Annatto and Color Removal

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Executive Summary

Annatto has a long history within the food industry, having been used in some form since the time of the Aztecs. Widely used as a commercial colorant in the dairy industry for nearly 150 years, annatto has frequently been used to give cheese the yellow to orange color that consumers desire. Despite this extensive history, several questions and concerns still remain regarding the use of this colorant. The concerns generally surround the methods used to whiten annatto colored whey, as the safety of benzoyl peroxide bleaching has been questioned by international end users. Additionally, international regulations on the use of coloring agents in baby formula products has also prompted companies to investigate alternatives to annatto.

In researching annatto, it became quite clear that this topic presents a number of challenges. Misinformation and ever changing international regulations have left many feeling confused. This document has been prepared with that in mind and is meant to be a resource for those who would like to know a bit more about the science behind annatto, color removal and annatto alternatives. By educating the industry and consumers, we will be better informed, and therefore, better able to handle these challenges and points of confusion as they come. The hope is that this document will provide you with the knowledge you need to make educated colorant decisions.
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Annatto and Color Removal

Introduction

Annatto may be added to cheese milk to give cheese a yellow color. Unfortunately, the annatto also is carried into the resulting whey. A customer preference for white colored whey means the whey from annatto colored cheeses may require some type of treatment to lighten or remove the color compounds. Bleaching by either benzoyl or hydrogen peroxide is the most common method for removing annatto color. Recently, a method for removing color through the use of the enzyme lactoperoxidase has become commercially available. Bleaching with peroxides can result in less than desired color removal, production of off flavors and possible exclusion of the product from certain international markets.

Sources of Color in Whey

There are four general sources of color in whey: carotenoids; riboflavin; Maillard reaction products; and annatto. The ability of peroxides to remove color from whey depends on the color compound present.

Carotenoids

Carotenoids are a group of pigments in the tetraterpenoid category that are built from four, 10-carbon terpene units and contain 40 carbon atoms. Found in fruits and vegetables, carotenoids range in color from yellow to orange, red and brown. More than 600 carotenoids occur in nature.

The carotenoid category can be divided further into xanthophylls, which contain oxygen and carotenes that do not contain oxygen. The word "xanthos" is Greek for yellow and reflects the fact that xanthophylls are often yellow. Bright orange carrots contain large amounts of carotene and are the basis for the term carotene. Because they do not contain oxygen, carotenes are soluble in fat and insoluble in water. Xanthophylls have oxygen in their structure and therefore are less hydrophobic (more soluble in water).

Carotenoids, in the form of beta-carotene (β-carotene, C_{40}H_{56}, molecular weight 537) (Figure 1) enter milk through forage eaten by cows. Incomplete conversion of β-carotene to Vitamin A in the mammary gland results in a yellow color in the milk. Very little of the carotenoids consumed by cows actually enter the milk and some compounds are colorless, nevertheless some color change in the milk due to forage is possible. The amount of carotenoids in milk is influenced by diet, breed of cow and season of the year. It is possible for the color in milk due to forage consumption to get into the whey that results from cheese manufacture. Carotenoids present in milk include: lutein; violaxanthin; antheraxanthin, zeaxanthin, neoxanthin and several forms of carotene. Beta-carotene comprises approximately 90% of the carotenoids present in milk.

![Figure 1. Structure of beta-carotene](image-url)

Hydrogen and benzoyl peroxide will bleach xanthophylls; however, regulations regarding bleaching xanthophylls in milk do not permit the use of hydrogen peroxide for bleaching milk intended for cheese or cheese products.
Riboflavin (Vitamin B<sub>2</sub>)

The greenish color of whey products (Figure 2) is due to the presence of riboflavin (Vitamin B<sub>2</sub>). The name riboflavin comes from the sugar "ribose" (Figure 3) and a ring structure that results in a yellow color when the molecule combines with oxygen (*flavus* is the Latin word for yellow). Riboflavin (C<sub>17</sub>H<sub>20</sub>N<sub>4</sub>O<sub>6</sub>, molecular weight 376) is water soluble and therefore unlike carotenes which are soluble in the lipid phase, riboflavin is present in the water phase. Riboflavin can be used as an orange-red food color additive.

Maillard Reaction Products

The Maillard reaction is a nonenzymatic browning reaction between an amino acid and a reducing sugar. In dairy products, the proteins and amino acids react with the lactose. The initial steps in the Maillard reaction, which result in colorless compounds, are favored by a lower pH. As the reaction continues, tan to darker brown colored compounds are formed (Figure 4). A higher pH accelerates the formation of darker colored compounds. Neither hydrogen nor benzoyl peroxide will remove color due to the Maillard reaction.

Annatto

Annatto is a colorant, derived from a shrub, that has been used to color a wide range of food products for over 200 years. There are references about the coloring of cheese in England with annatto dating back to 1796. Annatto also is used in cosmetics and to dye leather and textiles and is the focus of color removal by hydrogen and benzoyl peroxides.

The main color compound of annatto is the carotenoid bixin. Bixin is an oil soluble, 25 carbon diapo carotenoid. The ability of annatto in a number of chemical forms to act as a pigment is unusual among the carotenoids.
Annatto Background

Annatto is the yellow/orange pigment traditionally used to color cheese. Annatto is an extract of the pericap or fruit wall of the shrub achiote (*Bixa orellana*) and is among the oldest colorant known to man. In fact, the shrub is nicknamed the lipstick tree because of its use as a body paint, especially for the lips, by the Aztecs. Achiote is derived from the word *āchiotl* which is Nahuatl word for shrub (Nahuatl is the Aztec language of Central Mexico). It is also known as uruku in its original Tupi language (one of the main ethnic groups of Brazilian indigenous people) and aploppas. The shrub is named for the scientist, Francisco de Orellana, who was in the Pizarro expedition that explored the upper Amazon.

Annatto shrub

The shrub is approximately 6.5 to 16.5 feet tall (2 to 5 meters) (Figure 5) and native to the American tropics. The Spanish introduced the shrub to Southeast Asia in the 17th century and commercial production of annatto was noted in Jamaica in the 1790's. Many tropical countries such as Bolivia, Brazil, Columbia, Dominican Republic, Ecuador, Guyana, India, the Philippines, Jamaica, Mexico, Peru, Kenya and Surinam now grow *Bixa orellana*. Latin America, and Peru and Brazil in particular, is the major producer of annatto followed by Africa and Asia.

The shrub has pink flowers and bright red spiny fruits (Figure 6) that contain red seeds. The inedible fruit is found in a burr-like pod (Figure 7) with two sections that contains 10 to 50 seeds which have a triangular shape and are about the size of grape seeds. The seeds are comprised of a series of layers. The innermost section or kernel contains oils, waxy material, minerals and alkaloids. The kernel is covered by a layer of cellulose and tannins while the outermost layer of the seeds has pigments, some oils and water. The pigment is present as an oily, resinous pulp covering on the surface of the seeds. The thin layer of soft, sticky bright red pulp that covers the seeds turns to a brownish red color when dried. Approximately 2% of the dried seed is pigment although the amount of pigment can vary from 1 to 4% depending on the variety of the shrub, growing conditions, methods used to harvest the pulp, etc.

Figure 5. The shrub achiote (*Bixa orellana*).

Figure 6. The flower and spiny fruit of the shrub achiote (*Bixa orellana*).
Annatto pigments

The oily, resinous material covering the seeds is removed to produce the annatto color. Two methods are used to remove the material. One process uses mechanical abrasion with suspension of the material in either vegetable oil or a dilute potassium hydroxide solution. The second process involves extraction of the pigment with one or more organic solvents. The extract then is further refined depending on the final application. Precipitation with acids, recrystallization and spray drying in either oil or water soluble forms are possible processes. The final annatto extracts may be water or oil soluble or suspensions of the pigment in oil.

The major pigment (+80%) of the seed coat is cis-bixin (C_{25}H_{30}O_{4}), along with traces of norbixin (C_{24}H_{28}O_{4}), bixin dimethyl ester and other apocarotenoids (compounds derived from the oxidative cleavage of carotenoids). Stable forms of bixin were first isolated in 1913.

Both bixin and norbixin are present in annatto in cis and trans forms (Figure 8). Cis and trans refers to the isomers forms of norbixin. Isomers are compounds that have the same molecular formula but different molecular structure. Cis and trans indicate the position of groups around a double bond. Although isomers have the same chemical formula, the compounds can have very different properties depending on whether the reactive groups are in the cis or trans position. Bixin and norbixin can be converted from the cis to the trans form by light and heat. The trans form of both compounds is more red than the cis form.

The seeds typically are harvested manually. Workers cut off the ends of the branches containing the pods. The pods are allowed to dry and then threshed and winnowed to release the seeds and remove unwanted material. The seeds are dried and packaged for shipment.

The pigment covered seeds can be ground into a powder for culinary uses. The paste, often mixed with other spices, is known as achiote, annatto, bijol or pimentão doce and is a slightly bitter, earthy/musky flavored product similar to paprika or saffron used to flavor many dishes in Mexican, Brazilian and Jamaican cooking. In fact, the pigment covered seeds have been used for more than 200 years in Central and South American cooking prior to their use as a colorant in Europe. An example of a traditional dish is cochinita pibil which contains pork with ground bixa seeds and bitter orange juice. Depending on country, achiote paste is used in products such as sausage, fish, sauces, snacks and confections.

Figure 7. An open pod with seeds from the achiote shrub (Bixa orellana).
The two main commercially produced pigments of annatto are norbixin and bixin. Norbixin is water soluble and used for coloring cheese. Bixin is oil soluble and typically used to color higher fat dairy products, cosmetics and leather.

**Annatto manufacture**

Bixin is traditionally made by abrading the pigment from the seeds followed by mixing the pigment in a suspending agent. Annatto in this form is considered to be native bixin. Commercial products are pigment suspended in oil preparations and typically contain 4 to 8% bixin.

Abrasion of the pigment from the seeds also can be done in dilute potassium or sodium hydroxide solutions at ambient temperatures. When dried, the resulting powder typically contains about 25% bixin and can be dissolved or suspended in oil or processed into other types of annatto products.

Processing bixin under hot, alkaline conditions results in the saponification of the methyl group of bixin and the formation of norbixin. A water soluble powder then can be produced that ranges from 1 to 15% norbixin. The norbixin pigment also can be precipitated with acid, filtered and dried to produce a granular powder containing approximately 25 to 50% norbixin.

Organic solvents also can be used to extract color from the seeds. The resulting microcrystalline bixin products can contain 80 to 97% bixin. Acetone, ethanol, ethylacetate, hexane, methanol, sodium hydroxide in alcohol, dichloromethanol, dichloroethane, light petroleum and mixtures of ethanol and chloroform have been used.

Emulsifiers may be used to prevent undesired precipitation of norbixin. Calcium in hard water or cheese can cause precipitation of the pigment. Norbixin also can react with proteins to form a peach-red color.

The color of bixin is dependent on pH and ranges from yellow-orange to pink at lower pHs. The pH does not affect stability of the color. Bixin is stable at temperatures less than 212°F (100°C) but relatively unstable at temperatures greater than 257°F (125°C). The pigment also is unstable to light. Cis-bixin is orange in color and insoluble in vegetable oil but soluble in most polar organic solvents. Heating...
converts cis-bixin into the isomer trans-bixin which is red and soluble in oil. Further heating of bixin (>158°F, 70°C) results in degradation of the compound. A yellow pigment referred to as \( \text{C}_{17} \left( \text{C}_{17} \text{H}_{20} \text{O}_{4} \right) \) (Figure 8), also known as McKeown’s pigment, results when bixin is heated.

