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

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.

Vitamins

Livestock

Summary of Petitioned Use

This limited scope technical report provides information to the National Organic Standards Board (NOSB) to support the sunset review of vitamins, listed at 7 CFR 205.603(d)(3) with the following annotation: "used for enrichment or fortification when FDA approved." This report focuses on the manufacturing processes and use of excluded methods to produce vitamins used in organic livestock production as a synthetic feed additive.

Vitamins were initially reviewed by the NOSB in 1995 (NOSB, 1995c). They were included on the National List of Allowed and Prohibited Substances (hereafter referred to as the "National List") with the first publication of the National Organic Program (NOP) Final Rule (65 FR 80548, December 21, 2000).

In September 2002, the NOSB recommended a new listing in the livestock portion of the National List (§ 205.603) in order to allow organic livestock operations to use all materials (including vitamins) allowed in organic handling (§ 205.605) that are subject to U.S. Food and Drug Administration (FDA) or the Association of American Feed Control Officials (AAFCO) regulations (NOSB, 2009). However, the NOP did not adopt that recommendation.

The NOSB has recommended the renewal of vitamins in 2005, 2010, 2015, and 2019 (NOSB, 2005, 2010, 2015, 2019b).

For this report, the NOSB made the following requests:

- Review and list livestock vitamin production fermentation processes that use excluded methods and identify GMO microbe strains.
- Review and list livestock vitamin production processes that use feedstocks produced with excluded methods. This report uses the terms "feedstocks," "precursors," and "fermentation media" for greater precision and clarity. These are all defined in the *Glossary*.
- Review of any novel (i.e. not fermentation, extraction, or chemical synthesis) processes to manufacture livestock vitamins that might incorporate excluded methods.

We have reframed these requests as focus questions with several parts, which we can more easily provide discrete responses to.

Identification of Petitioned Substance

We identified 15 vitamins that are used for animal nutrition and meet the National List annotation at 7 CFR 205.603(d)(3) and NOP Guidance 5030, *Evaluating Allowed Ingredients and Sources of Vitamins and Minerals For Organic Livestock Feed* (NOP, 2013):

- vitamin A (retinol)
- vitamin B$_1$ (thiamine)
- vitamin B$_2$ (riboflavin)
- vitamin B$_3$ (niacin)
- vitamin B$_5$ (pantothenic acid)
- vitamin B$_6$ (pyridoxine)
- vitamin B$_7$ (biotin)
- vitamin B$_8$ (inositol)
- vitamin B$_9$ (folic acid)
- vitamin B$_{12}$ (cobalamin)
- vitamin C (ascorbic acid)
- choline
- vitamin D (cholcalciferols)
- vitamin E (tocopherols)
- vitamin K (menadione)

We have included monographs with names and other identifiers, chemical formulas, and molecular structures for each of these vitamins. We have also included manufacturing processes for those vitamins as well. The monographs support the summary answers to the focus questions.

A summary of the regulatory status of specific vitamin sources recognized as allowed by the U.S. Food and Drug Administration (FDA) and the Association of American Feed Control Officials (AAFCO) along with International Feed Numbers (IFN) for cross-referencing to foreign regulations is in *Table 2* of the *Appendix*.

The NOSB reviewed two full scope technical reports for vitamins used both in livestock and in processing/handling in 2015 (NOP, 2015a, 2015b). Readers can access those reports for more information on these vitamins and the basis for the NOSB's previous recommendations. This is a limited scope technical report that aims to answer specific focus questions. We explored specific manufacturing processes, as well as agricultural sources of individual vitamins. Additionally, we researched the use of microorganisms to produce commercial sources of feed-grade vitamins, using both methods that are allowed and excluded. These questions will be answered in summary form.

History of use in organic

Synthetic vitamins and minerals used as feed additives for livestock production were allowed for use by state, private, and international organic standards prior to the passage of the Organic Foods Production Act (OFPA) of 1990. They were included in the original round of petitions that the NOSB considered in 1995.

In 1995, the NOSB recommended that the NOP add synthetic nutrient vitamins and minerals approved by the Food and Drug Administration (FDA) to the National List (NOSB, 1995a). Both were added to the original National List in 2000 (65 FR 80548, December 21, 2000). The annotation reads, "As feed additives... Vitamins, used for enrichment or enforcement when FDA approved."

Folic acid (vitamin B_9) was petitioned separately for use in livestock (NOSB, 1995b).

Nutrient vitamins and minerals were also added to the National List at the same time as synthetic non-organic, non-agricultural ingredients used in organic processed products (65 FR 80548, December 21, 2000). While human food use is outside the scope of this review, information on sources and manufacturing processes for food-grade vitamins is included when needed to address the NOSB's focus questions.

Vitamins used as feed additives are allowed in organic livestock production as described at 7 CFR 205.603(d)(3). The substances are subject to review under the sunset provision of the Organic Foods Production Action (OFPA) (7USC 6517(d)). OFPA explicitly authorizes synthetic vitamins to be on the National List (7 U.S.C. 6517(c)(1)(B)(i)).

In 2013, the NOP issued Guidance 5030, *Evaluating Allowed Ingredients and Sources of Vitamins and Minerals For Organic Livestock Feed*, to help certifying agents and material review organizations evaluate what sources of vitamins and other non-organic feed additives and supplements are permitted in organic livestock feed (NOP, 2013).

Background

Vitamins are defined as "[o]rganic compounds that function as part of enzyme systems essential for the transmission of energy and the regulation of metabolisms of the body" (AAFCO, 2022). The requirements are minute compared with other nutrients such as carbohydrates, fats, and proteins, with daily requirements measured in micrograms to milligrams (McDowell, 2000; Micronutrient Information Center, 2023). Nutritional requirements for specific vitamins vary by species and other factors. Specifically, ruminants such as cattle and sheep can biosynthesize various specific vitamins in the gut, while monogastric animals such as pigs and poultry cannot (Cherian, 2020; Combs, 2012; Maynard & Loosli, 1956; McDowell, 2000; Morrison, 1951). Species requirements for specific vitamins are mentioned in greater detail in the monographs.

FDA and AAFCO regulation of vitamins

Animal food and feed ingredients and additives are regulated:

- at the federal level by the FDA under the Federal Food, Drug, and Cosmetic (FD&C) Act (21 USC 321 *et seq*.)
- at the state level by the various feed control officials.

Most state laws are modeled on the Association of American Feed Control Officials (AAFCO) model law (AAFCO, 2022; US FDA, 2023). The FDA recognizes AAFCO's *Official Publication* as containing the most complete list and descriptions of animal food ingredients (US FDA, 2023).

Any substance that is either directly or indirectly added to or expected to become a component of animal food—including vitamins—must be used in accordance with a food additive regulation unless it is Generally Recognized As Safe (GRAS) for that use (US FDA, 2023). Following FDA reforms to the GRAS affirmation procedures in 1997 (62 FR 18938, April 17, 1997) and 2016 (81 FR 54960, August 17, 2016), petitioners are no longer required to go through federal rulemaking to have the FDA declare a substance GRAS.

Petitioners of animal food additives who seek GRAS status have three options:

- Voluntarily petition the FDA and AAFCO under the provisions of 21 CFR 571 and the AAFCO Definitions Committee procedure (AAFCO, 2022; Koutsos & Haynes, 2023);
- Voluntarily notify the FDA that the substance is GRAS and receive a letter from FDA of no questions (US FDA, 2010);
- Self-determine that a substance is GRAS by a review of publicly available scientific data and the opinion of an expert panel (US FDA, 2017).

FDA GRAS notification is a voluntary program (Gaynor & Cianci, 2005; Koutsos & Haynes, 2023). Substances, including various vitamins used before the 1958 amendments to the FD&C Act, are exempted from new rules by virtue of a substantial history of consumption by a significant number of the targeted animals and humans eating the products of those animals prior to January 1, 1958 (21 CFR 570.30(c) & 570.3(3)). The FD&C Act requires the FDA to review and approve any substances that may be added to food—including food for animals—prior to it being marketed, unless it is considered GRAS by qualified experts using scientific procedures [21 U.S.C. 321(s) and 348].

A detailed description of the AAFCO definition process is contained in their *Official Publication* (AAFCO, 2022). The requester of a definition identifies an animal food ingredient and proposes a definition. The request also includes detailed, specific information about the ingredient, including data on a new animal food ingredient's manufacturing process, safety, efficacy, and analytical methods. The requester then submits this data to FDA's Center for Veterinary Medicine for scientific review. If the FDA has no questions or objections, the petitioner then submits information to the AAFCO Ingredient Definition Committee. The committee assigns an investigator that evaluates the request and FDA review and recommendation. The investigator may ask for additional information from the requester. Once the request is complete, the investigator prepares a report for the Ingredient Definitions Committee. The Ingredient Definitions Committee makes a recommendation to the AAFCO Board, which decides whether the definition can be added to the *Official Publication* (AAFCO, 2022).

Self-declared GRAS notices still require analytical, efficacy, and safety data. Vitamins and other animal feed additives can be marketed without review by an expert panel, but such an approach still involves the state-by-state review of the data and approval by each state feed control official (Koutsos & Haynes, 2023).

Excluded methods

The USDA organic regulations state that "[t]o be sold or labeled as '100 percent organic,' 'organic,' or 'made with organic (specified ingredients or food group(s)),' the product must be produced and handled without the use of excluded methods." [7 CFR 205.105(e)]. The regulation defines excluded methods 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. [7 CFR 205.2].

This technical report evaluates whether any vitamins used as livestock feed additives are produced by excluded methods.

The definition of *excluded methods* focuses mainly on the use of recombinant DNA (rDNA) technologies used to genetically modify plants grown as agricultural crops for the food and feed supply. The examples given in the definition, such as gene doubling, gene deletion, removing a gene from a donor organism and inserting it into a recipient organism, and changing the positions of genes can also be performed on other organisms besides plants.

Micro- and macro-encapsulation refers to the use of synthetic polymers in the delivery systems for various fermentation organisms. Microencapsulation involves the encapsulation of a single cell in a polymeric semipermeable membrane (Gibbs et al., 1999; Nedovic et al., 2011; Ray et al., 2016). Macroencapsulation fills a membrane with multiple cells in a polymeric matrix. The organisms themselves are not necessarily made by excluded methods, but such delivery systems are excluded. Many micro- and macro-encapsulated bacteria can be used in various food applications, including nutrient vitamins and minerals (Gibbs et al., 1999; Nedovic et al., 2011; Ray et al., 2016).

Advances in nanotechnology have created the possibility to nanoencapsulate vitamins (Katouzian & Jafari, 2016). Fat-soluble vitamins appear to be particularly promising for nanoencapsulation (Panigrahi et al., 2019). Even though nanoencapsulation is not mentioned in the definition, we consider it to be implicitly excluded as a form of micro-encapsulation.

The NOSB offered several final recommendations to the NOP regarding the use of genetically modified organisms and other excluded methods in organic production and handling.

- In 1995, the NOSB recommended a definition of genetic engineering (NOSB, 1995a).
- The NOSB recommended that genetically engineered organisms and their products be prohibited for organic production and handling at their next meeting (NOSB, 1996).
- The USDA issued the first proposed rule in 1997, which defined "genetic engineering" as the NOSB recommended, but the proposal for public comment did not adopt their recommendation to prohibit the use of genetic engineering in organic production and handling (62 FR 65850, December 16, 1997).
- The NOSB reaffirmed its recommendation in 1998, following publication of the first proposed NOP rule (NOSB, 1998).
- The USDA revised the second proposed rule for the NOP to define the new term "excluded methods" and used the NOSB's recommendation for the definition of "genetic engineering" in that proposal for public comment (65 FR 13512, March 13, 2000).
- The final and current definition of "excluded methods" in the NOP rule is identical to the NOSB's recommended definition of genetic engineering (65 FR 80548, December 21, 2000).

In 2019, the NOSB issued a Formal Recommendation on induced mutagenesis and embryo transfer (NOSB, 2019a). This recommendation also stated that "induced mutagenesis developed through exposure to UV light, chemicals, irradiation, or other stress-causing activities" should remain on the most current "To Be Determined (TBD)" chart for future discussion and review. The NOSB's most recent version of their document *Excluded Methods Determinations* is referenced in this technical report (NOSB, 2022). That recommendation includes Appendix A, which identifies new and emerging technologies that were not considered at the time that the NOSB made the original recommendation to the NOP. These technologies are mostly applied to plants, but some also have applications to other taxonomic kingdoms.

Availability of information for this report

Much of the information about vitamin production is proprietary and not publicly available (Shurson & Urriola, 2019). Knowledge of whether a given batch or lot of vitamins is made by methods included or excluded in organic standards requires traceback to the source and the full disclosure of manufacturing process at that source.

We found it uniquely challenging to answer NOSB focus questions for vitamin C because:

1. It appears in many different forms.
2. It can be made by several different manufacturing processes.
3. Manufacturers are not required to disclose to the public how they make vitamin C.
4. The facilities where the vitamins are made are not subject to third-party verification of claimed manufacturing processes.
5. The distributors that supply vitamin C are not required to disclose their sources.
6. It is by far the single largest vitamin market.
7. The supply chain may involve sourcing vitamin C from multiple manufacturers that make it by different processes.
8. The technology used to make vitamin C appears to be changing rapidly.

Given the rapid adoption of various techniques used to genetically manipulate organisms, it is not possible to predict what potential sources produced from excluded methods will begin production on a commercial scale or when they will enter the market. When we discovered processes that involved excluded methods, we searched the literature to see if such methods are currently being used. In some cases, claims made on the internet by multiple sources lacked information about the companies that used the excluded methods, or which specific vitamins are currently produced by such methods. In some cases, the sources referred to vitamins for human consumption, but there was no evidence that such vitamins were being marketed as animal feed additives.

