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Colorants in cheese manufacture: Production, Chemistry, Interactions and Regulation

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ABSTRACT

Colored Cheddar cheeses are prepared by adding an aqueous annatto extract (norbixin) to cheese milk; however, a considerable proportion (~20%) of such colorant is transferred to whey, which can limit the end use applications of whey products. Different geographical regions have adopted various strategies for handling whey derived from colored cheeses production. For example, in the USA, whey products are treated with oxidizing agents such as hydrogen peroxide and benzoyl peroxide to obtain white and colorless spray-dried products; however, chemical bleaching of whey is prohibited in Europe and China. Fundamental studies have focused on understanding the interactions between colorants molecules and various components of cheese. In addition, the selective delivery of colorants to the cheese curd through approaches such as encapsulated norbixin and micro-capsules of bixin or use of alternative colorants, including fat- soluble/emulsified versions of annatto or beta-carotene, have been studied. This review provides a critical analysis of pertinent scientific and patent literature pertaining to colorant delivery in cheese and various types of colorant products on the market for cheese manufacture, and also considers interactions between colorant molecules and cheese components; various strategies for elimination of color transfer to whey during cheese manufacture are also discussed.

Keywords: Colored cheeses, Cheddar cheese, annatto color, bixin, norbixin

1. Introduction

Color is one of the most important food characteristics that influences consumer preference, taste perception and thereby purchasing choice (Sukkwai et al., 2018). It may be considered one of the most appealing attributes of foodstuffs, and is a basis for evaluation of food freshness and quality (Zulueta, Esteve & Frígola, 2007). Traditionally, colorants have been used as food additives to make food more attractive and appear healthier, and even today many products available in the food market, such as snacks, pastries, puddings, liquors, sauces, dairy, ready-to-eat foods and ready-to-drink beverages contain added colors, either naturally or chemically produced.

Of these, natural food colorants are preferred, not only for their ability to make food appealing, but also in offering health benefits (Delgado-Vargas & Paredes-Lopez, 2003; Shim, et al., 2011; Gengatharan, Dykes & Choo, 2015). In addition, studies have shown that natural colors are as effective as the colorants derived from chemical synthesis; moreover, they are safe and healthy and confer additional functional properties, e.g., antioxidative, antimicrobial and surface-active properties to the products to which they are added (Delgado-Vargas et al., 2003; Carocho, Barreiro, Morales & Ferreira, 2014; Rodriguez-Amaya, 2016).

The main colorant used in the manufacture of cheese and butter is the natural color annatto, and it is also used in other food products such as margarine, ice cream, soft drinks, sugar confectionary and fish products. *Bixin* is a coloring compound found in the seeds of annatto (*Bixa Orellana* L.) plants. More than 80% of *Bixin* consists of *cis*-bixin [methyl hydrogen (9'Z)-6, 6'-apocarotene-6, 6'-dioate) (Balaswamy, Rao, Satyanarayana, & Rao, 2006, Scotter, Wilson, Appleton, & Castle, 1998). However, the colorant used for cheese-making is mainly composed of the water-soluble component norbixin, which imparts the desired "yellow/orange" color to Cheddar and other cheese varieties (Campbell, Boogers & Drake, 2014; Cardeñosa, Lunar & Rubio, 2011). Although Cheddar cheese containing annatto is usually orange in appearance, commercially it is referred to as Red Cheddar; therefore, in this review, it will be referred to as red Cheddar. When used, annatto is usually added to the milk; however, during cheese production, around 10-20% of it is transferred into the cheese whey (Kang, Campbell, Bastian, & Drake, 2010; Smith, Li & Drake, 2014; Düsterhöft, Engels, & Huppertz, 2017). The carryover of annatto to whey has no direct impact on the flavor of derivative ingredients produced from whey, but must be eliminated from whey to obtain white and colorless spray-dried products (Campbell, Miracle and Drake, 2011).

Different approaches are used to eliminate the impact of colorant contained in bulk whey. The most common approaches in certain countries such as USA include decoloration with the bleaching agents benzoyl peroxide and hydrogen peroxide (Croissant, Kang, Campbell, Bastian & Drake, 2009; US FDA, 2011a,b; Fox, Smith, Gerard & Drake, 2013). However, decolorization using oxidation processes may affect flavor and functionality of whey protein ingredients (Zhang, Campbell, Drake & Zhong, 2015; Jervis et al., 2012). It may also result in oxidized pigment residues in the derived ingredients (Smith, Li, & Drake, 2014).

Although these two chemicals are permitted for bleaching of liquid whey in the United States, they are prohibited in Europe and Asia. Moreover, due to increasing regulation of these bleaching agents, alternatives such as the enzyme lactoperoxidase have been investigated (Kang et al., 2010; Campbell, Kang, Bastian & Drake, 2012). Furthermore, stringent regulatory requirements for use of whey-based ingredients in infant formula applications do not permit the presence of norbixin in these dairy ingredients (Carter, Park, & Drake, 2017).

The global demand for cheese and dairy products continues to increase, in particular due to the versatility of cheese in diverse culinary applications, its nutritional and sensory properties, and also due to westernization of diet in many regions in Asia. Retail sales of cheese for 2017 in the United States exceeded $23 billion (Mintel Group Ltd., 2018), of which Cheddar accounted for 29% of production, second after Mozzarella (ADPI, 2018). Similarly, the global market for whey products also continues to grow due to its nutritious value and versatile uses, particularly in infant milk formulae and sports nutrition applications. Thus, this creates a high demand for whey proteins derived from many diverse processes, including acid whey and sweet whey from cheese or rennet casein production. This also can include whey obtained from cheese-making processes in which annatto has been added to obtain ‘red’ cheese (Zhang, et al., 2015). The commercial value of colored whey is lower than that of “white” whey, but this may be improved on development of sustainable and universally acceptable means of color removal.