**Commercial annatto products**

Commercially available annatto is considered to be a generally microbiologically clean product and resistant to microbial growth. Annatto is purchased based on the percent bixin (cis) content or “points”. Color will vary depending on the variety of plant, growing area, climate/weather conditions, manufacturing methods and storage conditions of the seeds.

Annatto as a commercial product is available in several forms. The four general types are: oil-soluble, water-soluble, emulsified, and solvent-extracted. The application determines the form of annatto to be used.

**Oil-soluble annatto** typically consists of bixin dissolved or suspended in oil. Bixin suspended in oil has a more orange color while bixin dissolved in oil is more yellow. Heating oil-soluble annatto results in the formation of \( \text{C}_{17} \) that has an even more yellow color. Food grade oils such as soybean, rapeseed, sunflower, etc. may be used. The bixin content of oil solutions of annatto typically range from 0.05 to 1.0% while oil suspensions have 0.1 to 8% bixin.

Oil-soluble annattos often are used to color foods with a high fat content such as processed cheese or shortenings. They also are used in baked goods, popcorn, sauces, dressings and cream desserts.

**Water-soluble annatto** consists of norbixin, often as a potassium or sodium salt, in either a liquid or powder form. Water solutions of annatto can contain 0.1 to 4.0% norbixin. The powder form of water-soluble annatto usually ranges from 0.1 to 8% norbixin.

Water-soluble annatto traditionally has been used to color cheese. A color chart for coloring cheese is given in Figure 9. Because water soluble annatto is not stable below pH 7, it cannot be used in clear, acidic beverages, however, it can be used with acidic products that have a solid structure. Additional applications include sausage casings, sausages, puddings, breakfast cereals, pet food, tomato sauce, smoked fish and chocolate fillings.

![Figure 9. National Cheese Institute cheese color standard.](image)

The powdered form of water-soluble annatto also can be used in powdered products. Examples of such products includes instant desserts and dip mixes.

**Emulsified annatto** may be soluble in both oil and water and typically is used with products that have both a water and oil phase. Depending on the emulsifier used the annatto product may have improved acid stability. Emulsified annatto typically is a liquid product containing 1 to 2.5% bixin/norbixin.

Emulsified annatto color can be used in products such as processed cheese, ice cream, soup and confections. If the emulsified annatto is acid stable it also can be used in juices, liqueurs, and jellies.

**Solvent-extracted annatto** has an annatto purity of >90%. The final application depends on the carrier used for the annatto. Typically, solvent-extracted annatto is used in formulating colorants with special
functions. Examples of special food systems would be acid and brine systems, products requiring a high degree of light tolerance and special seasoning blends that contain other colorants such as paprika and carmine.

The INS number for annatto is 160b and refers to annatto, bixin and norbixin. Other synonyms for annatto include CI Natural Orange 4, CI 75120, Achiote, Achiotl, Achote, Arnatto, Beni-No-Kí, Bija, Rocou, Roucou, Roucoyer, Orlean, Orleanstraugh, Terre orellana, Urucu, Urucum, L. Orange or by other international code numbers.

Flavor compounds
There has been very little research on the flavor compounds present in annatto despite its use as a flavoring for centuries. When used as a spice, also known as achiote, the entire seed with the annatto containing pulp on the surface, typically is ground into a powder or paste. The flavor contributed by the seed versus the annatto containing pulp needs to be considered when evaluating the contribution of annatto coloring to flavor of a product. It is thought that factors such as growing locations and soil also influence the flavor compounds present in annatto.

Achiote (annatto seeds/pulp) is said to be sweet with a slight peppery flavor. It gives food a yellowish red color and is sometimes called "Poor Man's Saffron". When cooking with annatto the red seeds may be added to a pan with oil and the seeds then removed. Alternatively, the seeds can be soaked in water and the water used or the seeds can be ground into a powder and added to food.

Researchers found 107 compounds when they examined the volatiles in commercial water or oil soluble annatto extracts. The majority of the compounds present in either type of annatto were sesquiterpenes (38% of volatiles in oil extracts and 89% of volatiles in water extracts). Most of the sesquiterpenes were in the form of β-humulene, a compound that is thought to be a major component of beer hops/flavor and is said to have a woody/spicy, dry, and somewhat bitter flavor, and a clove-like odor.

In addition to sesquiterpenes, there were monoterpenes and arenes. Other specific compounds of note included: ρ-xylene (rho-xylene), toluene, α- and β-pinene, γ-elemene (gamma-elemene) and spathulenol. Along with their possible flavor contributions, several of the compounds found in annatto extracts exhibit antimicrobial, anticarcinogenic or antioxidant activities.

Very little information is available on the composition of the non-pigment material of annatto extracts. Several carotenoids, fatty acids in the form of oils, and resins with a bitter flavor have been found. It is believed that lignocellulose, proteins, fatty acids and other cell components also are present.

Use of annatto in foods
Annatto is a commonly consumed colorant in foods. Bixin, which is oil soluble typically is used in higher fat foods while norbixin with its ability to bind to protein is used in high protein products. Additives such as emulsifiers may be used in conjunction with annatto to produce a colorant that is more stable to the effects of other food components such as acids, metal ions and salts.

Countries may regulate the amount of annatto used in specific food products. Some examples of the maximum level of annatto permitted in certain food products by the European Commission is given in Table 1.
Table 1. Examples of maximum level of annatto permitted in several food products by the European Commission*

<table>
<thead>
<tr>
<th>Product</th>
<th>Maximum permitted level (mg/kg)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoked fish</td>
<td>10</td>
</tr>
<tr>
<td>Margarine, other fat emulsions and fats essentially free from water</td>
<td>10</td>
</tr>
<tr>
<td>Liqueurs</td>
<td>10</td>
</tr>
<tr>
<td>Desserts</td>
<td>10</td>
</tr>
<tr>
<td>Flavored processed cheese</td>
<td>15</td>
</tr>
<tr>
<td>Ripened orange, yellow and broken white cheese; unflavored processed cheese</td>
<td>15</td>
</tr>
<tr>
<td>Edible cheese rinds and edible casings</td>
<td>20</td>
</tr>
<tr>
<td>Extruded, puffed and/or fruit flavored breakfast cereals</td>
<td>25</td>
</tr>
<tr>
<td>Mimolette cheese</td>
<td>35</td>
</tr>
<tr>
<td>Red Leicester cheese</td>
<td>50</td>
</tr>
</tbody>
</table>

* Adapted from Scotter, M. 2009.
**as 100% bixin or norbixin

US regulations

The use of annatto in foods in the United States is covered in 21 CFR73.30 (Code of Federal Regulations) which describes color additives that are exempt from certification. Annatto and β-carotene are exempt from certification although color additives that are not subject to certification must still be declared on a food label. Annatto extract is considered safe, in general, to color foods when used in amounts consistent with good manufacturing practice. Labels should contain the information that the color is derived from annatto seeds.

International regulations

New regulations in the European Union (EU) do not permit color in whey for use in infant formula (Standard for infant formula and formulas for special medical purposes intended for infants CODEX Stan 72-1981). The use of whey/whey based ingredients that have annatto as a carryover component would not be allowed under this regulation.

"Colourings shall not be present in ingredients supplied to Baby Nutrition. Regulation (EU) 1333/2008 in combination with Regulation (EU) 1129/2011 does not permit the carryover of a food additive including food colours to infant formulae, follow on formulae, processed cereal based baby food and baby food for infants and young children".

The CODEX Standard for Infant Formula references the carryover of food additives (such as added color) into ingredients for infant products. Only food additives that are listed in the document may be present in the foods. When there is carryover of an unlisted additive then:

"the food into which the food additive is carried over does not contain the food additive in greater quantity than would be introduced by the use of the raw materials or ingredients under good manufacturing practice, consistent with the provisions on carry-over in the Preamble of the General Standard for Food Additives (CAC/STAN 192-1995)."

Regulations in China are changing rapidly, therefore it is important to routinely check standards for what is and is not permitted. Permitted food additives are found in the National Food Safety Standard for Uses of Food Additives (GB 2760), however, infant formula may be
covered under different regulatory standards in China.

**Issues with use of annatto**

Annatto, including the seeds, pulp and leaves, has both proponents and detractors regarding its alleged effects on human health. There is scientific support for some of the claims while others are largely unsubstantiated.

**Health benefits**

Health benefit claims for annatto involve the ingestion of greater amounts of annatto than typically available in foods. In general, claims have been made that annatto is antibacterial, antifungal, antiinflammatory and high in antioxidants. The seeds are said to be good for heartburn and stomach problems, liver and kidney issues and helpful in clearing mucus (expectorant) from lungs due to bronchitis, asthma, etc. The seeds also are claimed to be helpful for diarrhea and may act as a cough suppressant. In addition, annatto seeds and leaves are claimed to be good diuretics.

Annatto contains a number of other compounds including salicylic acid, ellagic acid and tocotrienols. Tocotrienols are members of the vitamin E family and are found in a number of products including vegetable oils, palm oils, wheat germ and certain seeds, nuts and grains as well as annatto. All vitamin E related compounds, including tocotrienols, are antioxidants and are thought to assist in prevention of cardiovascular diseases and cancer.

A commercially available extract of annattotocotrienol has been used in studies on breast cancer. A study using mice indicated that the compound slowed the progression of breast cancer by delaying mammary tumor development. In addition, the size and number of tumors were reduced.

An ethanol extract of annatto also has been evaluated for antimicrobial activity. It was found to inhibit Gram-positive bacteria including *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus faecalis*. The extract was not inhibitory for *Escherichia coli, Serratia marcescens, Candida utilis* or *Aspergillus niger*.

Medical studies have found that a water extract of the seeds had antihypertensive properties while the water/alcohol extracts had an analgesic and anti-inflammatory property. In addition, annatto was found to be hypoglycemic for rats with severe diabetes mellitus.

**Adverse effects**

There are numerous claims of people having adverse reactions to annatto. A number of internet websites are devoted to problems said to be caused by ingestion of even small amounts of annatto.

Some incidences of problems with ingestion of annatto have been documented in medical journals. Problems included hives, irritable bowel syndrome and non-IgE mediated intolerance. Only one case of IgE mediated intolerance resulting in anaphylaxis, reported in 1991, has been documented. Journals with published studies or accounts of issues with annatto include Journal of Clinical Gastroenterology, Allergy, British Journal of Dermatology, Archives of Toxicology and Annals of Allergy, Asthma and Immunology. Butter, cheese and cereal containing annatto have been documented in some cases as causing problems within 4 hours of ingestion. It is thought that problems with allergic reactions to annatto may be confined to people with an uncommon hypersensitivity who already have problems with skin diseases such as urticaria and angioedema.

Much more numerous are anecdotal accounts of medical and behavior problems thought to be caused by consumption of annatto. Some web sites have very extensive lists of possible problems, however, typically concerns center on headaches, irritability, restlessness, sleep disturbances, behavioral changes in children including head banging and arthritis. Adverse reactions are believed to be delayed as compared to artificial dyes and some people believe the reactions may not occur on the same day. Studies have not provided conclusive results about the role of annatto and adverse reactions.
Annatto Chemistry

There are several important aspects to annatto chemistry. Chemical analysis can be for annatto compound itself in pure extracts or annatto within a food system. The ability to define the exact color compounds that are captured by analysis influences results for annatto interactions and the partitioning and color changes exhibited by annatto in cheese and whey systems.