Another challenge is the changing structure and performance of the vitamin industry. While not as opaque as the flavor industry, the vitamin industry protects many of their proprietary production processes by trade secrets. The global vitamin industry is dominated by Chinese enterprises that operate under the protection and direction of the Chinese government. American feed manufacturers filed a U.S. antitrust action against the Chinese enterprises involved in price-fixing that was appealed to the U.S. Supreme Court (*Animal Science Products, Inc., et al. v. Hebei Welcome Pharmaceutical Co. Ltd. et al.*, S.Ct. 2017). After being returned to the lower court, the case was dismissed because Chinese law requires anticompetitive behavior that is illegal in the U.S. (*Animal Science Products, Inc., et al. v. Hebei Welcome Pharmaceutical Co. Ltd. et al.*, 2nd Cir. 2021).

The vitamin monographs provide the most up-to-date information on the primary producers and prevailing production practices reported in the industrial literature. While we tried to find out what we could about the manufacturing processes used by these companies, many of the patents and much of the literature on such processes is in Chinese and has not been translated or peer reviewed. We were also unable to find a database equivalent to the GRAS notification system, or information resources comparable to those provided by the European Union for genetically modified microorganisms used to manufacture animal feed ingredients in China. We relied on secondary sources for information from China that, in some cases, could not be confirmed or verified by readily available and accessible published documentation.

Focus Questions Requested by the NOSB

1. What livestock vitamins are currently produced from fermentation? Which of these vitamins may be produced from microorganisms developed using excluded methods?

The vitamin monographs show what vitamins may be derived from multiple agricultural sources or produced by multiple industrial processes. Some of the vitamins identified as having a high probability of originating from a genetic engineered microorganism or feedstock may also have other sources. Some vitamins identified as low probability for originating from excluded methods may be the subject of ongoing research and development that is close to commercialization.

Vitamins made by fermentation with production organisms that are not developed by excluded methods

We found no commercial sources of vitamins exclusively made by non-genetically engineered fermentation organisms. In other words, all vitamins produced through fermentation are at high risk of being produced with excluded methods (see *Table 1*). While some sources might not be produced from genetically engineered organisms, such claims could not be categorically verified for any vitamin.

Several vitamins produced without excluded methods appear to be close to becoming commercially available, but we could not confirm that such methods are in production. These include:

- vitamin B_3 (niacin)
- vitamin B_5 (pantothenic acid)
- vitamin K (menadione)

Vitamins made by fermentation, with production organisms that are developed by excluded methods

Vitamin B_2 and vitamin B_{12} are almost entirely produced by fermentation. Most vitamin C is produced by chemical synthesis with a precursor that is made through a fermentation process.

Vitamin B_2 (riboflavin) and vitamin B_{12} (cobalamin) are made with production organisms developed by excluded methods (see *Table 1* below). The remaining B-complex vitamins are targeted for the development of genetically engineered processes to replace total chemical synthesis. The respective vitamins' monographs provide more details.

Table 1: Vitamin manufacturing processes. Adapted from Bonrath, et al., 2019 and NOP, 2015a.

Vitamin	Common names	Primary manufacturing process	Agricultural?	GE source microorganism?	GE source feedstock
A	retinol	total chemical synthesis	No	No	No
B_1	thiamine	total chemical synthesis	No	No	No
B_2	riboflavin	fermentation with excluded methods	No	High probability	High probability
B_3	niacin	total chemical synthesis	No	No	No
B_5	pantothenic acid	total chemical synthesis	No	No	No
B_6	pyridoxine	total chemical synthesis	No	No	No
B_7	biotin	total chemical synthesis	No	No	No
B_8	inositol	isolated from corn	Yes	No	High probability
B_9	folic acid	total chemical synthesis	No	No	No
B_{12}	cyanocobalamin	fermentation with excluded methods	No	High probability	High probability
Choline	choline	total chemical synthesis	No	No	No
C	ascorbic acid	fermentation with excluded methods	No	High probability	High probability
D	cholecalciferol	total chemical synthesis	No	No	No
E	tocopherols	isolated from soybeans	Yes	No	High probability
K	menadione	total chemical synthesis	No	No	No

As indicated *Table 1*, only three vitamins have a high risk of being derived directly from excluded methods because they are produced with genetically engineered organisms (vitamin C, vitamin B_2, and vitamin B_{12}).

Two vitamins (vitamin E and vitamin B_8) are isolated from crops that are commonly genetically engineered for pest related purposes (corn and soybean).

2. What livestock vitamins are produced through chemical synthesis? Which of these vitamins use chemical ingredients that may be produced using excluded methods?

The following vitamins are currently produced by total chemical synthesis without any fermentation steps:

- vitamin A (retinol)
- vitamin B_1 (thiamine)
- vitamin B_3 (niacin)
- vitamin B_5 (pantothenic acid)
- vitamin B_6 (pyridoxine)
- vitamin B_7 (biotin)
- vitamin B_9 (folic acid)
- choline
- vitamin D (cholcalciferols)
- vitamin K (menadione)

Vitamin C (ascorbic acid) may be produced by any of several different processes. Most commercially available sources of Vitamin C involve chemical synthesis that involves a precursor chemical produced by microbial fermentation. The various organisms, use of genetic engineering, and other technically feasible methods are presented in greater detail below.

3. Are excluded methods used to produce livestock vitamins in novel ways, which are not otherwise addressed in Focus Questions #1 and #2?

Yes.

- Natural vitamin E and tocopherols are extracted from soybeans. Most soybeans produced in the U.S. are genetically modified.
- Vitamin B_8 (inositol) is isolated from corn steep liquor from the wet milling process. Most corn produced in the U.S. is genetically modified.
- Corn, rice, and other crops have been genetically modified to be biofortified with vitamin A, folate (vitamin B_9), and vitamin C. Genetically modified biofortified crops have not yet been commercially released in the U.S.

Vitamin Monographs

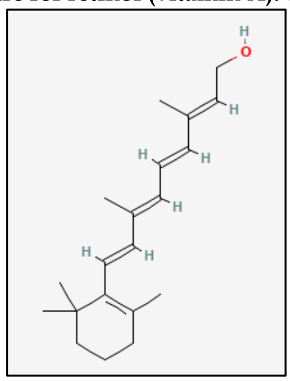
Below are monographs of each of the fifteen vitamins considered in this report. The sources used for the identity, chemical names, molecular formula, molecular structure, and numerical codes are recognized authorities on the subject (AAFCO, 2022; Combs, 2012; McDowell, 2000; *Merck Index Online*, 2023; Micronutrient Information Center, 2023; US NLM, 2023; Zempleni et al., 2014). The monographs include provitamins recognized as effective sources of vitamin activities (AAFCO, 2022; Combs, 2012; McDowell, 2000).

Each monograph includes the most current information for the prevailing manufacturing process for commercial production of each vitamin found in academic, encyclopedic, and trade publication sources using the Agricola, ChemSpider, Google Scholar, PubMed, the Micronutrient Information Center of the Linus Pauling Institute, and SciFinder search engines. We prioritized articles and other source material published open access.

Vitamin A

Common name: Retinol.
IUPAC name: (2E,4E,6E,8E)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-ol.
Other names: Retinal; retinoic acid.
CAS number: 68-26-8
EC (formerly EINECS) number: 200-683-7 (retinol); 204-844-2 (retinol acetate); 234-328-2 (vitamin A).
International Feed Numbers: 7-05-142 (vitamin A acetate); 7-05-143 (vitamin A palmitate); 7-26-311 (vitamin A propionate)
FDA GRAS: 21 CFR 582.5933 (vitamin A acetate); 21 CFR 582.5936 (vitamin A palmitate)
Provitamins: [1] ß-carotene and other carotinoids.*Alternate forms:* Vitamin A acetate; vitamin A palmitate; vitamin A propionate; cod liver oil, salmon oil, salmon liver oil; shark liver oil; tuna oil.
Molecular formula: $C_{20}H_{30}O$
Picture of molecular structure:

Figure 1: Molecular structure for retinol (vitamin A). Taken from US NLM, 2023.



Manufacturing process

Most commercial sources of vitamin A are produced by chemical synthesis (Bonrath et al., 2023). The Grignard process was first used to synthesize vitamin A in the 1940s (Milas, 1945). Arens and Van Dorp first synthesized various sources of vitamin A in 1946 (Parker et al., 2016). Hoffmann-La Roche manufactured the first commercial source of synthetic vitamin A in 1948 (Bonrath et al., 2023).

Agricultural sources

All plants contain carotenoids and retinoids that can be metabolized into vitamin A (Cherian, 2020; McDowell, 2000). Fresh pasture and forage are excellent sources of ß-carotene (National Research Council, 2001). Supplemental vitamin A is unnecessary in dairy cattle on pasture or properly conserved forage, and may even lead to toxic excesses (National Research Council, 2001). These sources of ß-carotene can all be organically produced. Corn gluten meal has been explored as a potential agricultural source for vitamin A (Wellenreiter et al., 1969). Most corn produced in the U.S. is genetically modified (USDA Economic Research Service, 2023).

Rice and corn have been genetically modified to have increased levels of vitamin A under the names “golden rice” and “orange maize,” respectively (Manjeru et al., 2019; Paine et al., 2005; Tang et al., 2009). Golden rice is developed through an entirely transgenic process (Paine et al., 2005). Orange maize was developed by a combination of classical selection, marker-assisted breeding, and transgenic techniques (Manjeru et al., 2019). Golden rice, orange maize, and other crops biofortified to increase vitamin A levels are being developed in a rapidly changing regulatory and market situation (Turnbull et al., 2021). We found no evidence that isolated vitamin A or any concentrated provitamins are made from agricultural sources.

[1] Provitamins are molecules that serve as precursors to vitamins. Living organisms convert provitamins into molecules with vitamin activity.

Fermentation (or synthesis methods) using excluded methods

Most of the research conducted on the potential microbial fermentation of carotenoids has used *E. coli* bacteria because it is a suitable host for the cloning and expression of foreign genes (Albermann & Beuttler, 2016). The provitamin A source, ß-carotene, can be produced by fermentation on a glucose and xylose substrate using a strain of the yeast *Saccharomyces cerevisiae* that has been modified using CRISPR genome editing technology (Sun et al., 2020). We found no evidence of current commercial production for this technology or estimates of when the technology is predicted to be commercialized.

Fermentation (or synthesis methods) using allowed methods

We found no sources of vitamin A made by allowed fermentation methods.

Other sources

Prior to industrial scale manufacturing from synthetic precursors made from compounds isolated from petrochemicals, livestock producers relied on vitamin A from natural sources (Maynard & Loosli, 1956; Morrison, 1951). Fish oils were a major source of supplemental vitamin A used in livestock feed prior to the invention of synthetic sources (Maynard & Loosli, 1956; Morrison, 1951). AAFCO continues to recognize cod liver oil, salmon oil, salmon liver oil, sardine oil, and shark liver oil as vitamin A sources sold as livestock feed additives (AAFCO, 2022). Livestock producers also historically used fish meal to meet their animals' Vitamin A requirements (Morrison, 1951). We found no evidence that such sources involve excluded methods.

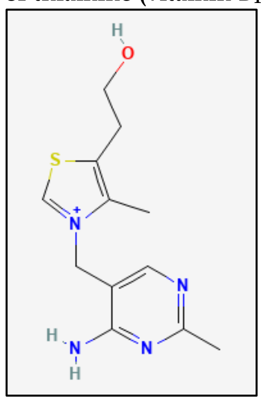
Various microalgae, such as *Arthrospira, Chlorella, Dunaliella, Haematococcus, Nannochloropsis* and *Odontella*, are the subject of research for potential production of ß-carotene and other carotenoids (Grama et al., 2016). The largest producer of ß-carotene from microalgae is BASF, which grows *Dunaliella salina* as the principal organism for its production in open pond culture. The review article notes that strategies to increase have focused on *E. coli* and *S. cerevisae* and fermentation processes. As of the publication date of the article, algae are considered potential platforms to genetically modify for carotenoid production (Grama et al., 2016).

We found no evidence that any microalgae source of ß-carotene or other carotenoids are commercially produced by any excluded methods.

Vitamin B_1

Common name: Thiamine
IUPAC name: 5-(2-Hydroxyethyl)-3-[(6-imino-2-methyl-1,6-dihydro-5-pyrimidinyl)methyl]-4-methyl-1,3-thiazol-3-ium chloride
Other names: Aneurine
CAS number: 59-43-8
EC number: 200-425-3
International Feed Numbers: 7-04-828 (thiamine hydrochloride); 7-04-829 (thiamine mononitrate)
FDA GRAS: 21 CFR 582.5875 (thiamine hydrochloride); 21 CFR 582.5878 (thiamine mononitrate)
Provitamins: Allithiamine; fursultiamine, sulbutiamine, benfontiamine.
Alternate forms: Thiamine chloride; thiamine hydrochloride; thiamine mononitrite.
Molecular formula: $C_{12}H_{17}N_4OS+$
Picture of molecular structure:

Figure 2: Molecular structure of thiamine (vitamin B_1). Taken from US NLM, 2023.



Manufacturing process

Researchers for the Merck Company reported the first successful synthesis of thiamine in 1936 using the substances 4-methyl-5-beta-hydroxyethylthiazole, 2,5-dimethyl-6-aminopyrimidine, 2-methyl-6-oxypyrimidine, 2-methyl-6-oxypyrimidine-5-methylene sulfonic acid in the presence of quaternary ammonia in a six step process (Cline et al., 1937; Williams & Cline, 1936).