This review provides a comprehensive analysis of the literature and patented technologies regarding cheese colorants, as well as interactions between colorant and cheese components. Moreover, it aims to identify new solutions to incorporate color into cheese, such as after whey removal, to obtain clear and ideal whey while yielding cheese with uniform color to satisfy both consumers and dairy companies.

2. Color in cheese

In the global cheese market, many cheese varieties have various shades of color, in particular orange, white, blue, and yellow hues. Those colors are obtained from different sources and, despite the high number of cheese varieties, only a few have a uniform and homogenous color distribution; in fact, most cheese color is present only on the cheese surface, with internal veins or a marbled effect (Fox, Guinee, Cogan & McSweeney, 2017). For the purposes of this review, the terms dye, colorant and pigment are used interchangeably to indicate natural annatto and alternative colorants. However, orange cheeses including red Cheddar, Gouda, Prato and Mimolette have their appearance because of the addition of annatto dye as a food colorant into the milk at the start of the cheese making process.

Moreover, depending on the country where this cheese is sold, shades can range from bright yellow to deep orange. Annatto color is also added to milk during the making of Red Leicester, Double Gloucester, red Cheddar, Mimolette or Cheshire, and Prato cheeses.

Within the European Union, permitted colorants are authorized by the EFSA (European Food Safety Authority) for use in cheese making. Furthermore, the European Parliament and Council Directive 94/36/EC on colors for use in foodstuffs lists the permitted food colors (Komisyonu, 1994). In the case of cheese and other milk products, only certain permitted dyes may be added, with maximum levels being specified in the Directive (Table 1).

For yellow cheeses with no added colorant, largely bovine milk cheeses, the hue is attributed to compounds arising from the animal diet, particularly β-carotene originating from pasture feeding (Winkelman, Johnson & MacGibbon, 1999; Nozière, et al., 2006; Calderón et al., 2007; McDermott et al., 2016). This may also result in seasonal variation in the color of cheese because of varying amount of carotenoids in the grass or supplementation of diets with silage or total mixed ration (TMR). Moreover, cheeses ripened for 9 months showed a deeper and more intense yellow than fresh cheeses (Buffa, Trujillo, Pavia & Guamis, 2001) although this may be related to loss of moisture during ripening. To overcome variation in cheese color, addition of colorants to cheese milk, such as annatto, has long been an accepted practice (Kang et al., 2010).

Cheddar cheese is widely produced in the US, Canada, Ireland, UK, Australia and New Zealand and, for its red variant, annatto colorant is added to the cheese milk prior to coagulation. Addition of annatto during cheese manufacture may traditionally have been used to give part-skimmed cheese an appearance similar to that of a full-fat equivalent, while more recently some consumers are of the impression that colored cheeses taste better or are more intensely ripened than their equivalent white counterpart (Fox et al., 2017). A recent consumer study (Speight, Schiano, Harwood, and Drake, 2019) identified color as one of the key attributes influencing consumer choice, with higher utility score (more attractiveness towards an attribute) for orange color in comparison to white Cheddar (Speight et al. 2019). However, white cheeses were considered to be more "natural" because of the absence of annatto.

Caprine milk, unlike bovine milk, contains no β-carotene. Any β-carotene consumed by a goat is immediately converted into Vitamin A, which has no color; therefore, the derived cheeses have a bright white hue even after ripening (Fraga, Fontecha, Lozada, & Juarez, 1998; Parkash & Jenness, 1968; Park, Juárez, Ramos, & Haenlein, 2007).

Blue-mould-ripened cheeses such as Danablu, Gorgonzola, Roquefort, and Stilton (Cantor, van den Tempel, Kronborg Hansen & Ardo, 2017) have a typical veined appearance due to development of *Penicillium roqueforti* during ripening. The microflora present are complex, comprising both fungi and lactic acid bacteria. Blue veining also depends on changes in level of salt and water activity, also on the concentrations of oxygen and carbon dioxide within the cheese mass (Prieto, Franco, Fresno, Bernardo, & Carballo, 2000; Florez & Mayo, 2006; Diezhandino, Fernández, González, McSweeney & Fresno, 2015; Duval et al., 2016).

In addition to the above categories, there are many different cheese products with green, brown, red or other colors resulting from added ingredients, i.e., herbs, sage, spices, port wine, brandy, or arising from specific processes, i.e., smoking (Hayaloglu & Farkye, 2011; Guillén, Palencia, Ibargoitia, Fresno & Sopelana, 2011). Such cheeses are often specific to certain geographical areas of production and may also be artisanal products (Chandan, 2014; McSweeney, Ottogalli & Fox, 2017).

3. Annatto

Annatto is a food colorant derived from the seeds of the *Bixa orellana* plant, and is identified as food additive E 160b as approved by the EU. It is a unique carotenoid that can act as a pigment in more than one chemical form and is available as both lipid-soluble (bixin) and water-soluble (norbixin) forms. Formulations of commercial annatto extracts are available in stabilized forms in different color ranges i.e., from red to orange to yellow for use in a variety of foods, including dairy, fish, confectionery, beverages, meat product, snack foods, and dry mixes (Coulson, 1980).