Analyzing for annatto color compounds
The analysis of annatto can be divided into two categories: analysis of the annatto color itself and; analysis for annatto in food systems. Bixin and norbixin have properties similar to other carotenoids; however, there are several differences that influence the analysis of these compounds.

Analysis of annatto color
The main techniques for analysis of annatto color compounds are spectrophotometry, nuclear magnetic resonance (NMR), chromatography and mass spectrometry (MS). Similar techniques are used to determine and measure annatto in food products. An in-depth review of the analysis of annatto is provided by M. Scotter (The chemistry and analysis of annatto food colouring: a review. 2009. Food Additives and Contaminants 26(8): 1123 - 1145).

Analysis for annatto in food
The presence of annatto or compounds resulting from the oxidation of annatto can be a problem for certain markets. The exact compounds that are detected and detection limits depend on the analytical method used.

Very few methods for detection/quantification of annatto in foods have been published before 1970. Methods typically involved extraction of the color with a solvent such as chloroform, benzene or ether followed by washing and adsorption of the color onto an inert material. Samples may or may not have been pretreated to prevent precipitation of the protein by the solvent. An example of such an analytical method would be the use of ethanol and a phosphate buffer to precipitate the proteins in whey followed by addition of dilute ammonium hydroxide to extract the annatto.

Extraction methods vary depending on the composition of the dairy product. Extractions typically use solvents such as ethanol:water:ammonia mixture, hexane, chloroform:acetic acid mixture, butylated hydroxyl toluene, methanol, petroleum ether, hydrochloric acid and acetonitrile and processes such as filtering, centrifugation and vacuum-assisted evaporation. Quantification of annatto in the extract then typically is done by high performance liquid chromatography (HPLC).

There are several problems with quantification of annatto regardless of the method used. Degree of annatto recovery from products, ability of the instrumentation to measure both cis- and trans- isomers of bixin and norbixin and separation of mixtures of bixin and norbixin are examples.

Issues with resolution of the peaks produced by HPLC remain even as more rapid methods for extracting annatto from food systems have been developed. Typically ultraviolet-visible spectrum spectroscopy (UV-VIS) or photodiode array are used to detect peaks produced by HPLC. The detection limit for bixin or norbixin is approximately 0.1 ppm (0.1 mg/kg).

An alternative methodology uses liquid chromatography-tandem mass spectroscopy (LC-MS/MS). In certain situations, LC-MS/MS can detect bixin at approximately 0.01 ppm (0.01 mg/kg), however, details of the method have not been provided in peer-reviewed journals and the method is subject to false negatives.

A new method for extraction and quantification of norbixin in whey recently has been published. The method is said to have a faster and less expensive extraction method with better sensitivity than previous methods (Campbell, R.C. 2014. Journal of Dairy Science 97(3): 1313 - 1318). The method has been evaluated using cheese milk,
fluid whey, whey protein concentrate and bleached whey (bleached by either hydrogen peroxide, hydrogen peroxide:lactoperoxidase combination or a commercial enzymatic method).

The method uses a solution of acetonitrile added directly to either fluid or rehydrated powders followed by mixing and centrifugation. The resulting supernatant is used for bixin quantification by HPLC with a photodiode array detector. The authors had a >90% recovery of norbixin from milk and whey. The limit of detection for norbixin in fluid products was 0.0027 ppm (2.7 µg/kg) and a quantification limit of 0.0035 ppm (3.5 µg/kg) both of which are lower than previously discussed methods.

**Quantifying color perceptions**

In addition to quantifying the amount of annatto present in a product, it can be important to determine the color of annatto as perceived by the human eye. Devices such as colorimeters assign numerical values to a color. Differences in the values allow comparisons of colors and color changes.

The Hunter L, a, b color scale is one such system for quantifying color that often is used with dairy applications. There are three values in the system that together quantify the color: L; a*; b* (Figure 10). The L value indicates lightness. The maximum value for L is 100, which indicates white. The minimum value for L is zero which would be black. The a* and b* axis's have no specific numerical limits. Positive a* is red and negative a* is green. Positive b* is yellow while negative b* is blue.

**Annatto reactions**

The polyene chain in annatto is responsible for a great deal of annatto’s reactivity. A polyene chain is a sequence of alternating double and single carbon to carbon bonds (Figure 11). Polyene chains absorb light in the visible region of the light spectrum resulting in compounds with color such as annatto. The bonds are susceptible to oxidation by oxygen and peroxides, reactions with acids and light, and instability related to temperature.
Oxidation of polyene bonds of annatto leads to loss of color which is very important to the whey industry. Oxygen is required and light acts as a catalyst for the reaction. Higher temperatures, presence of metal ions, greater intensity of light and greater availability of oxygen increase oxidation of annatto and loss of color. Studies have indicated that norbixin as measured by a Hunter Colorimeter becomes lighter, less red and more yellow with oxidation.

Annatto, especially in the form of norbixin, is more susceptible to oxidation in the powder form because of the increased surface area. Some researchers have concluded that light is the most effective agent for causing loss of annatto color followed by benzoyl peroxide. Contact with air is not considered very effective for oxidizing annatto. The presence of an antioxidant such as ascorbyl palmitate protects annatto from loss of color in the presence of light.

Bixin and norbixin have good thermal stability during food processing as compared to other carotenoids. Temperature induced changes in cis-bixin can occur during the extraction of bixin from the seeds/pulp and conversion into a commercial product.

Carotenoids are known to combine with proteins which stabilizes the carotenoid molecule. In addition, annatto will react with carboxyl groups (-COOH). Norbixin contains a carboxyl group, therefore, the molecule also can complex with divalent metal ions. Norbixin then is able to bind with the carboxyl group of another molecule thereby forming a stable complex. Such a complex can protect norbixin from oxidation and help retain the original color. While such a reaction may be desired for some products, it may be a problem when color removal by bleaching is desired.

Annatto in cheese and whey
The chemistry of annatto in cheese and whey is not clear. Annatto in cheese largely is combined with casein. A portion of the annatto, however, will be present in the resulting whey. Adding to the confusion is the complex mixture of pigments that may be present in annatto and research that does not necessarily differentiate between annatto, bixin and norbixin.

**Partitioning of annatto**
Depending on the study, 11 to 26% of the annatto (norbixin) added to cheese milk is present in the resulting whey. An example of the differences between whey from cheese with and without added annatto is given in Figure 12. Lower levels of norbixin addition to cheese milk had slightly higher percentages of norbixin in the whey as compared to higher norbixin addition levels. Older studies have reported that approximately 20% of the norbixin added to cheese milk partitioned into the whey. More recent research has found that approximately 10% of the norbixin added to the cheese milk will be found in the resulting whey.

![Figure 12. White (left) and annatto (right) colored whey.](image)

A color difference is evident in whey protein concentrates (WPC) manufactured from whey resulting from cheese manufactured with added annatto. Figure 13 is an example of a WPC34 powder made from annatto containing whey.
Figure 13. A whey protein concentrate powder manufactured from whey containing annatto.

One of the problems with determining the fate of annatto in cheese manufacture is the extraction of annatto from milk, cheese and whey. Annatto must be extracted from the product before it can be analyzed through techniques such as HPLC. Current extraction processes recover less than 100% of the annatto in the product. The percentage of annatto recovered is known as the extraction efficiency and must be considered when doing mass balances on the partitioning of annatto into cheese and whey. Studies cite an extraction efficiency of approximately 89% for cheese milk.

An additional problem is the loss of norbixin during cheese manufacture. Some researchers believe that heat and light during cheese make cause norbixin to be destroyed. It has been noticed that norbixin loss is more pronounced during manufacture of fat-free cheese as compared to full-fat cheese and it was thought the more opaque full-fat milk protects norbixin from destruction by light as compared to the more translucent skim milk. Experimental data estimates that approximately 7.5% of the norbixin is lost in full-fat cheese milk while fat-free cheese milk looses almost 12% of the original added norbixin.

Interactions with milk and whey components

There is a lack of information on the status of norbixin in whey. Although norbixin will combine with casein it is unclear whether norbixin has affinity for any specific whey components. If norbixin is able to bind with whey proteins to form a stable complex then removal of the color by bleaching with peroxides is not possible. Many people believe that annatto in whey powder is associated with the whey proteins and therefore cannot be removed.

The ability of norbixin to bind to whey proteins and caseins has been evaluated using specific milk proteins and whey protein isolates. The dicarboxylic groups of norbixin were important in binding norbixin to whey proteins. The β-sheet portion of α-lactalbumin was found to be the binding site for norbixin while α-helix structures were preferred for α- and β-caseins. The binding of norbixin was said to change the conformation of caseins and whey proteins, however, the denaturation temperature was not affected. Casein was found to have a stronger binding affinity for norbixin than the whey proteins.

When specific proteins were evaluated, norbixin was found to have a greater affinity for bovine serum albumin than either α-lactalbumin or β-lactoglobulin. Among the caseins, norbixin bound better to κ-casein than α- or β-casein.

When norbixin in whey from cheese manufacture has been evaluated annatto was not typically bound to protein but rather distributed between the water or serum phase and milk fat globule membrane material. Researchers found approximately 40% of the annatto in the water phase and 60% in the milk fat globule membrane material which is present as micelles in the whey.

Effects on flavor and functionality of whey products

Very limited research has been done on the effects of annatto on the flavor and functionality of whey products. One study found that annatto did not affect the solubility or heat stability of a WPC65 as compared to a WPC65 that did not contain annatto.

Flavor differences were not apparent in fresh, liquid whey or freshly manufactured WPC65 powder made with and without annatto. Analysis for volatile compounds found that fresh whey with annatto...
contained higher amounts of limonene and α-pinene than whey without annatto. Both limonene and α-pinene are present in commercial annatto extracts.

The WPC65 containing annatto also had higher concentrations of decanal, p-xylene, 2-butanone and pentanal than the WPC65 without annatto. The differences were thought to possibly be a result of an antioxidant effect of annatto.

**Color changes**

Annatto can produce a pink color in cheese. The color may be the result of norbixin precipitation by hydrogen sulfide. Other researchers have attributed the pink or peach-red color to a reaction of norbixin with protein. Exposure to ultraviolet light also can result in pink color in cheese. The pink color may be protected from further color change by phospholipids and β-casein and is stable to oxygen, light and pH changes.

Annatto also can cause a pink color in products containing bleached whey powder. Whey that previously has been bleached white and dried can become pink when incorporated into other products such as ice cream. The precise cause of the pink color is not known but factors such as pH, heat treatments to the whey and brand of annatto used are believed to be important in determining whether the defect occurs.

There have been some indications that heat treatment of the whey is a very important factor in determining whether a pink color defect will occur in ice cream. Whey ingredients that have received a more intense heat treatment in an effort to improve water binding can produce a pink color in ice cream that does not occur with whey ingredients that have not had such a heat treatment are used. It has been postulated that there is a change in the whey proteins that alter the chemistry of the ice cream mix such that the annatto becomes pink. Titratable acidity and pH do not seem to be important factors in the pink ice cream problem.

**Color Removal (Bleaching) by Peroxide Compounds**

Color removal by peroxides, also known as bleaching, has been done for more than 40 years by the dairy industry in the United States. Hydrogen peroxide and benzoyl peroxide are the only chemical compounds currently allowed in the United States for bleaching whey by oxidation. CODEX regulations recently have changed to permit the use of benzoyl peroxide for bleaching.