The following year, researchers reported thiamine synthesized by IG Farben using the Grewe process (Todd & Bergel, 1937). The Grewe process involves a synthetic diamine derived from either acrylonitrile or malononitrile (Eggersdorfer et al., 2012). The process has evolved from the first synthetic sources to improve efficiency and lower costs. One of the more efficient current processes involves continuous production with eight steps, involving synthetic reactions of compounds derived from petrochemicals, including 2-cyanoacetamide, dichloroethane, acetamidine, methanol, and ammonia, among others (Jiang et al., 2023). The Grewe process remains the prevailing method of commercial industrial manufacturing (Eggersdorfer et al., 2012; Jiang et al., 2023).

Agricultural sources

Whole grains and hay are the main agricultural sources of thiamine. Sources include wheat, rice, oats, barley, rye, corn, and alfalfa (Maynard & Loosli, 1956; Morrison, 1951; National Research Council, 1994, 1998, 2001; Schaible, 1970). Corn and alfalfa are commonly grown from varieties that have been genetically modified. No evidence was found that thiamine is isolated and commercially produced from such sources.

Fermentation (or synthesis methods) using excluded methods

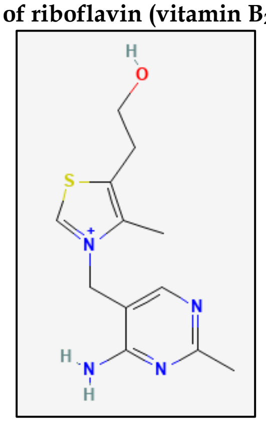
Researchers have studied yeast (*S. cerevisiae*) as a likely host to genetically engineer for extracellular vitamin B_1 production because of its already high content of thiamine (Rocchi et al., 2023). However, there is no evidence that any genetically engineered strains are currently used in the commercial production of thiamine.

Fermentation (or synthesis methods) using allowed methods
Brewer's yeast (*S. cerevisiae*) is the richest natural source of thiamine (Combs, 2012; Maynard & Loosli, 1956; McDowell, 2000; Zempleni et al., 2014). Nutritional yeast improved by classical methods can be added to feed rations to increase thiamine content (Maynard & Loosli, 1956; McDowell, 2000; National Research Council, 2001). We found no evidence that thiamine isolated from yeast is sold as an animal feed additive.

Other sources
None found.

<u>Vitamin B$_2$</u>
Common name: Riboflavin
IUPAC name: 7,8-dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4-dione
Other names: Beflavin, Flavaxin; Lactoflavin; Lutavit® (BASF); Ribosyn; Ribovel; vitamin G; vitasan B$_2$.
CAS number: 83-88-5
EC number: 201-507-1
International Feed Numbers: 7-03-920
FDA GRAS: 21 CFR 582.5695; 21 CFR 582.5697 (riboflavin-5-phosphate).
Provitamins: None identified.
Alternate forms: Vitamin B$_2$ supplement; riboflavin-5-phosphate.
Molecular formula: $C_{17}H_{20}N_4O_6$
Picture of molecular structure:

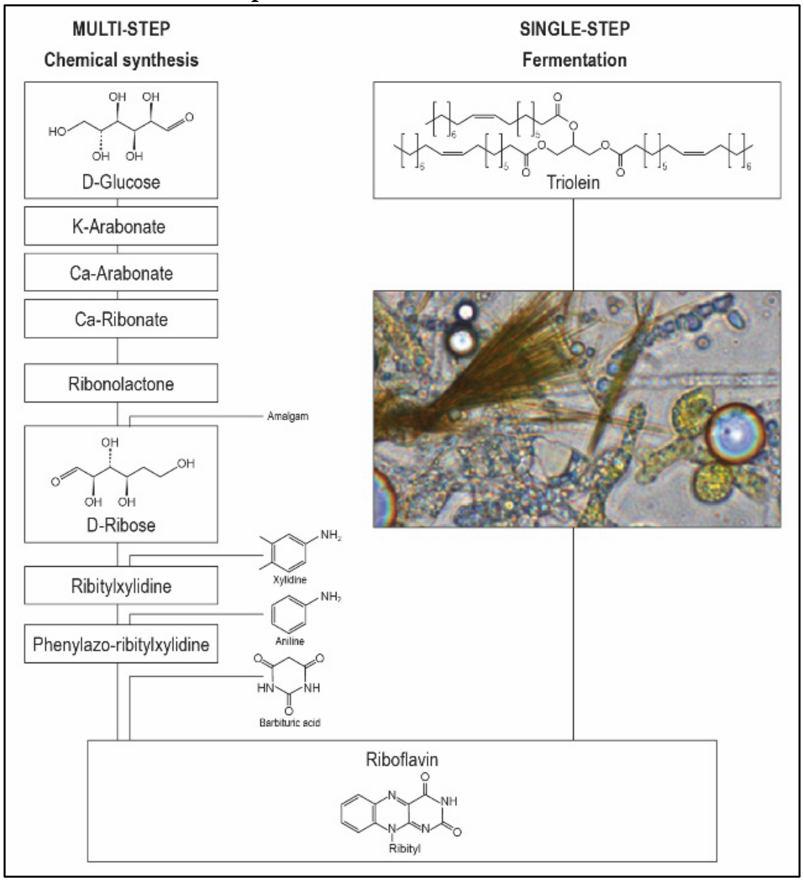
Figure 3: Molecular structure of riboflavin (vitamin B$_2$). Taken from US NLM, 2023.



Manufacturing process
Commercial production of vitamin B$_2$ through chemical synthesis does exist, although these sources contribute a relatively minor supply compared to those which use fermentation production methods (Chu et al., 2022). The ability to produce pure forms of riboflavin is possible using chemical synthesis methods. However, fermentation methods to produce riboflavin are more cost-effective than total chemical synthesis and are used to manufacture the global supply (Averianova et al., 2020; Chu et al., 2022).

The difference between direct microbial fermentation and indirect microbial biosynthesis involves different pathways to yield the end-product of riboflavin (see <u>*Figure 4*</u>, below). Microbial fermentation involves microorganisms—usually *Bacillus subtilis* or *Ashbya gossypii*—that have been genetically modified to overproduce riboflavin. The fermentation cycles for the process are relatively long, require specific growth media to support the overproduction, and have yield limitations (Averianova et al., 2020; Chu et al., 2022). Optimal yield depends on relative carbon and nitrogen concentrations, and the levels of 13 mineral nutrients (Averianova et al., 2020) A broader range of microorganisms can produce precursors in a shorter time that can be used to produce synthetic riboflavin (Averianova et al., 2020). These organisms may be classically selected, mutated by various methods, or genetically modified by excluded methods (Averianova et al., 2020).

Figure 4: Flowcharts of riboflavin production methods. Taken from Schwechheimer, et al., 2016.



Agricultural sources

Alfalfa hay is a rich source of riboflavin historically fed to animals (Maynard & Loosli, 1956). Whey and distiller's solubles are also commercially important feedstuff sources of riboflavin (McDowell, 2000). Riboflavin can be concentrated from milk, eggs, and liver by hydrolysis with nitric acid (Pasternack & Brown, 1943). We found no evidence that any commercially available sources of concentrated vitamin B_2 are isolated from an agricultural source by this or any other method.

Fermentation (or synthesis methods) with excluded methods

Vitamin B_2 used in human food and animal feed production is exclusively produced by bacteria or yeast that are made with excluded methods (Averianova et al., 2020; Hanlon & Sewalt, 2021; Oehen et al., 2011; Revuelta et al., 2017; Stahmann, 2011). By the year 2000, biotechnological sources of fermentation organisms were fully competitive with the existing chemical synthesis processes for industrial production and biotechnologists were rapidly innovating advances to overtake and replace chemical synthesis (Stahmann et al., 2000). Non-GMO sources of vitamin B_2 were reportedly unavailable in Europe by Fall 2007 (Oehen et al., 2011). As of 2020, the main manufacturers of vitamin B_2 were BASF, DSM, Hubei Guangji Pharmaceuticals, and Shanghai Acebright Pharmaceuticals (formerly Desano), with more than 70% of all production going to the animal feed market (Averianova et al., 2020).

The microorganisms that are currently used to manufacture riboflavin on an industrial scale for human food and animal feed include the bacteria *Bacillus subtilis,* and the yeasts *Ashbya gossypii, Saccharomyces cerevisiae,* and *Candida famata* (Averianova et al., 2020; Hohmann, Bretzel, et al., 2020; Revuelta et al., 2017; Stahmann, 2011). These production strains were all genetically modified using recombinant DNA techniques to increase riboflavin production and yield (Averianova et al., 2020; Hohmann, Bretzel, et al., 2020; Schwechheimer et al., 2016).

Genetically modified *B. subtilis* was reported to account for most of the commercial production of vitamin B_2 (Kirchner et al., 2014). Genetically modified *A. gossypii* also produces a significant amount of commercial production (Schwechheimer et al., 2016).

A series of patents show the various ways in which *B. subtilis* was genetically modified to overexpress riboflavin. The earliest patents began with strains of *B. subtilis* that had been modified by induced mutation to overproduce riboflavin. The inventors amplified the genes responsible for overproduction through rDNA techniques using *Escherichia coli* as the intermediate organism (Hohmann et al., 1998; Perkins et al., 1999). The vitamin industry continues to improve *B. subtilis* yield and overproduction, while lowering costs through various rDNA techniques (Averianova et al., 2020; Hohmann, Bretzel, et al., 2020; Kirchner et al., 2014; Revuelta et al., 2017).

The lack of transparency makes it difficult to determine which vitamins are produced by excluded methods. Riboflavin from unauthorized genetically modified *B. subtilis* appeared in the European market in 2014 (Barbau-Piednoir et al., 2015). The U.S. does not require comparable premarket notification and approval, and has less oversight over genetically engineered microorganisms than the E.U., in general (Hanlon & Sewalt, 2021). As a result, it is difficult to verify whether or not excluded methods are being used with any specific supply chain of vitamin.

Before the introduction of genetically modified *B. subtilis*, riboflavin was produced by mutant strains of *A. gossypii* (Kato & Park, 2012; Stahmann et al., 2000). Researchers published the genomic sequencing of a riboflavin over-producing mutant strain of *A. gossypii* in 2004, making genetic manipulation technically feasible (Karos et al., 2004). Researchers began to reach their limits using induced mutagenesis to increase riboflavin yields in *A. gossypii* (Averianova et al., 2020). As *A. gossypii* production lost market share to recombinant *B. subtilis*, developers began to employ various bioengineering strategies such as genetically engineered deletions, insertions, and substitutions (Averianova et al., 2020).

Three specific strategies that researchers pursued for both *B. subtilis* and *A. gossypii* were (Schwechheimer et al., 2016):

- Overexpression by amplifying the sequence responsible for increasing identified pathways for riboflavin overproduction.
- Disruption or knock-out of genes that inhibited the biosynthesis of a precursor to riboflavin.
- Underexpression of a genetic sequence that produces a catalyst for conversion.

These techniques increased yields over mutated strains by between 1.4 times to over a 10-fold increase (Schwechheimer et al., 2016). Genetically engineered strains have completely replaced wild-type and mutated strains as a result of their productivity advantage and lower costs (Averianova et al., 2020).

BASF submitted a dossier supporting the safety and efficacy of riboflavin produced by the genetically modified strain of *A. gossypii* DSM 23096 to the European Food Safety Authority's Panel on Additive or Substances used in Animal Feed (EFSA FEEDAP et al., 2018a). The specific information on the original host organism, the donor organism, the genetic modification process, and manufacturing process—presumably including the growth media—were all redacted from the EFSA's publicly available documents (EFSA FEEDAP et al., 2018a).

Specific information on fermentation media used for commercial production strains is often not publicly available and appears to be a proprietary trade secret. The literature generally reports that genetically modified *B. subtilis* used to overproduce riboflavin is grown experimentally in laboratory conditions using glucose, fructose, or molasses as the main energy source (Averianova et al., 2020). Glucose and fructose are made from the corn wet milling process (Rausch et al., 2019). Soy protein hydrolysate is used in some fermentation media as a protein source (Averianova et al., 2020). Most soybeans grown in the U.S. are genetically engineered (USDA Economic Research Service, 2023). Various amino acids are also used in the fermentation media (Averianova et al., 2020). It is not clear whether these amino acids are chemically synthesized, isolated from natural sources, or produced by organisms genetically modified by excluded methods.

Fermentation (or synthesis methods) using allowed methods

Timeline:

- The first microorganism used to produce commercial riboflavin from fermentation was *Clostridfum acetobutylicum* (Meade et al., 1945, 1947; Stahmann et al., 2000). Prior to this, riboflavin was chemically synthesized.
- *Eremothecium ashbyi* and *A. gossypii* soon displaced *C. acetobutylicum* as the main production organisms (Piersma, 1946; Stahmann et al., 2000; Tanner et al., 1948).
- *A. gossypii* became the preferred production organism because *E. ashbyi,* was genetically unstable (Stahmann et al., 2000). Fermentation with classically improved *A. gossypii* competed with chemical methods for the riboflavin market between 1946 and 1968 (Stahmann et al., 2000).
- In 1974, Merck began production of riboflavin with *A. gossypii* strains developed by mutagenesis (Revuelta et al., 2017).
- Researchers completed genomic sequencing of *A. gossypii* in 2004 (Dietrich et al., 2004).
- The sequencing along with various other molecular tools—including electro-transformation, recycling selectable markers, regulatory promoters, and insertional mutagenesis—made it feasible for research teams and manufacturers to improve production through genetic engineering, with a ten-fold increase in yield reported by 2006 (Revuelta et al., 2017).
- Strains that were genetically modified were scaled up rapidly and have completely displaced classically selected riboflavin producing organisms (Averianova et al., 2020; Schwechheimer et al., 2016).