The characteristic colorant compound present in annatto seeds (> 80% of the total carotenoid content) is the lipid-soluble diapocarotenoid 9' – *cis* – bixin. The water soluble form of this compound is the dicarboxylic acid 9' – *cis* – norbixin. Both norbixin and bixin are found in *cis* (α) and *trans* (β) geometrical isomeric forms. Smaller quantities of both *trans*-bixin and *cis*-norbixin are present in commercial bixin color solutions (Scotter et al., 1998), depending on the extraction conditions, e.g., process type and temperature used (Raddatz-Mota et al., 2017).

Under specific conditions of temperature and pH, bixin can be hydrolyzed into norbixin, and saponified into the potassium salt of norbixin (Figure 1). Saponification during the aqueous alkali extraction of norbixin removes the methyl group from the lipid-soluble fraction bixin. An intense red color indicates the presence of larger quantities of bixin, while a yellow to orange color indicates that norbixin is predominant in the annatto extract (Santos et al., 2014). The physico-chemical properties of annatto are summarized in Table 2.

Norbixin and bixin are the major carotenoids responsible for the yellow-red-orange color**,** although the water-soluble form, norbixin, is the main carotenoid used in cheese manufacture (Smith, Li, & Drake, 2014). Bixin is the nonpolar form of annatto and, because of its nonpolar nature, it is used to color high-fat dairy products such as butter (Lancaster and Lawrence, 1995).

However, the presence of highly conjugated π-bond structures in bixin and norbixin molecules make them susceptible to oxidation and reduction reactions. Oxidation of these compounds bleaches the color. However, the process of bleaching and oxidation of pigments is nonspecific, potentially also resulting in lipid oxidation (Carter & Drake, 2018), which may also adversely affect the flavor of derived dairy ingredients (Kang et al., 2010). Binding to milk proteins, i.e., β-casein or β-lactoglobulin, improves the oxidative stability of these colorant molecules (Govindarajan & Morris, 1973).

3.1. Manufacture of annatto and functional use

There are three main commercial processes that are commonly used to extract the pigment from the pericarp of dried annatto seeds: (i) direct extraction into oil; (ii) direct extraction into aqueous alkali; or (iii) indirect extraction using solvents. Depending on the methods employed, the products obtained have different amounts of bixin and/or norbixin and can be further processed into solution, suspension or powder for different purposes (Figure 2; Preston and Rickard, 1980; Wallin, 2006; PubChem, n.d.; Green, 1995). Direct extraction of the pigment compound into alkali or oil is a natural and environmental friendly way of manufacture, as it involves only mechanical shear. Conversely, extraction with solvents gives a better yield of pigment but may raise safety concerns due to potential chemical residues. Bixin can be obtained from direct extraction in oil as well by indirect extraction in solvents. Solvent-extracted bixin is in concentrated *cis*- form with little quantities of *trans* and *cis*-norbixin. However, extraction of annatto into aqueous alkali yields norbixin as the primary coloring molecule (Santos et al., 2014).

Therefore, annatto formulations for use as food additives vary due to the nature of the extraction method. Lipid-soluble annatto (bixin, dissolved in oil), water-soluble annatto (the dissociated form of norbixin in alkaline solution, usually potassium or sodium hydroxide) and emulsified annatto (norbixin and/or bixin in association with an emulsifier) are the chemical forms of annatto used to give various color shades in food matrices. Ultimately, the relative proportion of bixin and norbixin in commercial formulations determines the tone and hue of the color in the finished food products (Smith, 2006). More details of manufacturing methods are presented below (extracted from Smith, 2006):

(1) Oil-soluble annatto. These colors are produced when bixin is dissolved in a high quality oil of high purity (normally soybean oil, rapeseed oil, sunflower oil, with low free fatty acids and peroxides). The dissolution of annatto in oil can change its color shade from orange to yellow. Prolonged heating can degrade the bixin molecule into a C-17 compound that gives an intense yellow color. The form of annatto dissolved in oil typically contains 0.05-1.0% bixin, while oil-based annatto suspensions generally contain 0.1-8.0% bixin (Smith, 2006). Oil-based annatto colorant formulations are usually used to color fat-rich foods, e.g., margarine/shortening, processed cheese, biscuit fillings, snack foods, popcorn and sauces, salad dressing and dairy cream desserts.

(2) Water-soluble annatto. These colorant extracts usually contain norbixin as the principal component, and are commercially available either as dilute liquid alkaline solutions or as a powder. The aqueous liquid extracts generally contain 0.1-4.0% norbixin, while powder forms usually contain 1-15% norbixin (Smith, 2006). The liquid formulations are used for coloring cheese as well as other food products such as breakfast cereals, buttermilk desserts, chocolate fillings, sausages, puddings, smoked fish, tomato sauce and pet food. However, the powdered forms of norbixin can be applied in powered products such as instant desserts, dip mixes, food powders and dietary fiber products. Upon reconstitution in water, these dried products become yellow or orange yellow in color.

(3) Emulsified annatto. The addition of suitable emulsifiers to bixin or norbixin results in colors which may be miscible in both oil and water phases. These types of formulations are particularly suitable in food products containing both water and an oil phase. With an appropriate choice of emulsifiers, a water-soluble form of bixin/norbixin with improved acid stability can be obtained. Emulsified annatto products are liquid and usually contain 1-2.5% as bixin/norbixin (Smith, 2006). These colorants are normally used in emulsified dairy and food products such as ice cream, processed cheese, soup and confectionary. Acid-stable emulsified annatto is also used in acidic beverages, transparent jellies, liqueurs and gelatinous desserts. As is the case with all the other carotenoids, annatto extracts are also susceptible to oxidative degradation (Scotter et al., 1998).