**Hydrogen Peroxide**

Hydrogen peroxide (H₂O₂), also known as dihydrogen dioxide and dioxidane oxidanyl, is a clear, colorless liquid having a slightly pungent odor and a molecular weight of 34.0. It is the simplest peroxide and has an oxygen-oxygen single bond (Figure 14). Hydrogen peroxide typically is found in liquid form for safety reasons. The boiling point of hydrogen peroxide is thought to be 302°F (150.2°C), however, it can undergo explosive thermal decomposition if heated to this temperature. Concentrated hydrogen peroxide is very reactive and has been used as a propellant for rockets.

![Figure 14. Structure of hydrogen peroxide](image)

Food grade hydrogen peroxide typically has a concentration of 30 to 50%. Hydrogen peroxide is safe and stable under recommended storage and handling conditions, although hydrogen peroxide will decompose by an exothermic reaction when exposed to soil and other foreign materials.
Benzoyl Peroxide
Benzoyl peroxide or dibenzoyl peroxide ($C_{14}H_{10}O_4$) is a colorless, crystalline solid with a molecular weight of 242.2 and consists of two benzoyl groups linked by a peroxide group. The structure of benzoyl peroxide is given in Figure 15. Benzoyl peroxide has a faint odor of benzaldehyde, is insoluble in water and melts with decomposition at temperatures of 217 to 223°F (103 to 106°C). The dry, concentrated form of benzoyl peroxide is a highly reactive, dangerous oxidizing material that may spontaneously explode. Commercial products may contain 15 to 35% benzoyl peroxide.

Figure 15. Structure of benzoyl peroxide.

Benzoyl peroxide for bleaching whey often is blended with carriers to facilitate use. The strength of the benzoyl peroxide and degree of water solubility will vary with the specific carrier used. Examples of carriers are: calcium sulfate, magnesium carbonate, starch, calcium phosphate and whey.

Conditions of use
Although hydrogen and benzoyl peroxides may be used to bleach whey, there are several restrictions to their use. The inherent advantages and disadvantages of each chemical may determine the suitability of either compound for a given application.

Hydrogen Peroxide
Hydrogen peroxide use as a bleaching agent in whey is covered by 21CFR 184.1366. Hydrogen peroxide may be used at a rate of <500 ppm (< 0.05%) and is considered effective at all temperatures and total solids levels.

Residual hydrogen peroxide must be removed by appropriate physical and/or chemical means during processing. Addition of catalase to eliminate hydrogen peroxide is required according to 21CFR 133.113. The amount of catalase added must be sufficient to eliminate any residual hydrogen peroxide and cannot exceed 20 ppm (0.002%) catalase based on the milk used.

Hydrogen peroxide is not a permitted bleaching agent for milk that is to be used in cheese manufacture. Hydrogen peroxide may be used as a preservative in milk for cheese manufacture at 0.05%. Hydrogen peroxide is thought to stimulate the lactoperoxidase system which can inhibit the growth of certain microorganisms.

Before March, 2011, the only permitted use of hydrogen peroxide as a preservative for whey was during electrodialysis where a maximum of 400 ppm (0.04%) hydrogen peroxide may be used. The Food and Drug Administration (FDA) in response to a petition, now permits a maximum of 0.001% (w/w) hydrogen peroxide for use in modified whey produced by ultrafiltration as long as the residual hydrogen peroxide is removed during processing by appropriate chemical or physical methods.

Advantages of hydrogen peroxide for bleaching include effectiveness over a wide range of temperatures and total solids levels. The corrosive nature of hydrogen peroxide, need to be inactivated with catalase, long hold times to remove color, and potential to cause oxidized flavors are disadvantages to its use.

Benzoyl peroxide
Benzoyl peroxide use is permitted under 21CFR 184.1157 for removing color in whey both from naturally occurring colored
compounds and annatto addition. Benzoyl peroxide for bleaching has no use rate limitation other than that dictated by good manufacturing practices. A typical use rate for benzoyl peroxide is <20 ppm (<0.002%). Most effective use conditions are 140°F (60°C) for 15 minutes at pH 6 to 7. Longer holding times are required if lower temperatures are used and below pH 5.5 there is progressively pinker color in the whey.

Benzoyl peroxide also may be used to bleach milk used for the manufacture of certain cheeses (21CFR 133). Swiss, Emmentaler, Cheddar, Asiago, Blue, Gorgonzola, Parmesan, Reggiano, Provolone and Romano are among the cheeses that may have the starting milk bleached by no more than 0.002% (20 ppm) benzoyl peroxide.

Benzoyl peroxide is effective at lower usage levels, does not require catalase addition and does not pit stainless steel. There are several disadvantages to its use. Oxidized flavors can be produced by benzoyl peroxide treatments. Commercial benzoyl peroxide products use a carrier and the carrier may be considered an allergen. Benzoyl peroxide will not reduce microbial populations or control acid production in whey.

The United States does not prohibit benzoyl peroxide use in bleaching whey or whey products that are for infants; however, other countries or groups such as the European Union exclude benzoyl peroxide use in such products. Although CODEX regulations recently have changed to permit benzoyl peroxide as a bleaching agent some countries are concerned about the use or presence of benzoyl peroxide in imported products. China and Japan, for example, ban the use of benzoyl peroxide in all whey products.

Reactions and conditions during chemical bleaching

Hydrogen and benzoyl peroxides have been used for decades to remove color from whey. Many aspects of their use have been determined; however, details are lacking in several areas. A colorimetric method for measuring the amount of annatto and other related colors in dry whey often is used to monitor the bleaching process and its effectiveness.

General Mode of Action

Chemical oxidizing agents, such as peroxides, eliminate color by reacting with the portion of the color molecule known as the chromophore. The chromophore is the part of the molecule that absorbs certain wavelengths of visible light and transmits or reflects other wavelengths thus resulting in the molecule's color. Typically when the double bond within the chromophore is oxidized (eliminated) the color molecule becomes colorless.

Before peroxides can react with compounds such as annatto, peroxides must break apart at the peroxide bond to form peroxide radicals. Heat or metal ions (copper, iron, manganese, for example) can cause the bond to break. Less energy is required to break the peroxide bond in benzoyl peroxide as compared to hydrogen peroxide and benzoyl peroxide breaks apart at all temperatures used to process milk/whey. Hydrogen peroxide needs more energy, supplied by use of higher temperatures or a catalyst such as a metal ion to form peroxide radicals. Iron containing proteins in milk/whey, such as lactoperoxidase, can be a source of the metal ions.

Hydrogen Peroxide

Hydrogen peroxide decomposes to carbon dioxide and water during bleaching (Figure 16). Excess hydrogen peroxide can be removed from the whey by addition of catalase. Heat also will eliminate residual hydrogen peroxide.

$$2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}$$

Figure 16. Decomposition reaction of hydrogen peroxide.
Although hydrogen peroxide is effective over a wide range of temperatures, temperature still has an effect on the ability of hydrogen peroxide to bleach annatto in Cheddar cheese whey. Reaction temperatures >165°F (74°C) do not increase either the rate or extent of color removal and instead cause protein denaturation. Oxidized flavors, apparent immediately after treatment, decrease following evaporation and drying.

Recent research has indicated that at 39°F (4°C) hydrogen peroxide was ineffective at removing annatto color. When used at 154°F (68°C), hydrogen peroxide removed annatto color with increasing concentrations of hydrogen peroxide removing greater amounts of color. Lipid oxidation resulted when ≥ 500 ppm (500 mg/kg) hydrogen peroxide was used.

In addition to residual fat, it has been found that pasteurization, percentage of total solids in the WPC and fermentation versus direct acid addition do not affect color removal by hydrogen peroxide. Storage of whey, protein content of the whey and presence of curd particles have an effect.

Additional research has found that storage of fresh whey at 37°F (3°C) for up to 48 hours had a negative effect on color removal as compared to whey that had not been stored. Retentate containing 80% protein had better color removal than retentate with 34% protein and color removal was more efficient with retentate versus whey indicating composition has an role.

Rehydrated WPC34 powder had the same degree of color removal as fresh liquid whey while rehydrated WPC80 had less loss of color as compared to fresh whey again implying composition has a role in bleaching. It has been thought that annatto cannot be removed once the whey has been dried but this study would indicate that this may not be the case.

Whey containing annatto has been bleached with hydrogen peroxide during ultrafiltration to produce WPC34. The norbixin concentration in the final WPC34 was reduced as compared to the starting whey. There also was less iron in the final product suggesting an interaction between the hydrogen peroxide and iron containing whey proteins. Hydrogen peroxide was not as effective at color removal as benzoyl peroxide.

**Benzoyl Peroxide**

Benzoyl peroxide reacts with oxidizable compounds and is converted into water soluble benzoic acid (Figure 17). Studies have indicated that when added to milk more than 91% of the benzoyl peroxide is converted to benzoic acid. Whey should not contain any benzoyl peroxide following bleaching as all of the benzoyl peroxide is converted to benzoic acid. Benzoic acid has been extensively studied as a food additive and may be used unconditionally at up to 5 mg/kg body weight. Benzoic acid has Generally Recognized as Safe (GRAS) status for several food products and is naturally present in several fruits such as cranberries and cloudberries.

![Figure 17. Decomposition reaction of benzoyl peroxide.](image)

Benzoyl peroxide will bleach carotenoids present in cream. Carotenoids were effectively destroyed by 9 ppm (0.0009%) benzoyl peroxide in cream at 125 to 145°F (52 to 63°C) for 1 to 2 hours. Vitamin A content of the cream was not significantly affected. Temperature affected the rate of color loss but not the final color, rather concentration of benzoyl peroxide used determined amount of color loss. Temperatures of 165 to 185°F (74 to 85°C) resulted in cooked and scorched flavors while 18 ppm (0.0018%) benzoyl peroxide caused oxidized and tallowy flavors.
The effectiveness of benzoyl peroxide for removing color in whey depends on the amount of benzoyl peroxide used, how it is applied, whey components present, exposure time and temperature. A comparison of hydrogen and benzoyl peroxides found both were effective, however, benzoyl peroxide was more effective at all temperatures evaluated. Oxidized flavors initially present dissipated following evaporation and drying.

Benzoyl peroxide readily removes annatto in solution. When milk proteins are present annatto is more resistant to bleaching and additional benzoyl peroxide and/or time is required. Whey with higher total solids, such as condensed whey, also needs greater amounts of benzoyl peroxide to remove color. It has been noted previously that once whey has been dried, the annatto color is believed to be highly resistant to bleaching. The annatto is thought to be bound to proteins in the whey and once dried the reaction cannot be reversed to free the annatto for bleaching. Again, there is some research using hydrogen peroxide that indicates that this may not necessarily be the case.

Benzoyl peroxide reacts quickly to remove color. Generally 30 minutes is sufficient for benzoyl peroxide to react with all of the color compounds. Additional time will not increase color removal.

Optimum pH for bleaching is approximately 6 to 7. Use of pHs below 5.5 are said to result in a more pink color. It may be that using benzoyl peroxide to bleach whey with a pH <5.5 is a factor in the production of off-color whey.

Color removal by benzoyl peroxide is very temperature dependent. Recommended bleaching temperatures are 131 to 149°F (55 to 65°C). If temperatures are too low, residual annatto color may remain regardless of reaction time. For example, whey bleached at 86°F (30°C) may never have the annatto color completely removed.

Recent research has questioned the effect of temperature on color removal by benzoyl peroxide. Researchers believed the effect of temperature was minor and that benzoyl peroxide came closer to removing all annatto color in whey than hydrogen peroxide. They concluded that ≤25 ppm (0.0025%) benzoyl peroxide was the most effective concentration with lipid oxidation occurring at ≥100 ppm (0.01%).