Biofermentation with classically selected microorganisms is technically feasible but not currently cost-competitive with genetically engineered strains (Averianova et al., 2020). We were unable to verify that any primary manufacturer is making vitamin B_2 with such strains.

The scientific literature reported using similar fermentation media for wild-type, mutated, and genetically engineered strains (Averianova et al., 2020; Schwechheimer et al., 2016).

Other sources

Riboflavin was first synthesized on a laboratory scale by 1935 (Eggersdorfer et al., 2012; Hohmann, Bretzel, et al., 2020; Yoneda, 2000). The original Kuhn and Karrer process involved D-ribose, 3,4-methylaniline, and barbituric acid with a yield of about 48% (Hohmann, Bretzel, et al., 2020). Total chemical synthesis based on the condensation of xylol and uracil rings was the prevailing manufacturing process from the mid-1930s to the late-1980s (Friedrich, 1988). The maximum yields for total chemical synthesis was about 60%, compared with about 80% by microbial fermentation with genetically modified *B. subtilis* (Chu et al., 2022; Eggersdorfer et al., 2012). Total chemical synthesis produced more industrial waste than fermentation (Averianova et al., 2020; Chu et al., 2022). The microbial production of D-ribose by *B. subtilis* was a key step towards replacing total chemical synthesis with production of microbial precursors (Hohmann, Bretzel, et al., 2020; Sasajima et al., 1976).

By the mid-1990s, total chemical synthesis was no longer cost-competitive with production of riboflavin using genetically modified microorganisms. BASF ended synthetic production in 1996 after bringing a fermentation plant online that used genetically modified organisms to produce riboflavin (Averianova et al., 2020). Subsequent attempts to create a market for synthetic non-GMO vitamin B_2 in Europe were unsuccessful as of 2011 (Oehen et al., 2011). We found no comparable attempt made for the North American market.

We found no other commercial sources of riboflavin (B_2) that could be independently verified as made without excluded methods.

<u>Vitamin B_3</u>

Common names: Niacin, nicotinic acid; nicotinamide.
IUPAC names: Pyridine-3-carboxylic acid (niacin); pyridine-3-carboxamide (nicotinamide)
Other names: Enduramide; nicobion.
CAS numbers: 59-67-6 (nicotinic acid); 98-92-0 (nicotinomide)
EC numbers: 200-441-0 (nicotinic Acid); 200-659-6 (nicotinamide)
International Feed Numbers: 7-03-219 (nicotinic acid); 7-03-215 (nicotinamide)
FDA GRAS: 21 CFR 582.5530 (nicotinic acid); 21 CFR 582.5535 (nicotinamide)
Provitamins: None identified.
Alternate forms: Menadione nicotinamide bisulfite (MNB).
Molecular formula: $C_6H_5NO_2$ (Nicotinic acid), $C_6H_5NO_2$
Picture of molecular structure:

Figure 5: Molecular structure of niacin (vitamin B_3). Taken from US NLM, 2023.

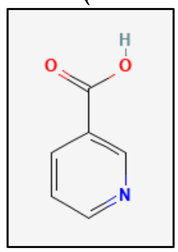
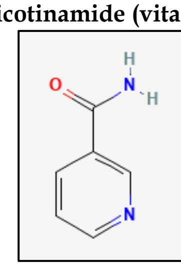


Figure 6: Molecular structure of nicotinamide (vitamin B_3). Taken from US NLM, 2023.



Manufacturing process

Chemical synthesis is the prevailing industrial method used to produce vitamin B_3. In 2014, the main manufacturer of niacin and nicotinamide was the Swiss company Lonza, with over half the market share for nicotinic acid, and about a third of the market share for nicotinamide (Blum, 2015). Both originate from the same petrochemical feedstocks of ammonia and various aldehydes.

Lonza also makes nicotinamide by (Blum, 2015):

1. Ammoxidation of 3-methylpyridine in the presence of a catalyst to make 3-cyanopyridine.
2. The 3-cyanopyridine is converted to nicotinamide by an enzymatic biocatalyst produced by an immobilized microorganism of the genus *Rhodococcus*. It is not a fermentation process because a live organism is not present.

The patent does not specify whether any of the *Rhodococcus* species used in the process have been genetically modified and they all appear to be naturally occurring strains (Heveling et al., 1998).

In another process, nicotinamide is made as follows (Blum, 2015):

1. 2-Methylglutaronitrile is converted to 2-methyl-1,5-diaminopentane.
2. Cyclic hydrogenation gives 3-methylpiperidine.
3. This is then dehydrogenated to 3-methylpyridine.
4. The 3-methylpyridine is ammoxidated and partly hydrolyzed to form nicotinamide.

Agricultural sources

Agricultural sources of niacin include whole grains and milling products that include bran and germ, such as rice bran, wheat bran, and corn gluten meal (Maynard & Loosli, 1956; McDowell, 2000).

Rice is a target crop for niacin biofortification using CRISPR genome editing technologies (Minhas et al., 2018).

We found no evidence that any commercial sources of nicotinamide or nicotinic acid are being isolated from agricultural sources that use excluded methods.

Fermentation (or synthesis methods) using excluded methods
One patented process to produce nicotinic acid involves a strain of *Escherichia coli* that has been genetically modified by rDNA techniques to increase production of quinolinic acid (Kim et al., 2016). The quinolinic acid is then decarboxylated to form nicotinic acid. The patent was assigned to CJ CheilJedang Corp (CJ Bio) in Korea (Kim et al., 2016). We were unable to determine whether excluded methods, such as the ones described in the patent above are currently used in the commercial industrial production of nicotinic acid.

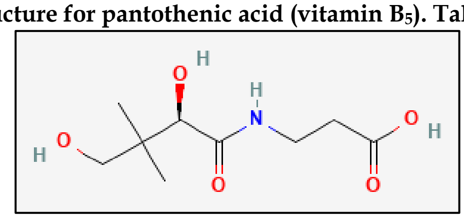
Fermentation (or synthesis methods) with allowed methods
Various fungal and bacterial cultures used to grow tempeh from soybeans can produce B-complex vitamins, including nicotinic acid and nicotinamide (Denter & Bisping, 1994). Various bacterial species used to produce nicotinic acid on a laboratory scale include *Escherichia coli* and *Brevibacterium ammoniagenes* (Chand & Savitri, 2016). *Lactobacillus* spp. and *Citrobacter freundii* have produced laboratory amounts of both nicotinamide and nicotinic acid. Natural strains have relatively low yields (Chand & Savitri, 2016). We found no evidence that nicotinic acid or niacinamide are produced by fermentation with allowed methods.

Other sources
Yeast and fish meal are feed ingredients high in niacin (McDowell, 2000). We found no evidence that excluded methods are currently used to produce yeast or fish meal as additives or from any other possible source.

Vitamin B_5

Common names: Pantothenic acid, D-pantothenic acid.
IUPAC name: 3-[[(2R)-2,4-dihydroxy-3,3-dimethylbutanoyl]amino]propanoic acid
Other names: Chick anti-dermatitis factor; CalPan; Calpanate.
CAS number: 79-83-4
EC number: 201-229-0
International Feed Numbers: 7-07-079 (calcium pantothenate); 7-01-229 (choline pantothenate)
FDA GRAS: 21 CFR 582.5212 (calcium pantothenate); 21 CFR 582.5772 (sodium pantothenate); 21 CFR 582.5580 (D-pantothenyl alcohol).
Provitamins: None identified. Some sources list panthenol as a provitamin, but it is considered a vitamin by FDA, AAFCO, and most livestock feed textbooks.
Alternate forms: Calcium pantothenate; choline pantothenate; D-pantothenyl alcohol (panthenol).
Molecular formula: $C_9H_{17}NO_5$
Picture of molecular structure:

Figure 7: Molecular structure for pantothenic acid (vitamin B_5). Taken from US NLM, 2023.



Manufacturing process
Pantothenic acid and its salts can be produced by either chemical synthesis or microbial fermentation (Müller et al., 2019). Synthetic pantothenic acid is made from the reaction of calcium ß-alanine with pantolactone at an elevated temperature. The resulting pantothenic acid can be crystalized as calcium pantothenate or sodium pantothenate in an alcohol solution (Müller et al., 2019).

Agricultural sources
Pantothenic acid is widely distributed in both plants and animals (Combs, 2012; Friedrich, 1988; McDowell, 2000). Feed milling by-products such as rice bran, wheat middlings, and corn gluten meal are excellent sources of vitamin B_5 in feedstuffs (Maynard & Loosli, 1956; McDowell, 2000). Proper preparation before storage of feedstuffs is important to conserve and optimize the effective vitamin B_5 content (Friedrich, 1988; McDowell, 2000).

Fermentation (or synthesis methods) with excluded methods
There are several patented microbial fermentation processes to make pantothenic acid from genetically modified microorganisms (Müller et al., 2019). The preferred production organism is *B. subtilis. E. coli* may also be genetically engineered through rDNA techniques to overproduce pantothenic acid and its intermediates (Yocum et al., 2002). The patents have been licensed to BASF (Müller et al., 2019).

Other microorganisms known to have encoding genes to produce pantothenate synthetase (PS)—the enzyme responsible for catalyzing the reaction of the precursors D-pantoic acid and ß-alanine—include *Cornyebacterium glutamicum, Bacillus thuringiensis, Bacillus cerus,* and *Enterobacter cloacae* (Tigu et al., 2018). The pantothenic acid produced from *E. coli* that had been genetically modified to amplify the *C. glutamicum* PS-encoding genes is believed to be the highest level reported by a fermentation process (Tigu et al., 2018).

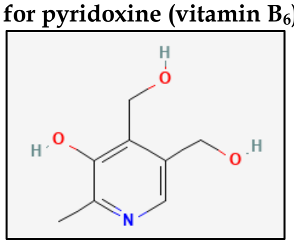
We were unable to obtain information that indicated whether a fermentation process using any of the experimental organisms made with excluded methods has been scaled up for commercial production.

Fermentation (or synthesis methods) with allowed methods
Researchers found *B. subtilis* to be the most efficient producer of pantothenic acid and its salts (Müller et al., 2019). Wild-type strains excreted less than 1 mg/L into the culture medium. Increasing yields required substantial genetic engineering. There is no evidence that any pantothenic acid or its salts are produced by fermentation with allowed methods.

Other sources
Yeast is a source of vitamin B_5. We found no other commercial sources that involved either allowed or excluded methods.

<u>Vitamin B_6</u>
Common name: Pyridoxine.
IUPAC name: 4,5-bis(hydroxymethyl)-2-methylpyridin-3-ol.
Other names: Pyridoxin; gravidox; hydoxin; adermin; bezatin.
CAS number: 65-23-6
EC number: 200-603-0
International Feed Numbers: 7-03-822
FDA GRAS: 21 CFR 582.5695 (pyridoxine hydrochloride)
Provitamins: None identified.
Alternate forms: Pyridoxine hydrochloride.
Molecular formula: $C_8H_{11}NO_3$
Picture of molecular structure:

Figure 8: Molecular structure for pyridoxine (vitamin B_6). Taken from US NLM, 2023.



Manufacturing process

Historically, DSM (formerly Roche) has been the leading manufacturer of vitamin B_6 but has steadily lost market share to companies in China and elsewhere in Asia (Eggersdorfer et al., 2012). Other manufacturers include (Bonrath et al., 2020):

- Jiangxi Tianxin Pharmaceutical Co., Ltd.
- Xinfa Pharmaceutical Co., Ltd
- Hubei Huisheng Pharmaceutical Co., Ltd.
- Huazhong Pharmaceutical Co., Ltd.
- Shanghai Hegno Pharmaceutical Co.

Since the 1960s, all producers of pyridoxine use the Diels-Alder method of chemical synthesis. (Bonrath et al., 2020; Eggersdorfer et al., 2012). The main chemical ingredients are D,L-alanine, oxalic acid, and ethanol (Bonrath et al., 2020).

None of the precursors named in the literature were described as coming from excluded methods.

Agricultural sources

Vitamin B_6 is widely distributed in various feedstuffs, but data on its bioavailability to various farm animals is limited. With that said, deficiencies are rare and supplementation is not needed unless symptoms develop (McDowell, 2000; National Research Council, 2012). Ruminal bacteria produce sufficient B_6 in healthy ruminants (National Research Council, 2001). Diets containing whole grain, milling by-products that are rich in the B-complex vitamins, and oilseed meal such as sunflower meal or soybean meal are usually sufficient in most feed rations (Combs, 2012; Maynard & Loosli, 1956; McDowell, 2000; National Research Council, 2012).

Fermentation (or synthesis methods) with excluded methods

We found no evidence that any commercially produced vitamin B_6 is produced by fermentation of microorganisms made with excluded methods.

Fermentation (or synthesis methods) with allowed methods

We found no evidence that any commercially produced vitamin B_6 is produced by fermentation of microorganisms.

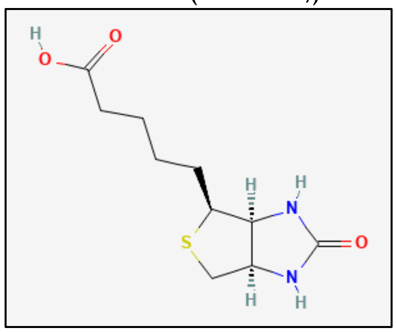
Other sources

None found.