3.2. Applications of Annatto, Bixin and Norbixin

According to the Directive 94/36/EC (Komisyonu, 1994) Annatto, Bixin, and Norbixin can be used in margarine, other fat emulsions, and anhydrous fats, flavored processed cheese, ripened orange, yellow cheese, unflavored processed cheese, edible cheese rind and edibles casings, Red Leicester cheese, and Mimolette cheese. Recommended levels of addition of annatto colorant in various dairy products are presented in Table 1.

3.3. Interactions of colorant with cheese components

Dairy processers predominantly use the water-soluble and dispersible norbixin annatto as cheese colorants. It is believed that, because of its water-soluble nature, norbixin is dissolved in the serum portion of the whey, and whatever color retained in the curd is because of entrapment of free water in protein matrix (Qiu, Smith, Foegeding, & Drake, 2015; Zhu and Damodaran, 2012).

Therefore, studies on the relative binding affinity of colorant with various components of cheese as well as of the serum phase such as milk fat globule membrane (MFGM), proteins and aqueous phase are important in order to understand colorant-protein phase interactions.

3.3.1. Colorant-aqueous phase interactions

If the colorant is dissolved in the aqueous phase and doesn't associate strongly with the proteins present in serum phase, it should be possible to completely remove norbixin from retentate by employing a series of protein purification (ultrafiltration) and washing (diafiltration) steps during the manufacture of whey protein concentrates (WPC) and isolates from the colored whey. However, most norbixin remains in the retentate, which suggests that, in whey, it might be tightly bound to whey proteins in the same way that it binds to caseins in cheese curd. This hypothesis is further supported by the fact that β-LG binds to retinol-molecules which have structural similarities to norbixin (Govindarajan and Morris, 1973; Hammond et al., 1975; Cho et al., 1994; Zhu and Damodaran, 2012).

3.3.2. Colorant-fat phase interactions

Zhu and Damodaran (2012), in their study concerning annatto in Cheddar cheese whey, suggested that norbixin molecules in solution, due to their amphiphilic nature, might be in the form of micelles dispersed in the aqueous medium, rather than in a "soluble" state. Therefore, should this hypothesis be correct, then the micellar norbixin would more likely to be associated with the MFGM particles than with globular proteins in Cheddar cheese whey (the association of norbixin with MFGM particles may occur *via* adsorption to the lamellar surface or *via* incorporation into the bilayer structure of the MFGM).

The above study reported that the original retentate and the diafiltered retentate were bright yellow but, when the MFGM particles were removed from the diafiltered retentate by selective precipitation at pH 4.2, the resulting supernatant was colorless. After freeze-drying, the MFGM powders were bright yellow, and the WPI was very white (Figure 3). This suggested that the annatto in the diafiltered retentate was primarily associated with the MFGM phase. Quantitative analysis showed that about 60% of annatto in whey was found in the MFGM phase, suggesting preferential binding to it, while the remainder was freely dispersed in the serum phase, either in solution or as micelles, which could be removed by diafiltration (Zhu and Damodaran, 2012). To confirm the presence of a colloidal (micellar) form of annatto in the aqueous serum phase and to understand its association with MFGM, a thorough study on particle size analysis and microstructural verification is required.

Moreover, according to Smith, Li and Drake (2014), nearly 81% of norbixin added to cheese milk was recovered in the curd portion of cheese. There could be a loss of quantity as well as pigmentation ability of norbixin due to exposure to heat and light during cheese-making. Higher losses of norbixin were observed during a fat-free cheese making process than that in making of a full-fat cheese; this was probably due to lack of opacity in the former because of a lack of fat. The opacity of whey originating from full-fat cheese protects norbixin from oxidation upon exposure to light.

Smith *et al.* (2014) further studied the partitioning of bixin and norbixin between cheese and whey fractions and found higher levels of norbixin (9.27%) compared to bixin (1.30%) in unseparated whey and a higher recovery of bixin (94.5%) in cheese than that of norbixin (80%). A very small proportion of bixin partitioned into the whey during cheese manufacture, because of its greater affinity towards the nonpolar fat component of cheese milk. Comparing the extraction efficiency (recovery) of norbixin from cheese and whey samples prepared from colorant-added unhomogenized and homogenized milk revealed nonsignificant differences; this indicates that greater surface area of fat globules due to homogenization did not affect partitioning of norbixin, possibly because of its hydrophilic nature, which suggests binding of norbixin to constituents of milk other than milk fat. After fat separation of whey, the losses were higher for bixin than norbixin, probably because of greater association of bixin with the fat phase, bixin being hydrophobic in nature (Smith et al., 2014). However, the findings reported by Smith et al. (2014) were partially in agreement with those reported by Zhu and

Damodaran (2012) and demonstrated that annatto is probably associated with the MFGM in the form of micelles dispersed in the whey. Therefore, norbixin is partially bound to fat globules, reflected by the decrease in norbixin levels upon fat separation of cheese whey originating from homogenized cheese milk compared to that from non-homogenized cheese milk.

The cheese obtained from milk with added bixin had lower L* and b* values, but higher a* values ($P<0.05$), indicating that cheese with bixin was more red and less yellow in comparison to cheese with norbixin (Smith et al., 2014) (Fig. 4). The whey obtained from cheese containing bixin had a lighter color, suggesting a possible use of bixin as an alternative colorant to produce red Cheddar, contingent on future studies necessary to optimize the concentration of bixin to optimize cheese color and to avoid the complication of requiring homogenization of cheese milk to create a stable dispersion of bixin in the serum portion of milk.