Additional research has found that the temperature effect appears to be dependent on the total solids/protein content of the whey. The effectiveness of benzoyl peroxide at 100 ppm (0.01%) in whey was not effected by use of either 41 or 122°F (5 or 50°C). When bleaching higher total solids/higher protein whey protein concentrate there was a significant difference in effectiveness with benzoyl peroxide removing more color at 122°F (50°C) as compared to 41°F (5°C).

Changes in color perception with bleaching
The changes in color resulting from bleaching of annatto by peroxides can be measured by instruments such as a Hunter Colorimeter. The Colorimeter scale measures lightness and color as it ranges from red to green and from yellow to blue and assigns numerical values to a color. The Hunter Colorimeter scale is given in Figure 10.

Research has indicated that the type of color change is different for hydrogen versus benzoyl peroxide. Typically hydrogen peroxide results in a whiter, more yellow (less blue) whey as compared to benzoyl peroxide. Conversely, benzoyl peroxide gives the greatest reduction in red color (more green). It is believed that hydrogen peroxide bleaches naturally occurring color pigments in the whey in addition to the annatto.

Color differences between bleached and unbleached products may not be evident when the product is in powder form. Researchers viewing WPC34 powders made from whey containing annatto indicated that there were no differences in the appearance of the powders regardless of whether or not the whey had been bleached with hydrogen or benzoyl peroxide or left as is.

Color differences however were very apparent when the powders
were rehydrated. Hunter Colorimeter values indicated the powders became less yellow with bleaching by benzoyl peroxide. Significant differences were not apparent for the unbleached control and hydrogen peroxide treated WPC34.

**Changes in norbixin concentration with bleaching**

The reduction in norbixin concentration by bleaching has not been extensively researched. Values in the literature indicate hydrogen peroxide can give a 44% reduction in norbixin concentration in WPC80 while benzoyl peroxide can result in a 92% reduction. Hydrogen peroxide was added at 500 ppm (0.05%) and benzoyl peroxide at 50 ppm (0.005%). Reaction conditions for either peroxide was 30 minutes at 151°F (66°C).

An additional study found hydrogen peroxide reduced the norbixin content of whey protein concentrate (80% protein) by 50% while benzoyl peroxide reduced the norbixin content in the same product by 92%. Benzoyl peroxide was used at 50 ppm (0.005%) and hydrogen peroxide at 500 ppm (0.05%). Both peroxides were in contact with the product for 30 minutes at 151°F (66°C).

Subsequent work concluded the opposite effect was true. Hydrogen peroxide was more effective at removing norbixin than benzoyl peroxide when higher levels of protein were present (for example WPC80). Unlike hydrogen peroxide, however, annatto destruction by benzoyl peroxide was not effected by either temperature or total solids.

The effect of protein concentration on annatto removal is thought to be a result of higher lactoperoxidase concentrations in higher protein whey products as compared to the original whey. Lactoperoxidase, in the presence of small amounts of hydrogen peroxide, will destroy norbixin, however, lactoperoxidase is inactivated by hydrogen peroxide concentrations of >100 ppm (0.01%). Because lactoperoxidase is concentrated along with other whey proteins during the manufacture of whey protein concentrates, there is a higher concentration of lactoperoxidase in the higher protein products which may allow some of the enzyme to survive exposure to hydrogen peroxide. Lactoperoxidase then is available to assist in color removal thereby increasing the efficiency of hydrogen peroxide.

Reductions in norbixin concentrations following bleaching with either hydrogen or benzoyl peroxide also have been seen with whey although percentage reductions were not available. Benzoyl peroxide gave the greatest reductions in norbixin concentration in whey regardless of the temperature used.

**Affects of bleaching on flavor**

Peroxides do not react exclusively with annatto but instead interact with any compound that will react with free radicals. Fats and proteins are the components in milk and whey most likely to be effected by peroxides.

Lipids are susceptible to oxidation by free radicals and therefore exposure to hydrogen or benzoyl peroxide can result in oxidized fat off flavors. Compounds associated with oxidized lipid off flavors include: hexanal; heptanal; and octanal.

Changes in the flavor of whey ingredients can be determined through flavor evaluation by panelists or by analytical methods. Panel evaluations give an indication of whether the flavors can be detected. Analytical evaluation determines the precise compounds that are present whether they are above or below the levels that can be detected by humans.

**Hydrogen peroxide**

Research has indicated that hydrogen peroxide generates more off flavors in whey, WPC34 and WPC80 than benzoyl peroxide. Heptanal, hexanal, octanal and pentanal, which are associated with cardboard or fatty oxidized flavors, were present in higher concentrations after hydrogen peroxide addition as compared to benzoyl peroxide. Sulfide compounds associated with oxidation of amino acids also were in greater concentration. Conversely, there was a decrease in cooked, milky and sweet notes in WPC34, WPC80.
and whey when bleached with hydrogen peroxide as compared to benzoyl peroxide. Although there was an increase in off flavor compounds between hydrogen and benzoyl peroxides, the difference was not necessarily significant.

Analysis of volatile compounds indicated hydrogen peroxide caused greater amounts of lipid oxidation and protein degradation than benzoyl peroxide. Compounds such as pentanal, hexanal, octanal, heptanal, nonanal, and 1-octen-3-one were present. Hexanal was the most abundant compound. Hexanal does not necessarily contribute a cardboard type flavor but rather is an indicator of lipid oxidation. Dimethyl sulfide and a cabbage/sulfur aroma also were present with hydrogen peroxide treated whey.

It has been noted that WPC80 with annatto that was not bleached had less cardboard flavor as compared to WPC80 without added annatto. It was hypothesized that annatto had an antioxidant effect that reduced oxidation of compounds in the WPC80.

**Benzoyl peroxide**

Benzoyl peroxide can oxidize milk fat resulting in tallowy, oxidized flavors. Flavor problems are more apparent with increasing temperature, contact time and benzoyl peroxide concentration.

Benzoyl peroxide used to bleach whey produced detectable cardboard flavor notes when used at 20 ppm (0.002%) while the cardboard flavor did not occur at 10 ppm (0.001%) use level. The off flavor was less than that found with hydrogen peroxide at 250 (0.025%) and 500 ppm (0.05%).

Research also has indicated that there are fewer oxidized flavors generated by the use of benzoyl versus hydrogen peroxide to bleach whey, WPC34 or WPC80. Flavor compounds resulting from both lipid (heptanal, hexanal, octanal and pentanal) and protein oxidation (dimethylsulfide and dimethyltrisulfide) were lower in concentration with the benzoyl as compared to hydrogen peroxide.

**Affects of bleaching on functionality**

The amino acids that make up proteins, especially those amino acids that contain sulfur, also are targets for hydroxyl radicals. Methionine, lysine, tyrosine, histidine and tryptophan are examples of amino acids that are especially susceptible to oxidation by peroxides. Changes to amino acids can alter protein properties such as solubility, heat stability, foamability, etc.

**Hydrogen peroxide**

Hydrogen peroxide can alter the functionality of whey proteins. The susceptibility of whey proteins to hydrogen peroxide depends on the specific protein, concentration of hydrogen peroxide, and reaction temperature, time and pH.

Hydrogen peroxide is known to inhibit browning in milk systems. Additional information on the mechanism of browning inhibition is lacking although it is likely hydrogen peroxide alters the ability of reactive groups on the proteins to interact with sugars thereby limiting the Maillard reaction.

When added to milk, hydrogen peroxide increased the amount of non protein nitrogen present while decreasing the concentration of whey proteins. Increasing the concentration of hydrogen peroxide and contact time increased the affect. Proteose peptones were the most susceptible to alteration by hydrogen peroxide. Immunoglobulins, bovine serum albumin and β-lactoglobulin were intermediate in susceptibility while α-lactalbumin was relatively unaffected by hydrogen peroxide treatments. Whey proteins were more susceptible to denaturation by hydrogen peroxide than casein. Hydrogen peroxide did not cause interactions between β-lactoglobulin and κ-casein.

Hydrogen peroxide at concentrations greater than 1,000 ppm (0.1%) in contact with whey at room temperature for three days caused a 5 to 8% decrease in the non-polar amino acids. Affected amino acid residues included aspartic acid, threonine, glutamic acid, methionine,
tyrosine, phenylalanine, histidine, lysine, tryptophan and arginine. Tryptophan with approximately 25% decrease in concentration, was most affected by exposure to hydrogen peroxide concentrations greater than 1,000 ppm (0.1%).

The number of free sulphhydryl groups within proteins increased with use of increasing hydrogen peroxide concentrations. Increasing the storage time beyond 24 hours did not significantly increase the number of free sulphhydryl groups. Relatively lower concentrations of hydrogen peroxide did not cause significant oxidation of the sulphydryl groups; however, relatively higher concentrations of hydrogen peroxide rapidly increased sulphhydryl group oxidation. It is thought that hydrogen peroxide is selective for sulfur containing amino acids and therefore reacts first with amino acids such as methionine. The structure of the amino acid is altered such that the sulphhydryl groups are exposed and then oxidized.

Hydrogen peroxide concentration, temperature and time are very important variables affecting whey protein denaturation. Higher concentrations of hydrogen peroxide, increased reaction temperatures and longer holding times all increased the amount of whey protein denaturation. Temperature has a very large affect on whey protein denaturation. The pH resulting in the greatest amount of protein denaturation depends on the specific whey protein although the affect of pH is minor compared to the other variables. For example, immunoglobulins and bovine serum albumin are more readily denatured at a lower pH while β-lactoglobulin denaturation is enhanced at a pH closer to neutral. Alpha-lactalbumin is unaffected by comparison.

The presence of sulfide compounds probably is related to the noted increase in heat stability of WPC80 treated with hydrogen peroxide. Researchers believed that increased denaturation of proteins resulted from altered sulfhydryl interactions and because the proteins were less organized, more time was required to align proteins to gel.

Whey at 77°F (25°C) with 5,000 ppm (0.5%) hydrogen peroxide has minimal whey protein denaturation. Alternatively, use of approximately 10,000 ppm (1%) hydrogen peroxide at 131°F (55°C) causes significant whey protein denaturation. Hydrogen peroxide concentrations greater than 1,000 ppm (0.1%) results in formation of methionine sulfone and cysteic acid that potentially can reduce the nutritional value of the whey proteins.

**Benzoyl Peroxide**

Unlike hydrogen peroxide, benzoyl peroxide is thought to lack the energy to be able to break the sulfur to sulfur bonds in amino acids. The addition of heat with benzoyl peroxide can provide the energy necessary for such interactions.

Benzoyl peroxide combined with heat can affect whey proteins. Beta-lactoglobulin, α-lactalbumin, proteose peptone, serum albumin and immunoglobulins were altered by use of benzoyl peroxide at 10,000 and 50,000 ppm (1 and 5%). The effect of benzoyl peroxide on casein was less apparent.

Very limited information is available on the effect of benzoyl peroxide on the functional properties of whey. Foaming properties of whey protein isolate (WPI) produced from sweet whey was not effected by either annatto addition or bleaching by benzoyl peroxide although there may be slight differences in nonprotein nitrogen, true protein and ash content of the WPI.

**General effects**

Researchers have indicated that there was no difference in gelling ability of WPC80 when bleached with either hydrogen or benzoyl peroxide. It was hypothesized that the two compounds interact differently with whey proteins although gel formation was not affected.