Vitamin B_7

Common name: Biotin
IUPAC name: 5-[(3aS,4S,6aR)-2-oxo-1,3,3a,4,6,6a-hexahydrothieno[3,4-d]imidazol-4-yl]pentanoic acid.
Other names: Factor S; biodermatin, ritatin; meribin, coenzyme R; lutavit H2, rovimix H2, vitamin H.
CAS number: 58-58-5
EC number: 200-399-3
International Feed Numbers: 7-00-723
FDA GRAS: 21 CFR 582.5159
Provitamins: None identified.
Alternate forms: None.
Molecular formula: $C_{10}H_{16}N_2O_3S$
Picture of molecular structure:

Figure 9: Molecular structure of biotin (vitamin B_7). Taken from US NLM, 2023.



Manufacturing process

Most biotin is now manufactured in China, with the primary producers being (Bonrath et al., 2022):

- Zhejiang Shengda Bio-pharm Co., Ltd.
- Zhejiang NHU Pharmaceutical Co., Ltd.
- and Xinchang Pharma ZMC

Historically, DSM (formerly Hoffmann-La Roche) is the leading manufacturer, and remains a significant producer (Bonrath et al., 2022). Most industrial processes used to produce biotin are based on the chemical synthesis invented by Goldberg and Sternbach (Goldberg & Sternbach, 1949a, 1949a, 1949b).

Agricultural sources

Biotin is found in most plant and animal sources, but feed quality varies (Maynard & Loosli, 1956; McDowell, 2000). Biotin deficiency is rare. Animals treated with antibiotics may need an extra supply of biotin, but otherwise supplementation is seldom needed (Cherian, 2020). Grains are relatively poor sources, while alfalfa, soy, and other legumes are more favorable to meet biotin requirements (McDowell, 2000; Morrison, 1951). Both soybeans and alfalfa may be produced with genetic engineering (USDA Economic Research Service, 2023). We found no evidence of commercial production of biotin isolated from crops grown using either allowed or excluded methods.

Fermentation (or synthesis methods) with excluded methods

Biotin is one of the B-complex vitamins targeted for biological production (Wronska, 2022). Research is still in the early stage. We found no sources of biotin manufactured by fermentation with excluded methods.

Fermentation (or synthesis methods) with allowed methods

Existing fermentation organisms are not competitive with chemical manufacturing processes (Wronska, 2022). We found no sources manufactured by fermentation with allowed methods or any evidence of research that seeks to produce biotin on an industrial scale by classical fermentation methods that would be allowed under the NOP.

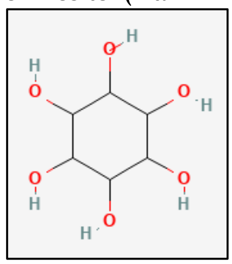
Other sources

We found no other sources.

Vitamin B_8

Common name: Inositol.
IUPAC name: Cyclohexane-1,2,3,4,5,6-hexol
Other names: Myoinositol; *myo*-Inositol; IP-6; i-Inositol; meso-Inositol; scyllo-inositol; cyclohexanol; (1R,2S,3r,4R,5S,6s)-1,2,3,4,5,6-Cyclohexanehexol
CAS number: 87-89-8
EC number: 230-024-9
International Feed Numbers: 7-09-354
FDA GRAS: 21 CFR 582.5370
Provitamins None identified.
Alternate forms: Inositol hexaphosphate (IP-6).
Molecular formula: $C_6H_{12}O_6$
Picture of molecular structure:

Figure 10: Molecular structure of inositol (vitamin B_8). Taken from US NLM, 2023.



Manufacturing process

Inositol is found in all plants and animals (McDowell, 2000). Corn processed by wet milling is historically a major industrial source of *myo*-Inositol (Hull & Montgomery, 1995; NOP, 2015b; US NLM, 2023). It can also be made from yeast in a fermentation process (Shirai & Yonehara, 1997). D-chiro-inositol can also be made from the antibiotic kasugamycin, which is a fermentation product (Kennington et al., 1992).

Agricultural sources

The predominant method to manufacture *myo*-inositol is from corn steep liquor obtained as a co-product of the wet milling process (NOP, 2015b). Most corn produced in the U.S. has been genetically engineered (USDA Economic Research Service, 2023). Various processes to isolate a purified inositol from corn have been used since the 1930s (Bartow & Walker, 1938).

The manufacturing process can be summarized as follows (Artz & Hach, 1952; Bartow & Walker, 1938; Elkin & Meadows, 1947; Thomas, 1948):

1) Corn is wet milled using various industrial processes that include sulfites, enzymatic reactions, and other synthetic and non-synthetic chemicals to produce the main products of oil, meal used as a protein and fiber source in livestock feed, and starch.
2) The process also creates a corn steep liquor of by-products that contain phytic acid. The phytic acid is recovered from the corn steep liquor by various means using enzymes and ion exchange.
3) The phytic acid is hydrolyzed in an acid, often sulfuric or hydrochloric acid, and then reacted with an alkaline solution, often calcium hydroxide to precipitate the inositol.
4) The precipitate has the lime and other impurities removed and is then ready for use.

Inositol is also found in lecithin, which is obtained from many different agricultural sources including as corn and soybeans (Schoeppe, 2021). Most soybeans grown in the U.S. have been genetically engineered (USDA Economic Research Service, 2023). Inositol may also be extracted from defatted rice bran (International Formula Council, 2011; NOP, 2012). While there is an abundant literature on the genetic modification of rice by excluded methods, we found no evidence that any rice grown to produce inositol uses such techniques, but also cannot rule out the possibility.

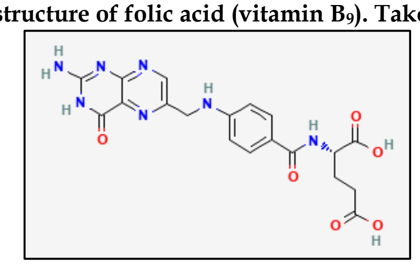
Fermentation (or synthesis methods) with excluded methods
Researchers are exploring several different microbial hosts as production organisms for overproducing inositol and phytic acid to replace plant sources (Borgi et al., 2015). We found no evidence that any are used to commercially produce inositol.

Fermentation (or synthesis methods) with allowed methods
Inositol can be made by fermentation of the yeast *Candida boidinii* (Shirai & Yonehara, 1997). There is no indication that any excluded methods were used. We found no evidence that any commercial production of inositol uses this yeast fermentation method.

Other sources
Pharmaceutical grade D-chiro-inositol can be made from kasugamycin (Kennington et al., 1992). Kasugamycin is a fungicidal antibiotic derived from a fermentation product of *Streptomyces kasugaensis* (Krieger, 2010). Scientist have genetically modified *Streptomyces lividans* and *Rhodococcus erythropolis* to express kasugamycin and increase yields (Kasuga et al., 2017). The target market is the pharmaceutical industry for human consumption. While it is possible that some product that does not meet pharmaceutical grade is diverted to the livestock feed market, we found no evidence that is occurring.

<u>Vitamin B$_9$</u>
Common name: Folic acid.
IUPAC name: (2S)-2-[[4-[(2-amino-4-oxo-3H-pteridin-6-yl)methylamino]benzoyl]amino]pentanedioic acid
Other names: Folate; folacin; vitamin B; vitamin M; (2S)-2-[(4-{[(2-Amino-4-oxo-1,4-dihydro-6-pteridinyl)methyl]amino}benzoyl)amino]pentanedioic acid– N -[4-<(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L -glutamic acid.
CAS number: 59-30-3
EC number: 200-419-0
International Feed Numbers: 7-02-066
FDA GRAS: 21 CFR 582.5676
Provitamins: None identified.
Alternate forms: None identified.
Molecular formula: $C_{19}H_{19}N_7O_6$
Picture of molecular structure:

Figure 11: Molecular structure of folic acid (vitamin B$_9$). Taken from US NLM, 2023.



Manufacturing process
The major producers of folic acid are the companies DSM, Changzhou Xinhong, Jiangxi Tianxin, Zhejiang Shengda, and Sri Krishna (Mair et al., 2019). All commercial folic acid and folates are chemically synthesized. Several different industrial processes are used. The original synthesis was by condensation of 2,5,6-triamino-4(3*H*)-pyrimidinone, *p*-aminobenzoyl-L-glutamic acid, and 2,3-dibromopropanol. These chemical feedstocks are still used with refinements to have more efficient production, increase yield, and enhance purity during the crystallization and purification steps of the process. Preparation of folic acid may involve the reactions of a wide variety of halogenated precursors and various aldehydes, include formaldehyde. About 75% of global production is used as a livestock feed additive (Mair et al., 2019).

Agricultural sources
Folates are found in legumes, grains, and leafy greens (Combs, 2012; Micronutrient Information Center, 2023). Natural sources of folic acid are not economically viable given current technologies and market conditions (Mair et al., 2019). Biofortification researchers have targeted genetic engineering various food

plants to overexpress folate as a potential natural source (Bekaert et al., 2008). Rice has been genetically modified to produce higher levels of folates (Storozhenko et al., 2007). Farmers are reluctant to accept and adopt biofortified plants for socio-economic, institutional, psychological-cognitive, and agronomic reasons (Samuel et al., 2023). We found no evidence of commercial adoption or planting of such varieties or that any folic acid or other folate compounds are extracted from plants grown by excluded methods in the US feed supply chain.

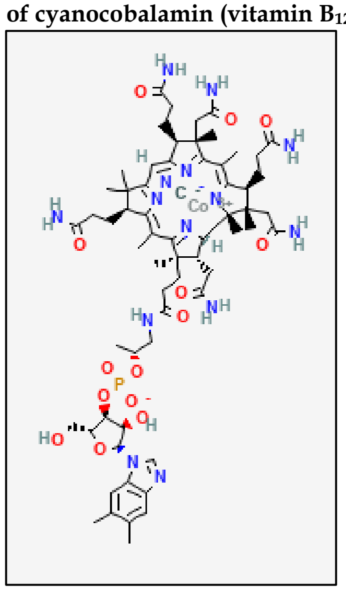
Fermentation (or synthesis methods) with excluded methods
Industrial production of folic acid or folates by the fermentation of microorganisms that have been genetically engineered is not yet economically viable (Mair et al., 2019).

Fermentation (or synthesis methods) with allowed methods
Industrial production of folic acid or folates by the fermentation of natural microorganisms that do not use excluded methods is not yet economically viable (Mair et al., 2019).

Other sources
We found no sources other than those listed above.

Vitamin B_{12}
Common name: Cobalamin
IUPAC name: Cobalt(3+); [(2R,3S,4R,5S)-5-(5,6-dimethylbenzimidazol-1-yl)-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl] [(2R)-1-[3-[(1R,2R,3R,5Z,7S,10Z,12S,13S,15Z,17S,18S,19R)-2,13,18-tris(2-amino-2-oxoethyl)-7,12,17-tris(3-amino-3-oxopropyl)-3,5,8,8,13,15,18,19-octamethyl-2,7,12,17-tetrahydro-1H-corrin-24-id-3-yl]propanoylamino]propan-2-yl] phosphate; cyanide.
Other names: Cyanocobalamin; vitamin B_{12} supplement
CAS number: 13408-78-1
EC number: 236-500-2
International Feed Numbers: 7-05-146
FDA GRAS: 21 CFR 582.5945
Provitamins: None identified.
Alternate forms: Vitamin B_{12} supplement.
Molecular formula: $C_{63}H_{88}CoN_{14}O_{14}P$
Picture of molecular structure:

Figure 12: Molecular structure of cyanocobalamin (vitamin B_{12}). Taken from US NLM, 2023.



Manufacturing process
More than 80% of the global production of vitamin B_{12} comes from China, with the leading manufacturers being Hebei Yuxing, Hebei, Huarong, and Ningia Kingvit (Hohmann, Litta, et al., 2020). The feed sector

accounts for about 30% of the market (Hohmann, Litta, et al., 2020). All industrial production of vitamin B_{12} is performed by fermentation (Burgess et al., 2009; Fang et al., 2017; Hohmann, Litta, et al., 2020). Manufacturing processes are explained in greater detail in the fermentation sections below. Cobalamin was first chemically synthesized in 1972 by a process that involved over 70 steps (Hohmann, Litta, et al., 2020; Martens et al., 2002). Manufacturers do not produce vitamin B_{12} by chemical synthesis because it is more expensive than fermentation (Burgess et al., 2009; Martens et al., 2002).

Agricultural sources

Cobalamin is produced *de novo* only by prokaryotes (Burgess et al., 2009; Fang et al., 2017). Animals store B_{12} in organ meats such as liver, and skim milk is another agricultural source (Combs, 2012). Sources of Vitamin B_{12} from slaughter by-products is prohibited in USDA organic production. We found no evidence of production of vitamin B_{12} from such sources.

Fermentation (or synthesis methods) with excluded methods

Pseudomona denitrificans—also known as *Ensifer adhaerens*—is the most important fermentation organism used to produce vitamin B_{12} on an industrial scale (Hohmann, Litta, et al., 2020). The other main species used in industrial production is *Proponibacterium shermanii,* sometimes classified as a subspecies of *Proponibacterium freudenreichii*. *Sinorhizobium meliloti* may also be used in industrial production (Fang et al., 2017). These have been developed by a combination of induced mutations and plasmid insertions that are excluded methods (Fang et al., 2017; Hohmann, Litta, et al., 2020).

The European Food Safety Authority Panel on Additives and Products or Substances used in Food Animals (EFSA FEEDAP) has reviewed vitamin B_{12} from various strains of *P. denitrificans*/*E. adhaerens* for safety and efficacy (EFSA FEEDAP, 2015; EFSA FEEDAP et al., 2018b, 2020, 2023). Most are declared to be genetically engineered according to the EU definition and thus produced from excluded methods (EFSA FEEDAP et al., 2018b, 2020, 2023). These reports redacted specific information on the genetic modification techniques used and production process, presumably including the fermentation media recipe (EFSA FEEDAP et al., 2018b, 2020, 2023).