3.3.3. Colorant-protein phase interactions

To study the relationship between norbixin and milk proteins, Zhang and Zhong (2013a) investigated the molecular binding between norbixin and WPI, sodium caseinate and purified whey proteins using Fourier transform infrared spectroscopy (FTIR), fluorescence quenching, circular dichroism (CD) and differential scanning calorimetry (DSC). The quenching of the intrinsic fluorescence (due to presence of tryptophan, tyrosine or phenylalanine in the structure) emitting from WPI and sodium caseinate was because of the formation of a complex with the norbixin molecule. Sodium caseinate had higher binding affinity for norbixin than WPI. Based on the estimated K_{SV} (Stern-Volmer quenching constant, a value that correlates the affinity between molecules), the relative affinity was higher between norbixin and WPI than bixin and WPI, because water-soluble norbixin interacts more strongly with WPI than the lipid-soluble bixin. Considering the individual whey proteins and caseins, the *Ksv* of BSA was highest for whey proteins because of its unique structure, with six binding sites (Dockal, Carter, & Ruker, 1999), while the binding affinity between κ-casein and norbixin is higher than for the other two caseins (α_s and β), possibly because of the hydrophilic nature of κ-casein. Even though BSA has a strong affinity towards norbixin molecules, the overall effect on binding properties of WPI was not high, because of the small proportion of BSA (6%) in WPI.

Overall, these findings suggest that caseins have stronger binding affinity towards norbixin as compare with whey proteins, indicating a higher probability of retention of norbixin in cheese curd than the whey stream (Kang et al., 2010). The effect of pH on binding affinity of norbixin with WPI and sodium caseinate was limited in the pH range of 5.5-10.3. Increasing ionic strength to 0.5 M NaCl at pH 6.4 enhanced binding of norbixin to sodium caseinate, possibly because of weakening of electrostatic repulsion between protein molecules and norbixin, facilitating permeation of colorant molecule to the interior hydrophobic structure of caseins. The CD and FTIR data indicated that norbixin caused conformational changes in the structure of whey proteins and caseins, presumably through complex formation (Zhang and Zhong, 2013a).

Moreover, norbixin precipitates in acidic conditions (pH lower than 5) and can interact with other food components (Shumaker and Wendorff, 1998). It has been suggested by Daly, McSweeney and Sheehan (2012) that the negatively-charged norbixin may bind to positively-charged sites available on whey protein and casein molecules. At alkaline pH, the negatively charged dissociated form of norbixin can possibly bind with divalent cations (e.g. calcium) and form insoluble salts, ultimately leading to appearance of a pink precipitate on the surface of cheese. In addition, decreasing pH (pH $\leq$ 5.0) can also cause formation of a pink precipitate of norbixin. Resolubilisation of this pink precipitate by restoring pH (to alkaline pH) may have been prevented because of its interaction with the phospholipid and β-casein (Govindarajan and Morris, 1973; Daly, McSweeney and Sheehan, 2012). Alkaline annatto extracts have shown a greater tendency to cause pink discoloration during processed cheese manufacture as compared to other annatto extracts (Shumaker and Wendorff, 1998). Emulsion-based annatto colorants used in processed cheese formulations were more susceptible to pink discoloration compared to suspension-based annatto colorants (Shumaker and Wendorff, 1998; Zeheren and Nusbaum, 2000).

3.4. Volatile compounds in annatto-colored cheese and whey products

Galindo-Cuspinera, Lubran and Rankin, (2002) characterized the volatile compounds present in commercial annatto extracts (both oil- and water-soluble) by GC/MS analysis. The most abundant volatile compounds present were sesquiterpenes, which constituted about 38% of the volatiles present in oil-soluble extracts and around 89% in water-soluble extracts. The compositional difference between oil and water extracts is potentially due to the low solubility of monoterpenes and sesquiterpenes in water (Robinson, 1991). Besides sesquiterpenes, other volatile compounds present in commercial annatto extracts include monoterpenes and arenes. *β*-Humulene is the major volatile present in annatto extracts, followed by p-xylene, toluene, *α*– and *β*- pinene, γ-elemene, and spathulenol. Differences between oil and water extracts are potentially due to differences in the extraction methods. The numerous odorants found in colorant formulations may influence food aroma as well as imparting additional bio-functional properties, e.g., anticarcinogenic, antimicrobial, or antioxidant activities (Galindo-Cuspinera et al., 2002).

Detection of aroma compounds was also undertaken in food matrices, such as WPC, to determine possible correlations between flavor of WPC and either annatto added to the milk or starter cultures used in the cheese manufacture (Campbell, Miracle and Drake, 2011).

Degradation compounds of lipid oxidation were present in all WPC samples, although at higher levels in WPC produced from whey produced using starter cultures compared to WPC produced from rennet casein whey. WPC with annatto had higher concentrations of volatile compounds such as pentanal, *p*-xylene, decanal and diacetyl in comparison with WPC without annatto. Moreover, hexanal levels suggested a strong relationship between starter cultures and annatto suggesting that annatto may have an antioxidant effect when present in whey made with starter cultures. However, no direct evidence was found to support the suggestion that either the intact or oxidized form of annatto contributes directly to the flavor of whey ingredients (Campbell, Miracle and Drake, 2011) and this should be a subject of future research. A parallel evaluation was performed on WPC functionality, and no significant differences were observed for WPC solubility at all pH levels tested or for heat stability, irrespective of WPC being produced either with starter culture or annatto (Campbell, Miracle and Drake, 2011). Qiu et al. (2015) compared the effect of microfiltration (MF) with bleaching treatment of the whey on the volatile compounds of whey products (Figure 5) and found a significantly lower amount of lipid oxidation products in the WPC obtained from unbleached MF-treated whey. This indicates that the bleaching process increases the oxidative load in the whey stream because of the non-specific nature of the oxidation process (either enzymatic or chemical). This study also established that the annatto colorant is associated with either the fat or milk fat globule membrane and, therefore, MF can be used as an alternative to bleaching to remove annatto from the whey.