Additional research with serum whey protein concentrate (whey proteins produced from milk rather than whey) indicates that foaming properties could be affected but the presence of lipids may obscure the differences. Foams produced from bleached serum whey protein concentrates with 80% protein were more stable than the same
concentrates that were not bleached. It was suggested that some denaturation of the protein due to bleaching improved foam stability. Some research has indicated that there are no differences in the solubility of WPC80s over a range of pH bleached with either hydrogen or benzoyl peroxides. Other researchers hypothesize that the presence of fat in the WPC80s limits the effects of bleaching on solubility. They did not detect differences at pH 6 or 7 but did note differences in solubility at lower pH (pH 3 to 5).

Effectiveness against microorganisms
Research has focused primarily on the effects of hydrogen peroxide on microorganisms. Relatively little information is available on the interactions of benzoyl peroxide with microorganisms associated with milk and whey.

Hydrogen Peroxide
The effectiveness of hydrogen peroxide in inactivating microorganisms depends on hydrogen peroxide concentration, microbial population, temperature, pH, inorganic ions, treatment time and exposure of microorganisms to additional treatments (chemicals, heat treatments, etc.). Oxidation and changes in the DNA and enzyme systems of the microorganisms are believed responsible for inactivation of the microorganisms although details are not known. The hydroxyl radical formed during the breakdown of hydrogen peroxide is thought to react with the free iron in the bacterial cell to create free radicals that damage DNA and cellular organs resulting in death. Hydrogen peroxide is more effective in inactivating spores than bacterial cells.

Hydrogen peroxide is most effective against anaerobic spore formers and coliforms. Anaerobes do not produce catalase to break down hydrogen peroxide. Lactic acid starter bacteria used in cheese manufacture can be inhibited by hydrogen peroxide concentrations as low as 5 ppm (0.0005%) although they may be considered intermediate in their susceptibility to inactivation by hydrogen peroxide. Aerobic spore formers are most resistant to hydrogen peroxide. Gram-negative bacteria are more sensitive to hydrogen peroxide as compared to gram-positive bacteria. Lipases and proteases in milk are not considered susceptible to inactivation by hydrogen peroxide.

Effectiveness of hydrogen peroxide for inactivating spores depends on temperature, pH, hydrogen peroxide concentration and number of spores. In general, time required for inactivation of spores increases with increasing concentration of spores, decreasing concentration of hydrogen peroxide, increasing pH and decreasing temperature.

The effectiveness of hydrogen peroxide for inhibiting microorganisms has generally been determined using milk rather than whey as the substrate. Milk is a more complex media than whey, therefore, it should be possible to extrapolate from milk to whey.

Lactic acid bacteria used for the production of cheese vary in their sensitivity to hydrogen peroxide. The effect of increasing hydrogen peroxide concentrations on inhibiting acid production is not linear and is affected by exposure time and the specific lactic acid bacteria present. Above a certain concentration of hydrogen peroxide no further reduction in acid production occurs. In general, hydrogen peroxide levels greater than 120 ppm (0.012%) have little additional effect on reducing acid production.

Some microorganisms, including some cheese starter culture bacteria, produce hydrogen peroxide that inhibits other microorganisms. Streptococcus mitis and Lactobacillus acidophilus are examples of such types of bacteria. Hydrogen peroxide, therefore, is not inhibitory towards these types of bacteria.

Some microorganisms produce catalase which breaks down hydrogen peroxide. Bacteria that can produce catalase when grown in milk include: Micrococcus spp., Staphylococcus spp., Bacillus spp., Corynebacterium spp. and Propionibacterium spp., Lactobacillus and Streptococcus spp. do not commonly produce catalase.

Bacteriophages of Lactococcus lactis ssp. cremoris inoculated into milk were not inactivated by exposure to 10,000 ppm (1%) hydrogen peroxide at 129°F (54°C) for 1 hour. Milk apparently provided a
protective effect since use of phosphate buffer in place of milk as a substrate led to inactivation of the bacteriophage.

Benzoyl Peroxide
Benzoyl peroxide decomposition is not significantly affected by the pH of whey. Benzoyl peroxide also is considered stable to heat treatments.

Studies have found benzoyl peroxide concentrations of up to 50,000 ppm (5%) are not effective in significantly reducing the acid producing activity of some lactic acid bacteria. Benzoyl peroxide, however, is often used in other products to limit microbial growth. A possible explanation for the lack of antimicrobial activity is the typical pH of whey. Benzoyl peroxide breaks down to benzoic acid. Benzoic acid in both the dissociated and undissociated forms inhibits microorganisms; however, optimum antimicrobial activity for benzoic acid is between pH 2.5 to 4.0. The pH of whey almost always is above pH 4.0 therefore benzoyl peroxide/benzoic acid is ineffective at controlling growth of cheese culture bacteria.

The bleaching of whey generally is done at one of two locations in the process. One location is when preheated whey is pumped into a storage tank and peroxide then added. The second location is the addition of peroxide at the hot well of the evaporator.

Additional comments and concerns with peroxide use
Several additional factors may need to be considered when deciding whether to use either hydrogen or benzoyl peroxide. Peroxide limitations, regulatory issues and international concerns may preclude the use of peroxides.

Peroxide Limitations
Peroxide limitations affect the ability of either hydrogen or benzoyl peroxide to bleach whey. As with any food ingredient, only food grade hydrogen or benzoyl peroxide should be used. Because less concentrated hydrogen peroxide is prone to decomposition, concentrated hydrogen peroxide (>35%) should be diluted to usable strength immediately before use. Contact with polyvalent metals such as copper and iron also will accelerate the decomposition of hydrogen peroxide.

Naturally occurring enzymes present in milk and whey, such as catalase and peroxidase, can decompose the hydrogen peroxide added to remove color or control microbial growth. Increasing temperatures increase the efficiency of hydrogen peroxide for inactivating microorganisms; however, the rate of hydrogen peroxide decomposition also increases.

For example, when hydrogen peroxide was added to milk containing...
catalase and peroxidase and held at 135°F (57°C) there was relatively little hydrogen peroxide decomposition as compared to holding at 100°F (38°C). It is thought that enzymes in the milk decomposed the hydrogen peroxide when incubated at 100°F (38°C) while the enzymes were inactivated by the storage temperatures of 135°F (57°C) thereby resulting in stable hydrogen peroxide concentrations. It has been noted that peroxide is more effective at temperatures above those typically used during starter culture growth in cheese manufacture.

Although peroxide will lighten coloring compounds such as annatto added to milk, peroxide will not remove brown color compounds resulting from caramelization or Maillard reactions. Hydrogen peroxide can inhibit browning but it cannot alter brown color compounds after their formation.

The use of hydrogen peroxide in milk as an alternative preservation method can oxidize proteins, aldehydes, ketones and vitamins A and C. In addition, hydrogen peroxide can split α_{s1-5} fractions from casein micelles resulting in longer clotting and setting times and softer curd during cheese manufacture.

**Catalase**

Catalase or hydrogen peroxide oxido-reductase is an enzyme used to remove hydrogen peroxide. Catalase catalyzes the conversion of hydrogen peroxide to oxygen and water (Figure 18). Crystalline catalase was originally isolated from beef liver in 1937 although catalase also is present in high concentrations in blood, liver, kidney and fatty tissues. The enzyme has a molecular weight of 225,000 daltons and is considered to be relatively stable and very active. One molecule of catalase can decompose 2,600,000 molecules of hydrogen peroxide at 32°F (0°C) in one minute.

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{O}_2 + 2\text{H}_2\text{O}
\]

**Figure 18. Reaction of catalase and hydrogen peroxide.**

Commercial catalase also is a fermentation product of *Aspergillus niger*. In liquid form, catalase is amber to dark brown in color with a fermentation odor. As a powder catalase has a tan color. Catalase is completely soluble in water. The enzyme typically is active from pH 2 to 7 and 32 to 149°F (0 to 65°C) and should be stored in a cool, dry location.

**International Concerns**

International concerns center on the use of benzoyl peroxide. Although CODEX recently approved both hydrogen and benzoyl peroxide as processing aids for use in bleaching whey, many Asian and European countries do not like the use of benzoyl peroxide. Some countries have regulations that are stricter than the CODEX standards and therefore the importation of whey and whey products bleached by benzoyl peroxide may not be permitted. Japan and China are examples of countries that do not allow the use of benzoyl peroxide in whey products. They also do not permit the presence of benzoic acid in dried whey products.

Benzoyl peroxide is approved in the US by the FDA as a GRAS substance and may be used at Good Manufacturing Practices (GMP) levels in whey products. Food Standards for Australia and New Zealand regulate the use of benzoyl peroxide in Australia and New Zealand and has set a maximum of 40 mg/kg (40 ppm, 0.004 %) (measured as benzoic acid) when used to bleach any food product. The Canadian Food and Drug Regulations has set a limit of 100 mg/kg (100 ppm, 0.01 %) for bleaching whey products that are to be dried. The CODEX Standard for benzoyl peroxide in either liquid or dry whey products (excluding whey cheeses) is also 100 mg/kg (100 ppm, 0.01 %).

Some countries do not permit the use of benzoyl peroxide in whey products for use in infant formula. The US does not have a regulation prohibiting use benzoyl peroxide whey products for infant formula. Canada and Europe do not permit benzoyl peroxide for bleaching whey products for infant formula.
The major decomposition product of benzoyl peroxide is benzoic acid. The safety of benzoic acid and its derivative benzoates (Figure 19) has been widely studied. Benzoates and the related salicylates are widely distributed in food plants and are present in prunes, tea, cloves, cinnamon and many berries such as cranberries. Benzoic acid and benzoates have been used as preservatives in food and beverages for approximately 100 years and are among the most commonly used additives.

![Figure 19. Structure of benzoic acid, sodium benzoate and aspirin.](image)

Originally, it was believed that benzoic acid related compounds did not cause adverse reactions when consumed. It is now apparent that a small percentage of the population is sensitive to such compounds. People with adverse reactions to benzoic acid related compounds typically have underlying diseases such as asthma. Asthmatics often are intolerant to aspirin (C$_9$H$_8$O$_4$), also known as 2-acetoxybenzoic acid or O-acetylsalicylic acid, which is very similar in structure to benzoic acid (Figure 19). The mechanism of the intolerance does not appear to be an allergy type but rather a pseudo-allergic response that relies on enzymes.

Adverse reactions to benzoic acid related compounds appear to be relatively rare. With the exception of asthmatics with aspirin intolerance reactions generally are mild with life-threatening reactions extremely rare.

The fruit juice industry is an example where the presence of benzoic acid has raised concerns related to the compound benzene. Under certain conditions benzoic acid can react with the ascorbic acid present in fruit juice resulting in the production of carcinogenic benzene (Gardner, L.K. and G.D. Lawrence. 1993. Benzene production from decarboxylation of benzoic acid in the presence of ascorbic acid and a transition-metal catalyst. Journal of Agriculture and Food Chemistry 41: 693-695). It is possible that the association of benzoic acid with benzene is a source of the concern for some groups when use of benzoyl peroxide is used for bleaching milk or whey.

Analyzing for peroxide use

Hydrogen peroxide decomposes into oxygen and water when used for bleaching therefore analysis focuses exclusively on determining the presence of hydrogen peroxide. Analysis for determination of bleaching by benzoyl peroxide may be based either on the presence of benzoyl peroxide or the by product of the reaction, benzoic acid.

Hydrogen peroxide analysis

Several methods are available for rapid, semi-quantitative determination of the hydrogen peroxide concentration in dairy fluids. The methods typically use a change in color of the testing material to indicate the presence and concentration of hydrogen peroxide.