Various recombinant strains of *P. freudenreichii* that were genetically modified by recombinant methods to increase the expression vectors of vitamin B_{12} precursors were able to increase production by half or over double (Piao et al., 2004).

The long fermentation cycles, complex and expensive media requirements, and the difficulty of genetically modifying the current host species have led researchers to focus on *E. coli* as the preferred host organism to genetically engineer for vitamin B_{12} production (Fang et al., 2017). We found no evidence that such systems are currently scaled up for industrial production of vitamin B_{12}.

Fermentation media used for the commercial production of vitamin B_{12} is proprietary and was redacted from regulatory documents (EFSA FEEDAP, 2015; EFSA FEEDAP et al., 2018b, 2020, 2023). Experimental literature lists the main component of most *P. denitrificans* as sucrose, with betaine also added (Martens et al., 2002). Both can come from sugar beets, much of which is genetically modified (USDA Economic Research Service, 2023). The main component of fermentation media for *P. freudenreichii* is glucose (Martens et al., 2002). Corn is a major source of glucose (Rausch et al., 2019). Most corn grown in the U.S. has been genetically modified (USDA Economic Research Service, 2023).

Fermentation (or synthesis methods) with allowed methods

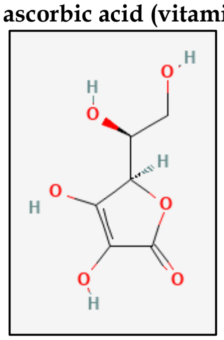
Various naturally occurring strains of bacteria were historically used to manufacture vitamin B_{12} and could conceivably be used at present. One *E. adhaerens* strain evaluated by EFSA FEEDAP was claimed by its applicant not to be genetically modified despite its resistance to 14 antibiotics of human and animal significance (EFSA FEEDAP et al., 2020). The panel concluded that the vitamin B_{12} was safe because it had no live production organisms or recombinant DNA detected in the most concentrated form of the additive (EFSA FEEDAP et al., 2020). While it is possible that some vitamin B_{12} is made using allowed methods, the specific strains are being used in industrial production in China are unknown (Hohmann, Litta, et al., 2020). In our opinion the vitamin supply chain would require greater transparency and traceability to be able to verify claims that excluded methods are not being used to produce any vitamin B_{12}.

Other sources
Vitamin B_{12} is present in the naturally occurring microorganisms used to make tempeh from soybeans (Denter & Bisping, 1994). While tempeh may be used as an animal feed in some local markets, we did not find information indicating that such a source is being used as an animal feed on a routine basis.

Feed grade vitamin B_{12} can be isolated from sewage sludge (Miner & Bernard, 1953). We found no evidence that any commercial sources of vitamin B_{12} currently on the market are produced by this method.

<u>Vitamin C</u>
Common name: Ascorbic acid.
IUPAC name: (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one
Other names: L-ascorbic acid; ascoltin; erythorbic acid; isoascorbic acid; D-ascorbic acid; hybrin; magnorbin.
CAS number: 50-81-7
EC number: 200-06-23
International Feed Numbers: 7-00-433 (ascorbic acid); 7-09-823 (erythorbic acid/iso-ascorbic acid)
FDA GRAS: 21 CFR 582.3041
Provitamins: Pubchem reports 2-keto-L-gulonic acid (2-KGA) to be provitamin C.
Alternate forms: L-ascorbyl—2-polyphosphate; L-Ascorbyl-2-sulfate; calcium ascorbate; calcium L-ascorbyl-2-monophosphate; erythorbic acid/iso-ascorbic acid; magnesium L-ascorbyl-2-phosphate; sodium ascorbate.
Molecular formula: $C_6H_8O_6$
Picture of molecular structure:

Figure 13: Molecular structure of ascorbic acid (vitamin C). Taken from US NLM, 2023.



Manufacturing process
Ascorbic acid can be chemically synthesized, produced by fermentation of naturally occurring microorganisms, and extracted and isolated from agricultural sources (Elste et al., 2020). We are including descriptions of some experimental sources where commercialization may soon be feasible or perhaps already in production. The survey is not exhaustive, and manufacturers may use methods other than the ones reported in the published literature. Elste *et al.* identify eight different basic routes for the industrial production of vitamin C, other than recovery from agricultural sources (Elste et al., 2020):

1. Chemical synthesis using the Reichstein process.
2. The two-step process using 2-keto-L-gulonic acid (2-KGA) produced by fermentation.
3. D-gluconic acid routes by fermentation with organisms like *Erwinia* spp. or *Corynebacterium* spp.
4. Direct vitamin C fermentation of with *Gluconobacter* spp.
5. Yeast fermentation with genetically engineered *Saccharomyces cervisiae.*
6. D-galacturonic acid route fermentation with *Aspergillus* spp.
7. Chemical synthesis using D-glucuronic acid route.
8. Microalgae fermentation with *Chlorella* spp.

Manufacturing processes for each of these sources are explained in further detail in the sections below. Most current commercial processes used to produce vitamin C involve a combination of chemical synthesis steps and the use of genetically engineered microorganisms (Elste et al., 2020; Yang & Xu, 2016).

Vitamin C is by far the vitamin imported in the greatest volume by the U.S. (Shurson & Urriola, 2019). About 15-20% of all vitamin C made is used for animal production (Elste et al., 2020). The vitamin supply chain for the U.S. feed industry is complex and difficult to trace (Shurson & Urriola, 2019). Feed mills and premix manufacturers purchase vitamin C and other vitamins from domestic distributors rather than directly from the primary manufacturers (Shurson & Urriola, 2019) Most distributors purchase vitamins from multiple sources based on current supply and demand conditions. Sources, and therefore the manufacturing process used to make the ingredient, can vary. Traceability is a concern not just for organic, but also for conventional producers (Shurson & Urriola, 2019).

<u>Reichstein process</u>

From the late 1930s to the early 1970s, most industrially produced ascorbic acid was produced by total chemical synthesis that followed the steps of a process published by Reichstein and Grüssner in 1934 (Eggersdorfer et al., 2012; Elste et al., 2020; Kuellmer, 2001; Reichstein & Grüssner, 1934; Yang & Xu, 2016). The Reichstein process began to be phased out in the 1990s in favor of fermentation processes. Almost all vitamin C is currently produced by fermentation (Elste et al., 2020; Yang & Xu, 2016).

Agricultural sources

Prior to the invention of the Reichstein process, ascorbic acid was isolated, concentrated, and crystallized from plant sources (Friedrich, 1988). Agricultural sources of vitamin C include acerola (*Malpighia emarginata*), citrus and other fruits, rose hips, and solanaceous and other vegetables (Combs, 2012; Micronutrient Information Center, 2023). A small amount of natural vitamin C is commercially produced from acerola and rose hips from *Rosa roxburghii* (Elste et al., 2020). Other possible sources include plums, black currants, oranges, broccoli, beets, apples, strawberries, blueberries, and cranberries. The vitamin C content of such isolated and recovered sources is 10-15%, with different excipients (Elste et al., 2020). These are sold for human consumption and used in personal care products (Elste et al., 2020; Hahn, 2018). We found no evidence that any such natural, agricultural source of vitamin C is sold into the feed additive market.

Vitamin C is a priority trait for plant biofortification given its importance for animal and human health, and the size of the market. Strategies to increase vitamin C in food and feed crops include a combination of both allowed and those methods whose status are to-be-determined. The literature is vast, difficult to summarize, and offers no conclusive evidence that any of the proposed strategies to introduce vitamin C in the animal feed chain or directly into the human food supply are currently in commercial use.

Fermentation (or synthesis methods) with excluded methods

Inventors have developed several fermentation processes to produce vitamin C precursors using genetically modified microorganisms improved through excluded methods. The routes are generally identified by the substrate upon which the microorganisms are grown.

<u>Two-step process</u>

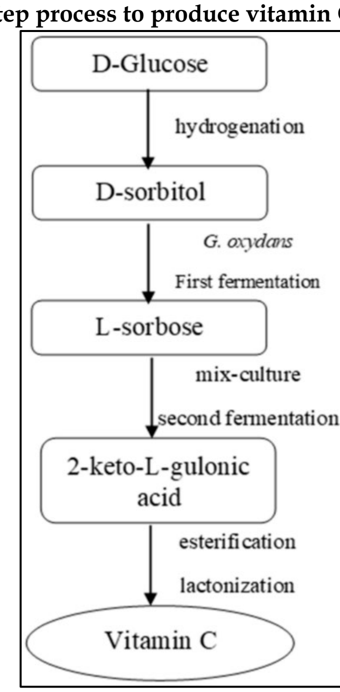
The two-step fermentation process has been the prevailing method of manufacturing since the late 1990s (Elste et al., 2020; Yang & Xu, 2016). The replacement of the Reichstein process coincided with the genetic improvement of microorganisms to increase production and yields of 2-KGA by direct fermentation. The two-step fermentation process now accounts for almost all vitamin C produced in the world (Elste et al., 2020; Yang & Xu, 2016). The two-step process is described in greater detail in both fermentation sections below.

Virtually all vitamin C on the world market as of the mid-2010s was produced by the two-step process that use strains of *Ketogulonigenium vulgare* that have been genetically modified to enhance production of 2-KGA from the fermentation of either L-sorbose or D-glucose (Cai et al., 2012; Yang & Xu, 2016). Much of the patent literature is in Chinese. It is beyond the scope of this report to translate and summarize the specific methods by which the various production organisms were genetically engineered. In some cases, the production organisms used appear to be protected as proprietary trade secrets and the specific genetic modifications performed are not always publicly available.

Fermentation cultures contain various other microorganisms, including *Bacillus megaterium, Bacillus subtilis, Bacillus thuringiensis, Bacillus cereus, Xanthomonas maltophilia,* and *Acetobacter* spp. (Elste et al., 2020; Yang & Xu, 2016). These may be classically selected, improved by induced mutation, or genetically modified (Elste et al., 2020; Yang & Xu, 2016). Both the species used and the methods by which they were selected and improved is often undisclosed and difficult to discover.

Figure 14 shows the two-step process used to manufacture vitamin C.

Figure 14: Flowchart of the two-step process to produce vitamin C. Taken from Tucaliuc, et al., 2022.



The state of the art to manufacture vitamin C has evolved rapidly since the 1990s when the first patents for genetically modified strains of 2-KGA producing microorganisms were granted (Elste et al., 2020; Yang & Xu, 2016). Producers continue to make improvements to fermentation microorganism strains used to produce 2-KGA through a combination of induced mutagenesis, rDNA techniques, and newer gene editing technologies (Tucaliuc et al., 2022).

<u>D-gluconic acid route</u>
The first application of genetic engineering to produce vitamin C by fermentation involved transgenic species of *Erwinia* and *Corynebacterium* that were genetically modified to overproduce vitamin C precursors on a substrate primarily composed of D-gluconic acid (Elste et al., 2020). Genentech and Biogen continue to research this route, but the results are still far from industrial production (Elste et al., 2020). We found no evidence that any commercial production comes from these genetically modified organisms.

<u>Direct fermentation</u>
Researchers at DSM invented a *Gluconobacter* strain by inserting a gene encoding L-sorbosone dehydrogenase (Elste et al., 2020). After the knockout of the 2-KGA forming enzyme, the genetically modified bacteria were able to yield 90% vitamin C directly rather than through a precursor. The direct fermentation route is reported as technically feasible (Elste et al., 2020). We found no evidence that this process has been scaled up or is in current production.

Yeast fermentation

Common brewer's yeast (*Saccharomyces cerevisiae*) offers an attractive platform to genetically modify as a host for fermentation production of vitamin C and its precursors. *Kluyveromyces* is another potential host platform that is the subject of research to genetically modify for yeast fermentation manufacturing of vitamin C (Elste et al., 2020). While technically feasible, we found no evidence that any vitamin C or its precursors are produced by genetically engineered yeast but cannot rule it out either.

Fermentation (or synthesis methods) with allowed methods

The two-step process originally relied on various naturally-occurring microorganisms discovered to directly produce 2-KGA through fermentation of L-sucrose as a substrate (Elste et al., 2020; Yang & Xu, 2016). However, yields with the naturally-occurring strains were low, and the industry prioritized and adopted genetic engineering strains that increased production many-fold over that of wild-type or naturally occurring strains and replaced production with classically selected strains (Cai et al., 2012).

The second step of the Reichstein process originally and historically used naturally-occurring fermentation organisms selected with allowed methods (Kuellmer, 2001; Reichstein & Grüssner, 1934). We were unable to confirm in the published literature that any specific industrial source still currently manufactures L-ascorbic acid in this way.

Microalgae process

It is technically feasible to produce vitamin C on an industrial scale using microalgae (Elste et al., 2020). *Chlorella* spp. have been the principal organisms of interest, but other genera, such as *Prototheca,* could be used. *Chlorella* can be produced agriculturally in pond culture or non-agriculturally through controlled fermentation. Most strain development for increased vitamin C expression occurs under controlled conditions and such strains may not be adapted to be scaled up for agricultural mass production. While it is possible to genetically modify *Chlorella* to increase production of vitamin C or its precursors, we found no mention of such a source in the literature. Genetic engineering of algae has bacteria and yeast as production hosts. We found no evidence that any commercial source of vitamin C comes from *Chlorella* or any other microalgae.