3.5. Color defects in Cheddar cheese

Annatto, as with all carotenoids, is sensitive to oxidation in foods, including cheese. Changes in pH and redox potential, light, oxygen, and temperature can generate color defects in cheese, including pink discoloration and color instability (Giuliano et al., 2003). In particular, because of the alternating sequence of single and double carbon to carbon bonds present in the polyene chain, annatto molecules are vulnerable to oxidation in the presence of oxygen and peroxides, and to instability because of temperature, light, and reactivity to acids.

Several external factors that interfere with food colorant stability and that adversely affect food attractiveness are the presence of light, air/oxygen, redox potential, pH, chemical structure, solvents, packaging materials and storage conditions (Jiménez-Aguilar et al., 2011; Zhu and Damodaran, 2012; Lemos, Aliyu, & Hungerford, 2012).

Color defects in both annatto-colored and mature white Cheddar ranging from pink to mud-brown have been observed sporadically. Moreover, bleaching of color in Cheddar can be observed after manufacture. Localized whey pockets entrapped between curd particles can cause bleaching, due to high acid production in the early stages of ripening (Daly, McSweeney and Sheehan, 2012). This bleaching effect diminishes with maturation and as pH increases.

Studies on the mechanisms of the development of these defects in Cheddar have not been conclusive. Oxidative browning may be linked with the activity of tyrosinase enzyme which catalyzes the oxidation of monophenols, particularly tyrosine to quinines leading to formation of red-colored dopaquinones and dopachromes *via* DOPA (3, 4-dihydroxyphenylalanine). These are subsequently are converted to a brown pigment melanin through a series of chemical reactions. Loss of pigmentation of the colorant molecule can also occur upon prolonged exposure to elevated temperature, light or to sulphur dioxide. The major thermal degradation products of annatto have been characterized as yellow-colored isomers and hydrolysis products of the *trans*-monomethyl ester of 4,8 – dimethyltetradecahexaenedioc acid (C17) (Scotter et al., 1998, Scotter, 1995; Preston & Rickard, 1980; Collins, 1992).

3.6. Regulations regarding annatto

Regulations for annatto use vary from country to country and have changed in recent years. Federal code 21 CFR73.30 (US FDA Code of Federal Regulations, 1963) regulates the use of annatto in foods in the United States and identifies color additives that are exempt from certification. Annatto and β-carotene are exempt from certification, but must still be declared on food labeling (Smith, 2014). As reported in the Code of Federal Regulations, annatto extract is considered safe, in general, for use in coloring foods, when used in amounts consistent with good manufacturing practice, although the source of the colorant formulation should be declared on labeling, e.g., derived from annatto seeds (Smith, 2014).

Annatto color added to milk is considered an additive and therefore is covered within the food additive European Union (EU) Regulation 1333/2008. Moreover, new regulations in the European Union (EU) do not permit color in whey destined for use in infant formula applications (CODEX Stan 72-1981; Smith, 2014). Food additives permitted in whey or whey based ingredients used in infant formulae are listed in the CODEX Stan 72-1981. Both EU Regulation 1333/2008, and 1129/2011, amending Annex II to Regulation on food additives, do not permit the carry-over of food additives e.g. food colors to various infant and baby nutrition products (Smith, 2014).

Given the dramatic growth of dairy and in consumption of infant milk formula in Asia, and particularly China, there is also a need to ensure that food products adhere strictly to regulatory standards in those countries. These regulations continue to evolve; therefore, it is advised to routinely check current standards for what is and is not permitted. Food additives permitted for use in infant formulae in China are listed in the National Food Safety Standard for Uses of Food Additives (2011: GB 2760).

4. Alternative cheese colorants

Many food colorant manufacture companies produce a wide range of natural food color preparations for application in food, drug and cosmetics industries. They also create targeted products for different purposes. Among natural colors are included annatto, saffron, paprika, beet red, β–carotene, turmeric, caramel, carmine, elderberry, anthocyanin, natural green extract (Products-Cyber color, n.d.). Organic colors include organic juice extract colors like blueberry and purple carrot or organic spices added for color like paprika and turmeric. Others color extracts derived from natural sources are β-carotene from beet and carrot.

Much recent work has focused on development of range of colorants for cheese applications that offer ease of use, dispersiblity in milk, and color stability, while still offering a clear whey with good flavor profile without requiring bleaching, and which are suitable for infant formula applications. A number of products are available commercially based on patented formulations comprising of β-carotene alone or combination of with paprika (see also Section 5.0).

