFCC19013		Culture of Hygrocybe punicea
TISTR 3245	ATCC 6275=QM 458 =IFO 6341	Leather
VTCC 30023	VTCC-F-0023	Unknown
VTCC 30024	VTCC-F-0024	Unknown
VTCC 30025	VTCC-F-0025	Unknown
BNCC185762		Unknown
VTCC 30030	VTCC-F-0030	Unknown
VTCC 30031	VTCC-F-0031	Unknown
TISTR 3089	ATCC 1414=NRRL 2270	Derived from ATCC 1015
TISTR 3106	UPCC 3074	Unknown

251

252
APPENDIX A

253
254 **Excluded Methods:**
255

Method and synonyms	Types	Notes
Targeted genetic modification (TagMo) syn. Synthetic gene technologies syn. Genome engineering syn. Gene editing syn. Gene targeting	Sequence-specific nucleases (SSNs) Meganucleases Zinc finger nuclease (ZFN) Mutagenesis via Oligonucleotides CRISPR-Cas system (Clustered regularly interspaced short palindromic repeats) and associated protein genes TALENs (Transcription activator-like effector nucleases) Oligonucleotide directed mutagenesis (ODM) Rapid Trait Development System	Most of these new techniques are not regulated by USDA and are currently difficult to determine through testing.
Gene Silencing	RNA-dependent DNA methylation (RdDM) Silencing via RNAi pathway RNAi pesticides	
Accelerated plant breeding techniques	Reverse Breeding Genome Elimination FasTrack Fast flowering	These may pose an enforcement problem for organics because they are not detectable in tests.
Synthetic Biology	Creating new DNA sequences Synthetic chromosomes Engineered biological functions and systems	
Cloned animals and offspring	Somatic nuclear transfer	
Plastid transformation		
Cisgenesis	The gene modification of a recipient plant with a natural gene from a crossable-sexually compatible-plant. The introduced gene includes its introns and is flanked by its native promoter and terminator in the normal-sense orientation.	Even though the genetic manipulation may be within the same species, this method of gene insertion can create characteristics that are not possible within that individual with natural processes; it can have unintended consequences.
Intragenesis	The full or partial coding of DNA sequences of genes originating from the sexually compatible gene pool of the recipient plant and arranged in sense or antisense orientation. In addition, the promoter, spacer, and terminator may originate from a sexually compatible gene pool of the recipient plant.	Even though the genetic manipulation may be within the same species, this method of gene rearrangement can create characteristics that are not possible within that individual with natural processes; it can have unintended consequences.
Agro-infiltration		In vitro nucleic acids are introduced to plant leaves to be infiltrated into them. The resulting plants could not have been achieved through natural processes and are a manipulation of the genetic code within the nucleus of the organism.
Transposons-Developed via use of in vitro nucleic acid techniques		Does not include transposons developed through environmental stress such as heat, drought or cold.
Induced Mutagenesis		Developed through in vitro nucleic acid techniques does not include mutagenesis developed through exposure to UV light, chemicals, irradiation, or other stress-causing activities.
Cell and Protoplast Fusion	donor and/or recipient cells are outside taxonomic plant family; and/or recombinant DNA technology is employed	See NOP Policy Memo 13-1.

256

257 **Methods Allowed:**
258

Method and synonyms	Types	Notes
Marker Assisted Selection		
Transduction		
Embryo rescue in plants		IFOAM's 2018 position paper on Techniques in Organic Systems considers this technique compatible with organic systems.
Embryo transfer, or embryo rescue, in animals		*use of hormones not allowed in recipient animals.
Transposons		Developed through environmental stress, such as heat, drought, or cold.
Cell and Protoplast Fusion	Recipient and/or donor cells are within the same taxonomic plant family; must be achieved without recombinant DNA technology	NOP Policy Memo 13-1; Definition of Modern Biotechnology

259
260 **Report Authorship**
261
262 The following individuals were involved in research, data collection, writing, editing, and/or final
263 approval of this report:
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266 • Doug Currier, Technical Director, OMRI
267 • Amy Bradsher, Deputy Director, OMRI
268
269 All individuals are in compliance with Federal Acquisition Regulations (FAR) Subpart 3.11—Preventing
270 Personal Conflicts of Interest for Contractor Employees Performing Acquisition Functions.
271
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