One method uses an analyzer that relies on hydrogen peroxide reacting with a phenolic derivative and the enzyme peroxidase to form a pink colored complex. The color is measured and the result compared to a standard curve. Results are available in approximately 6 minutes. Range of this particular analyzer is 1.5 to 25 ppm (0.00015 to 0.0025%) hydrogen peroxide with a resolution of 0.1 ppm (0.00001%) hydrogen peroxide.

Another method uses tests strips which are similar to those used for pH determination. The test strips are placed in the solution to be tested and the strips change from white to blue in the presence of hydrogen peroxide. The resulting color is compared to a color scale to give an indication of the hydrogen peroxide concentration. Measuring range is said to be 1 to 100 mg hydrogen peroxide/L sample.
Benzoyl peroxide analysis
An older method for benzoyl peroxide analysis involves dissolving the sample in acetone followed by addition and mixing of a potassium iodide solution. The solution then is titrated with a sodium thiosulfate solution.

The current method for determining the concentration of benzoyl peroxide involves HPLC with a UV detector and ethyl ether for the extraction. The method has a detection range of 0.2 to 1.7 mg per sample with a limit of detection of 0.01 mg per sample. Extraction of benzoyl peroxide from the whey is done with ethyl ether with a peroxide recovery rate of 97%.

Benzoic acid analysis
Benzyol peroxide may not be present even when it has been added to milk or whey, therefore, analysis for the reaction end product benzoic acid is chosen when attempting to determine if peroxide has been used. Initial methods of analysis were based on the determination of benzoic acid in flour where benzoyl peroxide is used for bleaching. Additional work has been done on detecting benzoic acid in cheese as a result of the bleaching of cheese milk.

Currently, HPLC/UV detector is the method for determination/quantification of benzoic acid in products such as milk and whey. Methanol, sulfuric acid, potassium hexacyanoferrate and zinc acetate in conjunction with filtering are used to precipitate and remove fat and protein such that a filtrate is produced that can be analyzed by HPLC. The limit of detection is 1 mg/kg sample.

Benzoic acid
Benzoic acid (C\textsubscript{7}H\textsubscript{6}O\textsubscript{2}), also known as phenylformic acid and benzenecarboxylic acid, is a colorless compound with a molecular weight of 122 and a melting point of 251.6°F (122°C). Its solubility in water is pH dependent. Benzoic acid is widely used as a preservative in acidic foods because of its antimicrobial activity when at a lower pH. Benzoic acid is effective against a wide variety of bacteria, yeasts and molds that are associated with food spoilage and poisoning. Benzoic acid occurs naturally in products such as fruits, vegetables, spices and nuts and at low levels in milk and other dairy products.

Benzoic acid in whey products
Whey that has been bleached with benzoyl peroxide will contain the reaction end product benzoic acid. There has been very limited research on the fate of benzoic acid in whey that is processed into products such as whey protein concentrates.

Whey that has been bleached with benzoyl peroxide may have significant differences in benzoic acid content. Differences in the amount of hippuric acid (a naturally occurring acid in fresh milk) in the starting milk, differences in lactic acid bacteria used to produce the cheese and resulting whey and the amount of benzoyl peroxide used are among the factors that influence final benzoic acid concentration of the whey.

When whey is processed into WPCs through ultrafiltration, larger molecular weight compounds, such as protein, are retained by the membrane while smaller molecular weight materials, such as lactose and minerals, enter the permeate stream. Benzoic acid is a relatively low molecular weight compound (molecular weight 122) and therefore should become part of the permeate thereby leaving the WPC with a lower benzoic acid content.

Research has indicated that benzoic acid, in fact, is removed by ultrafiltration. A WPC34 which has less permeate removed as compared to a WPC80, had a higher concentration of benzoic acid (Table 2). Conversely, the permeate resulting from production of a WPC80 contained greater amounts of benzoic acid than the permeate from a WPC34.
Table 2. Benzoic acid concentration in retentate and permeate from whey bleached with benzoyl peroxide during whey protein concentrate production*.

<table>
<thead>
<tr>
<th>Ultrafiltered stream</th>
<th>Benzoic acid concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retentate</td>
</tr>
<tr>
<td>WPC34</td>
<td>278</td>
</tr>
<tr>
<td>WPC80</td>
<td>60</td>
</tr>
</tbody>
</table>

*Adapted from Listiyani (2011)

Additional sources of benzoic acid

Benzoic acid is produced by lactic acid bacteria. Unfortunately, the presence of benzoic acid due to microbial activity can cause problems for some end users where they may incorrectly assume that the benzoic acid is the result of bleaching of the whey product rather than a by product of fermentation of the milk by bacteria.

Benzoic acid occurs naturally at low levels in milk. Several strains of lactic acid bacteria are able to convert hippuric acid (C₉H₉NO₃), a natural constituent of fresh milk, to benzoic acid during fermentation of milk. One molecule of hippuric acid converts into one molecule of benzoic acid. The structure of hippuric acid, also known as N-benzyolglycine and benzoylaminoacetic acid, is given in Figure 20.

A few studies have determined the levels of benzoic acid typically present in dairy products. A summary of the results is given in Table 3. Considerable variability in benzoic acid content was possible for a given product. The variability in benzoic acid concentration may be due to factors such as method of acidification (fermentation or direct acid addition), variations in the manufacturing procedures, type of lactic acid bacteria used, natural variation in the hippuric acid content of milk and degree of hippuric acid hydrolysis.

Products that have been produced through direct acidification by acid rather than through fermentation by lactic acid bacteria will not have increased levels of benzoic acid. Any benzoic acid present in direct acidified products will be from the benzoic acid in the original milk.

Table 3. Benzoic acid content of dairy products*.

<table>
<thead>
<tr>
<th>Product</th>
<th>Benzoic acid content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw whole milk</td>
<td>Trace</td>
</tr>
<tr>
<td>Raw skim milk</td>
<td>Trace</td>
</tr>
<tr>
<td>Pasteurized skim milk</td>
<td>4 – 6</td>
</tr>
<tr>
<td>Fermented skim milk</td>
<td>30 – 60</td>
</tr>
<tr>
<td>Sour cream</td>
<td>11 – 18</td>
</tr>
<tr>
<td>Buttermilk</td>
<td>11 – 20</td>
</tr>
<tr>
<td>Kefir</td>
<td>10 - 23</td>
</tr>
<tr>
<td>Yogurt</td>
<td>16 – 56</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>2 – 18</td>
</tr>
<tr>
<td>Cheddar cheese</td>
<td>17</td>
</tr>
<tr>
<td>Mozzarella</td>
<td>26</td>
</tr>
<tr>
<td>Variety of cheeses</td>
<td>Trace - 35</td>
</tr>
<tr>
<td>Whey</td>
<td>12 - 13</td>
</tr>
<tr>
<td>Whey powder</td>
<td>23 - 75</td>
</tr>
</tbody>
</table>

*Adapted from Sieber (1995) and Chandan (1977).
The partitioning of benzoic acid during production of cheese, whey and whey protein concentrate also has been studied. Figure 21 is an outline of the development and distribution of benzoic acid during production of cottage cheese cultured with lactic acid bacteria. Benzoic acid partitioned with the serum (whey) portion of the milk and was present in the whey that drained from the curd.

In another study, benzoic acid distribution between curd and whey during cheese manufacture was 9% in the curd and 91% in the whey. A similar pattern was noted for hippuric acid where 7% of the hippuric acid remained in the curd while the remaining 93% entered the whey.

A study of the manufacture of lactic acid casein, a product produced using methods similar to those used for cottage cheese, found washing of the curd with water removed 90% of the benzoic acid present. Essentially 90% of the benzoic acid produced therefore was present in the acid whey. When the acid whey was processed into a whey protein concentrate, the researchers found 95% of the benzoic acid went into the permeate.

Figure 21. Outline of benzoic acid distribution in cottage cheese. Benzoic acid concentration is given in ppm. Adapted from Chandan, R.C. (1977).
Biochemical Bleaching Compounds

Another category of bleaching compounds are the biochemical agents which includes enzymes such as lipoxygenases. Lipoxygenases were originally known as carotene oxidases because of their ability to bleach carotenes. The use of lipoxygenases as bleaching agents has been patented and commercial products are now available.

General mode of action
Lipoxygenases work by accelerating the oxidation of xanthophylls into colorless compounds. Lipoxygenases are enzymes, therefore, only a small amount of the compound is required and the reactions are specific to the color compounds. The specificity of the enzyme limits their interactions with other components such as lipids thereby reducing the potential for generation of off-flavors.

Milk and whey have a lactoperoxidase system that consists of a group of compounds that together have antimicrobial properties. Lactoperoxidase, thiocyanate and hydrogen peroxide are required and together they are bacteriocidal and have been used to used to preserve milk without refrigeration.

Lactoperoxidase is relatively heat stable with an upper temperature limit of approximately 158°F (70°C) although the enzyme appears to have greater heat stability when in products such as milk and whey. Lactoperoxidase contains one heme group (1 iron/molecule of lactoperoxidase), has an optimum pH range of 5.5 to 6.3 and is less stable to heating when at a pH of less than 5.3. It is thought that the enzyme is active in pasteurized milk and whey.

The lactoperoxidase system can be used to bleach whey if a small amount of hydrogen peroxide is added to initiate the process. The lactoperoxidase system reaction is given in Figure 22. Hypothiocyanate is a strong oxidizer and will react with the double bonds in norbixin to eliminate the yellow/orange color.

\[
\begin{align*}
H_2O_2 & + SCN^- \rightarrow OSCN^- + H_2O \\
\text{Hydrogen peroxide} & \text{Thiocyanate} & \text{Hypothiocyanate} & \text{Water} \\
\end{align*}
\]

Figure 22. Lactoperoxidase reaction

The lactoperoxidase system in whey requires less than 0.5 mM of hydrogen peroxide to become active. The level of hydrogen peroxide is sufficient to allow lactoperoxidase to regenerate and produce the hypothiocyanate ion required for bleaching.

The iron in the lactoperoxidase molecule is very important in generating the free radicals required for bleaching. Hydrogen peroxide, thiocyanate (SCN⁻) and lactoperoxidase react together to form a superoxide radical (HO₂⁻) that then can react with the double bonds in annatto to remove color (Figure 23). If the appropriate concentration of hydrogen peroxide is present then the iron in lactoperoxidase can regenerate and return to the original 3+ charge.

\[
\begin{align*}
\text{Fe}^{3+} + H_2O_2 & \rightarrow \text{Fe}^{2+} + \text{HO}_2^- \\
\text{iron (III)} & \text{hydrogen peroxide} & \text{iron (II)} & \text{superoxide radical} \\
\end{align*}
\]

Figure 23. Formation of superoxide radical.

The reaction typically requires 20 to 45 minutes at 104° F (40°C). Although required to initiate the process, large amounts of hydrogen peroxide will react lactoperoxidase and inactivate the enzyme. Limiting hydrogen peroxide to < 10 ppm (0.001%) is recommended. If the concentration of hydrogen peroxide decreases to a level that does not continue to generate hypothiocyanate, the color removal process will proceed at a much slower rate.

Although lactoperoxidase is present in milk and whey, variability in the amount of enzyme present can make it difficult to determine the
appropriate amount of hydrogen peroxide to use. The problem is less noticeable in products such as whey protein concentrates since the proteins and therefore the lactoperoxidase is concentrated during the production process. Addition of lactoperoxidase to the system eliminates problems with differences in initial enzyme concentrations.