4.1. Saffron

Use of Saffron (*Crocus sativus L.* stigmas) as a natural colorant in cheese is limited; saffron has a characteristic bitter taste arising from picrocin, whereas the aroma mainly comes from safranal (Carmona et al., 2006). The compounds responsible for its color are water-soluble carotenoids, known as crocetin esters. One of the best-known cheeses with added saffron is Piacentinu Ennesse (a hard cheese variety produced from ovine milk), in which saffron adds color as well as flavor and aroma. However, the addition of saffron to milk is more difficult than addition to water, due to the presence of fat emulsions and colloidal casein suspensions, and also due to inherent variations in physico-chemical properties of milk, as influenced by breed, feeding, milking system or lactation stage. For example, higher fat content results in a yellower color in cheese samples prepared from milk with added saffron (Licón et al., 2012b). These variations in color of saffron-added milk could be attributed to a proportionate increase in colorant concentration in the serum phase with increasing fat concentration. Water-soluble compounds of saffron, e.g., crocetin esters, can interact with hydrophilic/amphiphilic components of the serum phase, e.g., whey proteins, MFGM proteins and phospholipids (Livney, 2010; Licón et al., 2012b). In order to understand the nature of interactions between various milk-saffron components, further research is required. Licón et al. (2012a) established that saffron addition to milk results in a lower uptake of salt into cheese, and more deformable and less elastic textural properties than control cheeses, without affecting compositional, microbiological, textural, and sensorial characteristics. Cheeses with saffron were less bright, less red and yellow than control cheeses, with significantly higher b* values (Hunter Lab system). Similarly, b* values for the cheeses with Saffron were similar to those of Cheddar cheese shreds colored with annatto, whereas a* values were lower and L* values were higher, giving a more yellow than red color (Colchin et al., 2001). Sensory evaluation also showed flavor differences between the cheeses (Licón et al., 2012a).

4.2. Paprika

Paprika extract is typically a powder with a deep red color and pungent flavor by grinding dried pods of sweet pepper (*Capsicum annum)*. Paprika contains mainly capsanthin and capsorubin carotenoids occurring primarily as esters of lauric acid (Delgado-Vargas and Paredes –López, 2003). According to the American Spice Trade Association (ASTA), paprika quality is specified as intensity of color, i.e., the absorbance of light at 460 nm in an acetone extract. The fat/oil-soluble version of Paprika extract (oleoresin) is obtained by using hexane as solvent. The most common food use of paprika is as a spice in savory products. The use of paprika powder and oleoresin is permitted as a general food coloring agent in the USA (US FDA, 1999; Delgado-Vargas and Paredes –López, 2003).

There are limited reports on use of paprika in natural cheese. A mixture of oil and paprika is periodically applied to the surface of the caprine cheeses *Ibores* (Mas et al., 2002) and *Majorero* (Fontecha, Peláez, Juárez, & Martín-Hernández, 1994). However, paprika is more commonly used in processed cheese varieties to adjust their color or flavor (Tamime, 2011).

4.3. β-carotene

The pigment β-carotene is naturally present in milk drawn from grass-fed lactating cows. Use of β-carotene to color cheese has been successful in the past (Chapman et al., 1980). β-carotene can be a useful cheese colorant molecule because of its natural presence in milk, and also offers a color which appeals to the customer and does not produce off-flavors during the ripening of the cheese (Berglof & Kjell, 1963; Chapman et al., 1980).

β-carotene can be synthesized chemically or extracted from natural sources using food-grade solvents. Additionally, β-carotene plays a significant role in human health because it is a precursor to vitamin A and, when ingested along with vitamin C and E, can prevent cardiovascular disease or cancer (American Dairy Products Institute, 2015). These additional health benefits also support the use of this substance over annatto.

Addition of β-carotene preparations to milk or water-based complex foods can be challenging because of their fat soluble nature (Klaui et al., 1970), and thus they should be added in a pre-emulsified form or should be added to a portion of cheese milk followed by a homogenization step. Manufacture of some dairy products e.g., cheese and butter leads to carry-over of constituents or food additives such as colorant molecules from milk to its derived products. Given that carotenoids such as β-carotene are fat-soluble, they tend to associate with milk fat components (Noziere et al., 2006), therefore resulting in less carry-over to the whey.

4.4. Lutein

Lutein (3,3'-dihydroxy-α-carotene) is a xanthophyll, a carotenoid found in green leafy vegetables and yellow carrots. Lutein is a natural yellow-red colored fat-soluble pigment with antioxidant properties. Regular intake of lutein helps to prevent age-related macular degeneration (AMD) disease (Sobral et al., 2016). Lutein, as a food colorant, has been successfully added to cream cheese (Tokusoglu, 2013), yoghurt (Domingos et al., 2014), Cheddar cheese (Jones, Aryana, & Loss, 2005) and yellow colored Brazilian Prato cheese (Kubo et al., 2013; Sobral et al., 2016). Sorbal et al (2016) successfully replaced annatto (bixin) with lutein without affecting the sensory quality and consumer acceptance of Prato cheese.

5. Patents covering cheese colorants

Much effort has been focused on developing patented technology platforms for obtaining uniform color distribution in cheese, including the development of new colorant solutions to achieve clear whey. In particular, a range of patents exists related to annatto colorants or other colorants/products suitable for use in cheese production, which will be considered here.

5.1. Annatto-based colorants

A process was developed for the uniform coloration of cheese that involves binding water-soluble annatto colorant with a renaturable casein carrier which has an affinity towards the curd portion during cheese manufacture, therefore, improved partitioning of color between curd and whey and resulting in whey with less or substantially no color contamination (Talbott, 2002;US 6458394 B1). Because the annatto colorant is delivered through a renaturable and hydrateable casein support, it is substantially uniformly and homogeneously distributed within the curd portion of the cheese. By using the renaturable affinity support, it is claimed that little, if any, of the water-soluble colorant is distributed in the whey fraction, which then can be used in other applications.

Hettiarachchy et al. (1987) describes a method for the loading and stabilization of natural pigment complexes including ethyl bixin, i.e., annatto (US Patent 4699664; EP 0200043 B1). The colorants are claimed to have improved stability against oxygen, heat, light and moisture degradation. The formulation was preparing by forming a complex between the pigment and a hydrocolloid such as pectin, gums and modified celluloses. Polyvalent metal cations are used to connect these two structural elements, and are soluble salts of calcium, magnesium, zinc, and copper. The stabilized colorants can be incorporated into cheese, beverages, and processed foods.