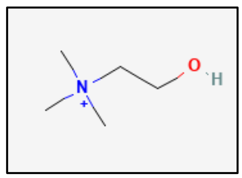
Other sources

Vitamin C has a wide variety of potential sources (Micronutrient Information Center, 2023). Given the size of the market and structure of the industry, intense research is devoted to develop other sources of vitamin C (Elste et al., 2020). We were unable to confirm whether specific sources are made from allowed or excluded methods. Such confirmations would require case-by-case and lot-by-lot review of each individual source of vitamin C.

<u>Choline</u>

Common name: Choline.
IUPAC name: 2-hydroxyethyl(trimethyl)azanium
Other names: Choline chloride (IFN 7-01-228).
CAS number: 62-49-7
EC number: 200-535-1
International Feed Numbers: 7-01-228 (choline chloride); 7-01-229 (choline pantothenate); 7-01-230 (choline xanthate), (choline bitartrate); 6-20-869 (cobalt choline citrate complex); 6-20-868 (copper choline citrate complex); 6-20-867 (ferric choline citrate complex); 7-01-230 (choline xanthate).
FDA GRAS: 21 CFR 582.5250 (choline bitartrate); 21 CFR 582.5252 (choline chloride).
Provitamins: None identified.
Alternate forms: Choline pantothenate (IFN 7-01-229); choline xanthate (IFN 7-01-230); choline bitartrate; cobalt choline citrate complex (IFN 6-20-869); copper choline citrate complex (IFN 6-20-868); ferric choline citrate complex (IFN 6-20-867); choline xanthate (IFN 7-01-230).
Molecular formula: $C_5H_{14}NO^+$
Picture of molecular structure:

Figure 15: Molecular structure of choline. Taken from US NLM, 2023.



Manufacturing process
Choline is a quaternary ammonia product that may be produced by extraction from agricultural sources, prepared by total chemical synthesis, or a combination of the two processes. Various processes to manufacture choline salts have been patented as early as the 1950s (Blackett & Soliday, 1956; Meyer, 1952). The normal process is by the reaction of triethylamine, ethylene oxide, and water (Atwater, 2001; Callen, 2011). The reaction generates choline hydroxide, which is basic (Blackett & Soliday, 1956; Callen, 2011). Hydrochloric acid is added to the basic solution to neutralize and crystallize it as a salt (Blackett & Soliday, 1956). Choline chloride can also be produced by the reaction of trithethylamine and chlorohydrin (Atwater, 2001). Choline bitartrate is produced by adding tartaric acid instead of hydrochloric acid to the basic choline solution (Callen, 2011).

Feed-grade synthetic choline chloride is imported to the U.S. from China on corn cobs as the excipient/ carrier (Shurson & Urriola, 2019). The cobs may be from genetically modified corn.

Agricultural sources
Choline is found in lecithin, and that has been the historical source. Soybeans are the primary source of most commercial lecithin, with other agricultural sources, including corn, being used (Schoeppe, 2021). Most commodity corn and soybeans grown in the U.S. have been genetically engineered(USDA Economic Research Service, 2023).

Fermentation (or synthesis methods) with excluded methods
We found no reference in the literature on the commercial or experimental production of choline by fermentation of organisms made with excluded methods.

Fermentation (or synthesis methods) with allowed methods
We found no reference in the literature on the commercial or experimental production of choline by fermentation of organisms made with allowed methods.

Other sources
While not an alternate source of choline itself, AAFCO recognizes the crystalline or anhydrous forms of betaine as partial substitutes for choline (AAFCO, 2022) since betaine is the primary product of choline oxidation in humans and animals. Betaine is derived from sugar beets. Much of the sugar beet production

in the U.S. has been genetically modified (Fernandez-Cornejo et al., 2016; USDA Economic Research Service, 2023).

We found no reference in the literature on the commercial or experimental production of choline by fermentation of organisms made from other sources.

<u>Vitamin D</u>

Common name: Ergocalciferol (vitamin D_2); cholecalciferol (vitamin D_3).
IUPAC name: ((3S,5Z,7E,20R,22E,24R)-9,10-Secoergosta-5,7,10,22-tetraen-3-ol (vitamin D_2); (3S,5Z,7E)-9,10-Secocholesta-5,7,10-trien-3-ol (vitamin D_3)
Other names: Viosterol; egerone; deltalin; ercalciol; doxercalciferol.
CAS numbers: 50-14-6 (vitamin D_2); 67-97-0 (vitamin D_3)
EC numbers: 200-014-9 (vitamin D_2); 200-673-2 (vitamin D_3)
International Feed Numbers: 7-03-728 (vitamin D_2); 7-00-408 (vitamin D_3)
FDA GRAS: 21 CFR 582.5950 (vitamin D_2); 21 CFR 582.5953 (vitamin D_3)
Provitamins: Numerous sterols that are transformed to vitamin D are broadly considered to be precursors of vitamin D. References consider 7-dehydrocholesterol to be the most significant in animals. These substances are transformed into vitamin D in the presence of ultraviolet light.
Alternate forms: Cod liver oil; herring oil; salmon oil; tuna oil.
Molecular formula: $C_{28}H_{44}O$ (ergocalciferol); $C_{27}H_{44}O$ (cholecalciferol)
Picture of molecular structure:

Figure 16: Molecular structure of ergocalciferol (vitamin D_2). Taken from US NLM, 2023.

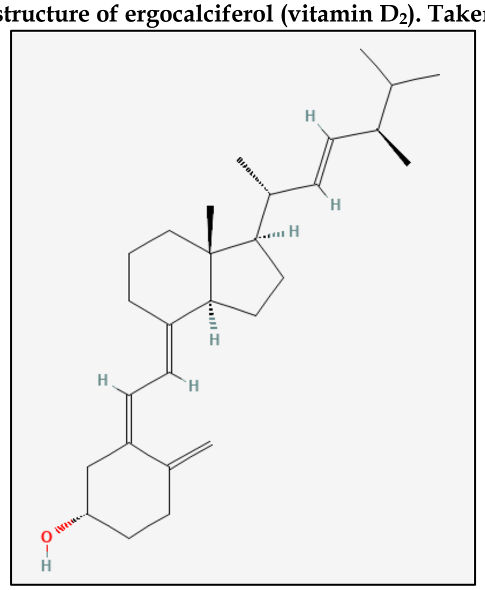
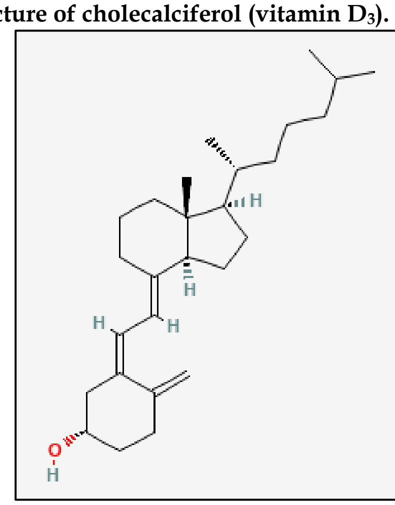


Figure 17: Molecular structure of cholecalciferol (vitamin D_3). Taken from US NLM, 2023.



Manufacturing process

Most commercially available vitamin D sold as a feed additive is manufactured by chemical synthesis (Bendik et al., 2019; Hirsch, 2000; Shurson & Urriola, 2019). A small amount of natural vitamin D is isolated from cod liver oil and other fish, but is primarily sold in vitamin supplements (Bendik et al., 2019).

The preferred source of vitamin D for animal feed is in the D_3 form. Poultry are able to process vitamin D_3, but D_2 has limited activity (National Research Council, 1994). Ruminants and swine are able to process vitamin D_2 made from irradiated yeast, but it fell out of favor as a feed additive many years ago (Hirsch, 2000).

About 80% of all vitamin D_3 is produced in China, with Europe and India each producing about 10% of the global supply (Shurson & Urriola, 2019). The main Chinese producers of feed-grade vitamin D are Zhejiang Garden, Zhejiang Medicine Co., Ltd. Xinfa Pharmaceutical Company, Zhejiang NHU Co., Ltd., Taizhou Hisound Chemical Co., Ltd., and Huazhong Pharmaceutical Co., Ltd. (Bendik et al., 2019; Shurson & Urriola, 2019) European producers include DSM, Solvay-Duphar, and Synthesia (Bendik et al., 2019).

The chemical synthesis process for cholcalciferol (D_3) involves the transesterification of 7-dehydrocholesterol with bromine, saponification, and treatment with ultraviolet light (Bendik et al., 2019) Vitamin D from China may be shipped in a porcine gelatin carrier, which has raised concerns about the African swine virus (Shurson & Urriola, 2019).

Agricultural sources

Ergocalciferol is formed only after the plant is harvested or otherwise injured and exposed to sunlight, and is not present in living plant cells (Cherian, 2020). Field hay that has been properly cured will have adequate vitamin D for ruminants, but not for poultry (Morrison, 1951). Alfalfa is a potential source of vitamin D, but none appears to be currently isolated. Alfalfa that is genetically modified for herbicide tolerance (HT) has been available to farmers in the U.S. since 2005 (Fernandez-Cornejo et al., 2016). While the adoption and planting of HT alfalfa has not been as widespread as that for corn, soybeans, cotton, canola, or sugar beets, a substantial amount of genetically engineered alfalfa is planted every year (Fernandez-Cornejo et al., 2016; USDA Economic Research Service, 2023).

Fermentation (or synthesis methods) with excluded methods

Brewer's yeast (*S. cerevisiae*) offers a promising platform to produce vitamin D (Kessi-Pérez et al., 2022). We found no sources of vitamin D produced by excluded methods.

Fermentation (or synthesis methods) with allowed methods

Irradiated yeast can produce vitamin D_2. We found no source of vitamin D that is produced by this method or meets this requirement.

Other sources

Livestock operations historically supplemented vitamin D from wild-caught fish, particularly cod liver oil (Combs, 2012; McDowell, 2000). AAFCO recognizes cod liver and other fish oils as a vitamin D source for livestock feed (AAFCO, 2022).

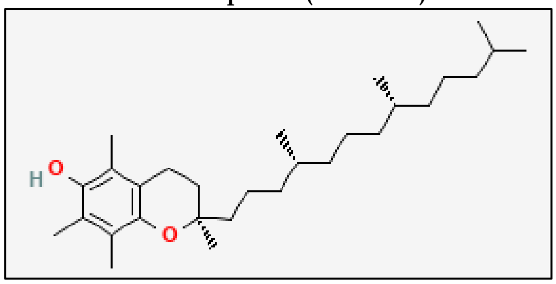
Vitamin D_2 can be produced by irradiating yeast (Hirsch, 2000). Other D-vitamins can be produced by the irradiation of various sterols (Bendik et al., 2019). Most of the literature refers to UV irradiation outside of the ionizing range prohibited by the NOP rule (7 CFR 205.105(f)). Sterols derived from animals may be derived from slaughter by-products (7 CFR 205.237(b)(5)). We found no evidence that any irradiated sources are still in production or are currently marketed.

Livestock and poultry can produce sufficient vitamin D through their skin when exposed to sunlight (Cherian, 2020; Combs, 2012; Maynard & Loosli, 1956; Morrison, 1951). Thus, outdoor access reduces or eliminates the need for dietary supplementation of vitamin D in animal feed (Maynard & Loosli, 1956).

<u>Vitamin E</u>

Common name: Tocopherol.
IUPAC name: (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol
Other names: α-tocopherol; ß-tocopherol; γ-tocopherol; δ-tocopherol; methyltocols; tocoferols.
CAS number: 59-02-9
EC number: 233-466-0
International Feed Numbers: 7-00-001 (tocopherol); 7-18-777 (α-tocopherol acetate)
FDA GRAS: 21 CFR 582.5890 (tocopherol); 21 CFR 582.5892 (α-tocopherol acetate).
Provitamins: None identified.
Alternate forms: α-tocopherol acetate.
Molecular formula: $C_{29}H_{50}O_2$
Picture of molecular structure:

Figure 18: Molecular structure of tocopherol (vitamin E). Taken from US NLM, 2023.



Manufacturing process

Vitamin E can either be isolated from natural plant sources or made in a synthetic form as α-tocopherol acetate (Bonrath et al., 2021). Global production of synthetic vitamin E was 75,000 tons/annum of synthetic tocopherol and 3,000 tons/annum vitamin E content from natural sources.[2] Use as an animal feed additive accounts for 85% of world consumption. Both natural and synthetic sources are used in animal feed, but the main animal feed additive product is a 50% adsorbate on a silica carrier. The synthetic form has higher purity, but lower biological activity. The main producers of natural vitamin E are ADM, Cargill, and AOM with smaller amounts made by Riken, ZMC, Vitae Naturals, and Matrix Fine Science. The main producers of synthetic α-tocopherol acetate are DSM, BASF, ZMC, Beisha, and Yimante (Bonrath et al., 2021).

Vitamin E was first synthesized in 1948 (Bonrath et al., 2021; Eggersdorfer et al., 2012). Total industrial synthesis of α-tocopherol acetate is based on a condensation reaction of 2,3,6-trimethylhydroquinone (TMHQ) with phytol, phytyl halides, or isophytol (Bonrath et al., 2021).

Catalysts used include *p*-toluenesulfonic acid, methanesulfonic acid, BF_3, $AlCl_3$, and $ZnCl_2$ (Bonrath et al., 2021; Eggersdorfer et al., 2012).

Synthetic vitamin E and natural sources of vitamin E are not identical and can be readily identified by analytical methods (Survase et al., 2006).