Research has indicated that the optimum concentration of hydrogen peroxide for lactoperoxidase activation is 20 ppm (0.002%). The enzyme was considered to be 99% effective for bleaching whey although off flavor generation similar to what occurs with use of hydrogen and benzoyl peroxides was noted both through sensory and instrumentation techniques.

Bleaching of whey and WPC80 by lactoperoxidase and hydrogen peroxide has been compared. Whey bleached at either 95 or 122°F (35 or 50°C) for 1 hour with lactoperoxidase had >99% of the annatto destroyed. Hydrogen peroxide (250 ppm, 0.025%) by comparison eliminated 32% of the annatto at the lower temperature and 47% at the higher temperature. Similar results were seen when bleaching WPC80.

Researchers believe the lactoperoxidase reaction was complete within 30 minutes. The reaction was not affected by fat separation but there was a slight increase in enzyme activity following pasteurization of the whey. Additional research indicated that lactoperoxidase activity was not affected by whey storage either at 39°F (4°C) or -4°F (-20°C) for up to 72 hours, vat-pasteurization of the milk, fat separation of the whey or liquid WPC80 production by membrane processing.

Lactoperoxidase, either native (naturally occurring in whey) or added exogenous enzyme, removed color in whey fastest with whey at a lower pH (5.5 versus 6.5) and higher temperatures (140°F, 60°C). A similar trend was apparent for liquid WPC80 (10% total solids) except that the effect of pH was reversed. When additional enzyme was not added, there was greater color removal at a higher pH (6.5 versus 5.5). With added exogenous enzyme the pH trend was the same as for whey. It is thought that thiocyanate required for the destruction of annatto by lactoperoxidase is removed during ultrafiltration to produce the WPC80 thereby altering the ratio of thiocyanate to hydrogen peroxide and altering the optimum pH for bleaching. In all cases, however, bleaching occurred faster in retentate than whey.

Studies conducted at 39°F (4°C) found that following 12 hours of reaction time lactoperoxidase with hydrogen peroxide removed 97% of the annatto as compared to hydrogen peroxide at 250 ppm (0.025%) which removed only 38% of the annatto.

Sensory analysis found that WPC80 bleached with lactoperoxidase had a distinct cabbage flavor while the hydrogen peroxide bleached product had a fatty flavor. Chemical analysis indicated the lactoperoxidase bleached product contained higher levels of heptanal, octanal, dimethyl sulfide and 2-pentylfuran than the WPC80 bleached with hydrogen peroxide. The off-flavors were thought to be a result of the lactoperoxidase system catalyzing the oxidation of residual lipids in the WPC80.

Additional research has indicated that bleaching with a commercial lactoperoxidase product resulted in a potato/brothy flavor in the WPC80. Color removal by hydrogen peroxide alone led to sulfur notes in the product.

**Commercial products**
Commercial lipoxygenase based products for whitening whey have become available in recent years. MaxiBright® from DSM Food Specialties is an example. The product uses a fungal peroxidase from a strain of *Aspergillus niger* that is specific for carotenoids. The enzyme requires activation with 0.5 to 1 mM hydrogen peroxide which is consumed in the reaction.

Company literature list the benefits of their peroxidase as faster bleaching at lower temperatures without the generation of off-flavors. The product can replace benzoyl peroxide thereby eliminating benzoate residues in the powder. They also list MaxiBright® as a sustainable and eco-friendly product.
MaxiBright® has an optimum pH range of 3.5 to 6.5. Hydrogen peroxide must be added to a level of 5 to 40 ppm (0.0005 to 0.004%). Company literature states that because there is no residual hydrogen peroxide, addition of catalase is not required. The product can be used over a temperature range of 41 to 122°F (5 to 50°C). Residual enzymes, including amylases, are present in the product.

Studies have found that MaxiBright® was able to remove 92% of the annatto in liquid WPC80 at 39°F (4°C). The lactoperoxidase naturally occurring in the product that was activated with hydrogen peroxide required 12 hours to remove 97% of the color.

**Alternative bleaching methods**

Ozone and ultraviolet light have been evaluated for their ability to eliminate annatto color in whey. Bleaching was done with whey that was then made into WPC80 through ultrafiltration.

Ultraviolet light is known to disrupt double bonds in the chromophore group in annatto thereby decreasing or eliminating the yellow/orange color. Annatto colored whey at 122°F (50°C) was exposed to ultraviolet light for 1 minute and then processed into WPC80. The resulting powder had increased cardboard and fatty flavors and decreased sweet aromatic notes as compared to the control. The powder also had unusual animal, mushroom and pasta flavors. Although ultraviolet light was able to reduce norbixin by 39% the resulting off flavors made ultraviolet light an unacceptable alternative for bleaching whey.

Ozone also interacts with the conjugated double bonds of chromophore groups in annatto. Ozone naturally decomposed to oxygen and an oxygen radical. The oxygen radical is the reactive molecule that attacks the double bonds thereby eliminating or decreasing the annatto color. Annatto colored whey at 122°F (50°C) was exposed to 2.2 grams/hour ozone for 1 hour and then processed into WPC80. The resulting powder had a 15% decrease in norbixin with flavor problems similar to the WPC80 that had been exposed to ultraviolet light. Given the low level of annatto reduction and resulting flavor problems, ozone was considered unacceptable for bleaching whey.

**Methods for removing color**

Previously discussed methods have focused on altering annatto color such that it becomes colorless. An alternative approach for reducing the color in annatto colored whey is to remove the color itself. Color removal methods typically use charged compounds that have the ability to adsorb components such as carotenoids, unsaturated fatty acids, milk fat globule membrane and glycerides in the whey.

Acid activated bentonite has been evaluated for ability to remove annatto color from whey. Bentonite is used to decolor vegetable oils and will remove hydroperoxides formed by oxidation, unsaturated fatty acids, glycerides, carotenoids and trace metals. Acid activation increases the ability of bentonite to adsorb molecules.

Annatto colored whey at 122°F (50°C) was mixed with acid activated bentonite (0.5% w/w) for 1 hour, the mixture centrifuged to remove the bentonite and any adsorbed material and the whey then processed into WPC80. The resulting powder had a 79% decrease in norbixin. The WPC80 had reduced sweet aromatic and cooked/milky flavors, a slight fatty flavor and no change in cardboard notes as compared to the unbleached control. Acid activated bentonite was considered to be an acceptable alternative to chemical bleaching with peroxides.

Zinc chloride and zinc acetate have been used to flocculate milk globule membrane material and annatto. The negatively charged phosphate groups of the membrane phospholipids result in repulsion of milk fat globule membrane material. Positively charge groups such as those provided by zinc can cross link with the membrane material resulting in flocculation. Researchers added zinc acetate (>20 mm zinc acetate) to whey at pH 5.2 and 86°F (30°C) for 30 minutes and found milk fat globule membrane flocculated. The material then was removed by centrifugation resulting in a clear, uncolored whey.
Chitosan also has been used to flocculate and remove milk fat globule membrane material and annatto. Chitosan is a natural polyglucosamine polymer derived from chitin that is found in crustacean shell. Chitosan is a polycationic polymer that should bind to the negatively charged milk fat globule membrane material.

The chitosan process starts with the addition of a chitosan solution to whey at 77°F (25°C). The pH of the whey-chitosan mixture is then adjusted to pH 4.5 which results in the flocculation of the chitosan, milk fat globule membrane material and annatto. The solution is allowed to set undisturbed so that the floc will settle. The supernatant then is withdrawn and microfiltered to remove any floc remaining in the whey. The permeate resulting from microfiltration is ultrafiltered/diafiltered to produce a WPC80. The resulting WPC80 has the clarity of a WPI and no annatto color evident.

There currently are two types of chitosan, KitoZyme and ChitoClear®, that have received GRAS status. KitoZyme is produced from the mycelia of the fungus Aspergillus niger and has GRAS status for use in the production of alcoholic beverages such as wine and beer where following its addition it is removed by physical separation processes such as centrifugation. ChitoClear® is derived from shrimp using the Primex production process and is said to meet a very high purity standard. ChitoClear® is intended to be used as a functional food ingredient, natural preservative and natural dietary fiber in a wide range of food products such as breads, cereals, vegetables, meats, cheese, milk desserts and yogurt.

**Alternatives to Annatto**

The presence of annatto in whey products can be a problem for certain applications such as infant formula. In addition, bleaching of annatto can be an issue for products to be exported to certain countries. Several companies recently have developed alternatives to annatto for use in cheese manufacture with the goal of producing a whey that either does not contain colorant or contains a colorant that is permitted in certain applications. As always, it is important to check the current regulatory status on the use of such alternatives when the whey product is destined for export.

**Beta-Carotene**

A β-carotene based product, under the trade name WhiteWhey™, is available from Chr Hansen. Beta-carotene is a naturally occurring pigment in milk and whey.

WhiteWhey™ is a patented β-carotene solution that is claimed to limit the transfer of the colorant into the whey. Typically 10 to 20% of the annatto added to cheese milk enters the whey stream. The WhiteWhey™ product is said to have a 1 to 3% color transfer into the whey. Figure 24 illustrates the differences between a WPC34 made from whey resulting from milk with WhiteWhey™ and a WPC34 made from whey containing annatto.

![Whey protein concentrate 34 made from WhiteWhey™ (left) and whey protein concentrate 34 made from whey containing annatto (right).](image)
**Carotenoid blends**

Another commercially available option is an encapsulated blend of red and yellow fat-soluble carotenoid colors of paprika and β-carotene. The patented product is known as SO-TEC™ Clear Whey. The product is produced by Socius and Cyber Colors. The color is said to remain within the cheese curd and not enter the whey stream thereby eliminating the need to bleach the resulting whey (Figure 25).

![Figure 25. Whey resulting from SO-TEC™ Clear Whey color addition to cheese milk (left) and whey resulting from annatto addition to milk for cheese manufacture (right). Color from riboflavin is present in both samples.](image-url)
Abbreviations

CFR  Code of Federal Regulations
EU   European Union
FDA  Food and Drug Administration
GMP  Good Manufacturing Practice
GRAS Generally Recognized as Safe
HPLC High Performance Liquid Chromatography
LC-MS/MS Liquid Chromatography-tandem mass spectroscopy
MS   Mass spectroscopy
NMR  Nuclear magnetic resonance
USDA US Department of Agriculture
UV-VIS Ultraviolet-visible spectrum spectroscopy
WPC  Whey protein concentrate
WPI  Whey protein isolate

Symbols

α   Alpha
β   Beta
κ   Kappa
γ   Gamma
ρ   Rho
μ   micron
ppm parts per million

Chemical Formulas

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<tr>
<th>Compound</th>
<th>Formula</th>
<th>Molecular Weight</th>
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<tr>
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<tr>
<td>Benzoic acid</td>
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<td>Benzoyl peroxide</td>
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<td>Beta-carotene</td>
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<td>Bixin (cis, trans)</td>
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<td>Carboxyl group</td>
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<td>Hippuric acid</td>
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<td>Water</td>
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Additional Resources


Websites

Center for Dairy Research (CDR)
www.cdr.wisc.edu

Code of Federal Regulations (CFR)
www.gpoaccess.gov/cfr/index.html

CODEX Alimentarius
www.codexalimentarius.net

Food and Drug Administration (FDA)
www.fda.gov

Food and Drug Administration (FDA)
Center for Food Safety and Applied Nutrition (CFSAN)
www.fda.gov/Food/default.htm