Agricultural sources

Agricultural sources of vitamin E are various oilseeds, including soybeans, safflower seeds, cottonseed, palm oil, and peanuts (Combs, 2012; McDowell, 2000; Morrison, 1951). Whole grains such as corn, wheat, and barley can contribute vitamin E to a feed ration (Maynard & Loosli, 1956; Morrison, 1951). Milling and bleaching significantly reduces the vitamin E content of feedstuffs (McDowell, 2000). Grazing animals can get adequate vitamin E from pasture and forage, but availability varies seasonally, with the highest levels occurring in early growth (Maynard & Loosli, 1956; McDowell, 2000; National Research Council, 2001). Alfalfa is rich in vitamin E (Maynard & Loosli, 1956).

[2] Reported as "t/a." We assume this is tons per annum.

Natural vitamin E is isolated and concentrated from plant sources on an industrial scale (Bonrath et al., 2021). The primary agricultural source is soybeans. The process for obtaining vitamin E from soybeans is as follows (Bonrath et al., 2021):

1) Soybeans are crushed and the oil is extracted either by cold-pressing or solvent extraction.
2) The oil is deodorized by distillation, removing various by-products, including 3-15% tocopherols.
3) The deodorizer distillates are treated with an alkaline solution.
4) The tocopherols are purified by one of several methods, including distillation, treatment with calcium chloride or hydrochloric acid, or esterified with excess fatty acids.
5) After removal of the free fatty acids, the tocopherols can be concentrated by adsorption to ion exchange resins.
6) The tocopherol solution is then methylated with formaldehyde under acidic conditions or various other catalysts.

Most soybeans produced in the U.S. have been genetically engineered (USDA Economic Research Service, 2023). Researchers are investigating ways to increase vitamin E content of agricultural crops through genetic engineering (Eggersdorfer et al., 2012).

Fermentation (or synthesis methods) with excluded methods

We found no process to manufacture vitamin E through fermentation using excluded methods.

Microbial production of vitamin E and the chemical precursors of tocopherol is an ongoing research priority for the vitamin industry (Eggersdorfer et al., 2012). None of the processes have been scalable to industrial production because of the complexity.

Fermentation (or synthesis methods) with allowed methods

We found no process to manufacture vitamin E through fermentation. Research on microbial production of vitamin E began in the 1970s using classical methods, but all the research summarized in the literature since around 2000 has been on the identification of pathways that can be genetically manipulated (Eggersdorfer et al., 2012).

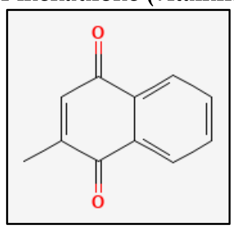
Other sources

Microalgae are considered a potential source of vitamin E (Durmaz, 2007). Production from such sources does not appear to be commercially feasible at present (Bonrath et al., 2021).

Vitamin K

Common name: Phylloquinone (K_1); menaquinone (K_2); menadione (K_3)
IUPAC name: 2-methylnaphthalene-1,4-dione; 2-methylnaphthoquinone.
Other names: Phylloquinone, menaquinone; the vitamin MK series (MK-1 to MK-15); vikasol, kaynone, juva-K; menaphthene.
CAS number: 58-27-5
EC number: 200-372-6
International Feed Numbers: 7-08-102(MPB); 7-03-078 (MSB)
FDA GRAS: 21 CFR 573.620 (MPB); 21 CFR 573.625 (MNB)
Provitamins: None identified. Some sources list menadione as a provitamin, but it is considered a vitamin by FDA, AAFCO, and most livestock feed textbooks.
Alternate forms: Menadione sodium bisulfite (MSB); menadione sodium bisulfite complex (MSBC); menadione dimethylpyrimidol bisulfite (MPB); menadione nicotinamide bisulfite (MNB).
Molecular formula: $C_{31}H_{46}O_2$ (menadione)
Picture of molecular structure:

Figure 19: Molecular structure of menadione (vitamin K). Taken from US NLM, 2023.



Manufacturing process

The preferred source of vitamin K used as feed is water-soluble menadione (K_3), with about 2,500 tons sold annually (Netscher et al., 2020). Vitamin K_3 is the simplest form and is readily ingested by monogastric animals (McDowell, 2000). Most vitamin K is fed to poultry and swine. Just under 80% of the global supply comes from China, with the rest of production divided between Europe and South America (Shurson & Urriola, 2019). The main Chinese producers are Mianyang Vanetta, Brother Enterprises, Chongqing Minfeng, and Yunan Luliang Peace (Netscher et al., 2020). Dirox in Uruguay is responsible for about 12% of the market (Shurson & Urriola, 2019). Oxyvit is the only vitamin K_3 producer in Europe (Oxyvit, 2023).

Typical manufacturing processes involve a combination of fermentation production of precursors followed by chemical synthesis, but total chemical synthesis is also possible (Netscher et al., 2020). Menaquinone is the primary precursor produced by fermentation (Berenjian et al., 2015). The fermentation process is discussed in the sections below.

The simplest and most common method of total chemical synthesis process to produce menadione (K_3) is to oxidize 2-methylnaphthelene with chromium (CrO_3) in acetic acid or $Na_2Cr_2O_7$ in sulfuric acid (Netscher et al., 2020). Because the process uses toxic and carcinogenic hexavalent chromium(VI) and generates large amounts of waste, the industry is actively looking for alternative processes that involve both chemical and biotechnological strategies (de Souza et al., 2022; Netscher et al., 2020). One review article identified 15 alternative processes for total chemical synthesis of menadione (de Souza et al., 2022). Alternative processes that use less toxic chemicals and generate lower volumes of waste have been challenging for manufacturers to scale up to industrial production (Netscher et al., 2020).

Menadione is complexed to make it stable while keeping it water soluble (Netscher et al., 2020). Vitamin K_3 for poultry feed is generally sold as menadione sodium bisulfite (MSB), menadione sodium bisulfite complex (MSBC), or menadione dimethylpyrimidol bisulfite (MPB) (McDowell, 2000; National Research Council, 1994; Shurson & Urriola, 2019). Menadione nicotinamide bisulfite (MNB) provides niacin (B_3) as well as menadione (K_3) (Oxyvit, 2023; Shurson & Urriola, 2019).

Agricultural sources

Green leaves are the best source of phylloquinones (K_1). Sun cured alfalfa hay has higher levels of vitamin K than dehydrated alfalfa meal, but both are suitable feed sources for poultry (McDowell, 2000). Bacteria in most mammals and all ruminants produce sufficient menaquinones (K_2) that supplementation is generally not necessary (McDowell, 2000).

Fermentation (or synthesis methods) with excluded methods

The industry is actively pursuing various strategies to bioengineer production of vitamin K and its precursors (Ren et al., 2020). Enhancing vitamin K_2 production from *B. subtilis* is the main strategy, with *E. coli* another host organism that has already been genetically modified to overproduce MK-8 (Ren et al., 2020). Another host organism being explored is *Lactococcus lactis* (Bøe & Holo, 2020). We could not confirm that any of these organisms are currently used in commercial industrial production of vitamin K for feed.

Fermentation (or synthesis methods) with allowed methods

Natural strains of *Bacillus subtilis natto* have the ability to produce a range of menaquinone homologues (Berenjian et al., 2015; Sato et al., 2001). Production takes place on a small scale and is generally marketed for human consumption. A mutated strain of *Bacillus subtilis* showed up to a 25-fold increase in production of vitamin K_2 grown on fermentation media of soy meal, yeast extract, glycerol, salt, and potassium phosphate compared to the natural strain (Benedetti et al., 2010). The product is sold as ViaMK7® by a subsidiary of LeSaffre (Gnosis, 2023).

Other sources

We found no other sources of Vitamin K.

Report Authorship

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Appendix

Table 2: Regulatory status of officially recognized vitamin and provitamin sources (AAFCO, 2022).

Vitamin / Ingredient Common Name	FDA 21 CFR §	AAFCO Definition	IFN
Vitamin A			
Carotene	582.5245	90.25	7-01-134
Cod liver oil	†	90.1	7-08-993
Cod liver oil with added vitamins A & D	†	90.2	7-08-047
Herring oil	†	90.25	7-08-048
Menhaden oil	†	90.25	7-08-048
Salmon liver oil	†	90.25	7-02-013
Salmon oil	†	90.25	7-08-050
Sardine oil	†	90.25	7-02-016
Shark liver oil	†	90.25	7-02-019
Tuna oil	†	90.25	7-02-024
Vitamin A acetate	582.5933	90.25	7-05-142
Vitamin A oil	†	90.3	7-05-141
Vitamin A palmitate	582.5936	90.25	7-05-143
Vitamin A propionate	†	90.25	7-26-311
Vitamin A supplement	†	90.14	7-05-144
Vitamin A&D oil	†	90.6	7-05-145
Vitamin B_1			
Thiamine hydrochloride	582.5775	90.25	7-04-828
Thiamine mononitrate	582.5878	90.25	7-04-829
Vitamin B_2			
Riboflavin	582.5695	90.25	7-03-920
Riboflavin supplement	†	90.13	7-03-921
Riboflavin 5-phosphate	582.5697	90.26	†
Vitamin B_3			
Menadione nicotinamide bisulfate	573.625	90.25	7-08-102
Niacin supplement	†	90.16	7-26-003
Nicotinamide	582.5535	90.25	7-03-215
Nicotinic acid	582.5530	90.25	7-03-219
Vitamin B_5			
Calcium pantothenate	582.5212	90.25	7-07-079
Choline pantothenate	†	90.25	7-01-229
D-Pantothenyl alcohol (Pantothenol)	582.5580	90.27	†
Sodium pantothenate	582.5772	90.27	†
Vitamin B_6			
Pyroxidine hydrochloride	582.5676	90.25	7-03-822
Vitamin B_7			
Biotin	582.5159	90.25	7-00-723
Vitamin B_8			
Inositol	582.5370	90.25	7-09-354
Vitamin B_9			
Folic acid	†	90.25	7-02-066
Vitamin B_{12}			
Vitamin B_{12} supplement	†	90.11	7-05-146
Vitamin C			
Ascorbic acid	582.5013	90.25	7-00-433
Calcium ascorbate	†	90.25	†
Calcium L-Ascorbyl-2-Monophosphate	†	90.25	†
Calcium L-Ascorbyl-2-Monophosphate	†	90.25	†

Vitamin / Ingredient Common Name	FDA 21 CFR §	AAFCO Definition	IFN
Erythorbic acid	573.300	90.25	7-01-230
Choline			
Betaine	†	90.17	7-00-722
Choline chloride	582.5252	90.25	7-01-228
Choline pantothenate	†	90.25	7-01-229
Choline xanthate	†	90.25	7-01-230
Vitamin D			
25-Hydroxyvitamin D3	573.550; 584.725	90.9	†
Cholcalciferol (D-activated animal sterol)	†	90.7	7-00-408
Ergocalciferol (D-activated plant sterol)	†	90.8	7-00-728
Herring oil	†	90.25	7-08-048
Menhaden oil	†	90.25	7-08-048
Salmon liver oil	†	90.25	7-02-013
Salmon oil	†	90.25	7-08-050
Sardine oil	†	90.25	7-02-016
Shark liver oil	†	90.25	7-02-019
Tuna oil	†	90.25	7-02-024
Vitamin A&D Oil	†	90.6	7-05-145
Vitamin D oil	†	90.5	7-05-141
Vitamin D_2 supplement	†		
Vitamin D_3 supplement	†	90.15	7-05-699
Vitamin E	†		
Tocopherol	†	†	7-00-001
α-Tocopherol acetate	†	†	7-18-777
Vitamin K			
Menadione	†	90.25	†
Menadione dimethylpyridinol bisulfate	573.620	90.25	7-08-102
Menadione nicotinamide bisulfate	573.625	90.25	†
Menadione sodium bisulfite complex	†	90.25	7-08-078

†Reference not found

Glossary

Bacterium – (*Pl. bacteria*) A single-celled prokaryotic microorganism that does not have chlorophyll.

Coenzyme – A protein substance that facilitates the action of an enzyme that is sometimes derived from a vitamin.

Co-factor - A non-protein substance that facilitates a biochemical reaction.

CRISPR (Clustered Regularly interSpaced Palindromic Repeats) – A gene editing technique that involves 1) a guide RNA to match a desired target gene and 2) an endonuclease (e.g. Cas9) that causes a double-stranded DNA break that allows modifications to the genome.

Culture – A microorganism or collection of specific microorganisms, their tissue, or an organ growing in or on fermentation media used to support their reproduction.

Current Good Manufacturing Practices – Systems that assure proper design, monitoring, and control of manufacturing processes and facilities.

Eukaryote – An organism that has cell nuclei. Includes protozoa, fungi, and most multicellular organisms.

Feedstock – (1) *Biol.* An energy or protein source added to fermentation media. (2) *Chem.* A raw material used to produce substances for chemical processes.

Fermentation medium – (*Pl. media*) A preparation that contains all the nutrients and water needed for a specific microorganism's cellular growth and reproduction.

Fungus – (*Pl. fungi*) A heterotrophic, eukaryotic, non-motile organism lacking chlorophyll that reproduces sexually through spores.

Homologous recombination-mediated gene targeting – A genetic modification technique that exchanges nucleotide sequences for two similar or identical DNA molecules on defined genes of interest.

Precursor – A compound that participates in a chemical reaction to produce another compound.

Prokaryote – An organism that lacks cell nuclei. Includes bacteria and blue-green algae.

Provitamin – A substance that an organism can convert into a vitamin.

Recombination – The process of creating a new assortment or combination of genes in progeny that did not occur in either parent.

Vitamin – An essential growth factor for organisms other than proteins, fats, and carbohydrates that cannot be met by internal metabolic processes.