Acid-soluble annatto colorant was developed in a powdered form by Schmidt (1985; US 4548822 A). The method involves mixing of an alkali-soluble annatto extract with an aqueous dispersion of a dextrinized starch derivative followed by drying.

Tan and Foley (2002) recovered valuable colorant compounds from *Bixa orellana* byproducts (US 6350453 B1). A byproduct of *Bixa orellana* seed components in the form of an oily material consisting of tocotrienol and geranylgeraniol was obtained after removal of the bulk of annatto color using aqueous or solvent extraction techniques. Furthermore, this byproduct contains healthy components such as tocotrienol and geranylgeraniol components, which act as antioxidants.

5.2. Carotenoid- based colorant

Johnson et al. (1992) developed a β-carotene emulsion formulation for coloring cheese. The formulation for coloring cheese curd without loss of color in the cheese whey comprises β-carotene, a fat, a caseinate and an aqueous solution of gelatin (CA 2052412 A1).

An improved colorant formulation for coloring cheese curd (Sexton et al., 2010; EP 2009066997) was developed. The formulation is a combination of an oil phase comprising of paprika, carotenoid, and a fat phase, and an aqueous phase comprising of sodium caseinate or acid casein. The colorant composition selectively colors the cheese curd while leaving the whey fraction uncolored.

An improved cheese colorant formulation was developed by Moeller et al. (2012) with the aim of providing cheese with the same color as that of annatto-colored cheese by delivering β-carotene in a structured carrier (US 20140113027 A1). The formulation is based on creating a liquid coloring mixture containing two separate preparations. For the first preparation, fat-soluble carotenoid is dissolved in an oil phase which is subsequently emulsified (O/W) in an aqueous phase using a suitable emulsifier; for the second preparation, water-dispersible carotenoid particles are encapsulated with a suitable hydrocolloid, to achieve dispersion in the aqueous phase and thereby be miscible within the continuous phase of the oil-in-water emulsified carotenoid preparation.

5.3. Other processing interventions

Kempeners and Köllmann (1991) developed a process (EP 0 492716 A1) for treating cheese with liquid (brine or a solution containing substances such as flavorants, colorants, enzymes, proteins, vitamins, minerals, etc) prior to ripening. The liquid is sprayed with a high-pressure nozzle (around 15 – 35 MPa) against the surface of cheese kept in a stationary condition such that the liquid penetrates the cheese surface. The diameter of nozzles is not more than 0.3 mm, and the penetration depth is dependent on the operating pressure and the position of the nozzles, as well as the injected medium. Increasing the temperature of cheese or injecting warm brine accelerated the diffusion process.

Damodaran (2014) patented a method (US 8771772 B2) of selectively separating milk fat globule membrane (MFGM) fragments and/or milk fat globules from whey. The method involves the addition of a whey-soluble zinc salt and adjustment of the pH of whey to less than 6.0. The zinc salt added to the whey precipitates milk fat globule membrane fragments and milk fat globules from the whey. Selective removal of MFGM from whey can also help in the removal of annatto, as the colorant molecules are strongly associated strongly with MFGM material (see Section 7 for more details).

6. Analytical methods for cheese colorants

Numerous analytical methods have been developed to determine annatto content in various food materials (Table 3). Spectrophotometric and HPLC analysis are the main methods of annatto color quantification in various food materials (Scotter, 2009) and, in addition to these methods, interactions between colorants and caseins can be evaluated *via* intrinsic fluorescence quenching, differential scanning calorimetry (DSC), Fourier transform infrared (FTIR) spectroscopy, and circular dichroism (CD) (Zhang & Zhong, 2013a). In general, the estimation of various binding parameters including binding/affinity constants, change in enthalpy ΔH and Gibb's free energy ΔG, and Stern-Volmer constants is important in understanding the stability of the colorant-protein complex (Zhang & Zhong, 2013a; Santos et al., 2014). Moreover, these parameters are good indicators of the type of interactions (e.g., hydrophobic, hydrophilic, electrostatic etc.) of colorant molecules with other constituents present in food matrices. For example, β-CN micelles obtained from camel milk interact with curcumin mainly through hydrophobic interactions, which increases the solubility of curcumin and its bioavailability and antioxidant activity (Esmaili et al., 2011). Curcumin is a natural spice with potential cancer-therapeutic attributes (Sahu et al., 2008).

Standard microcopy equipment used for elucidating cheese microstructure includes light microscopy (LM), confocal scanning laser microscopy (CSLM), and electron microscopy such as scanning (SEM) and transmission electron microscopy (TEM) (Everett and Auty, 2008; El-Bakry and Sheehan, 2014). However, it is not possible to observe the presence of colorant in the cheese microstructure unless a probe (e.g., fluorescent probe) is used or an advanced analytical tool (e.g., Raman spectrometer; XRF etc.) is combined with these techniques to detect such molecules (Burdikova et al., 2015).

7. Color removal *via* microfiltration

Microfiltration (MF) is a pressure-driven membrane separation technique that selectively retains larger components of the feed and concentrates them in the retentate, while low molecular weight species/soluble compounds pass into the permeate (Soodam and Guinee, 2018). The process involves passage of the liquid components under relatively low pressure (~100 kPa) across a semipermeable membrane with pore sizes ranging from 0.2 to 5 μm (Olesen and Jensen, 1989). MF can also be used to recover micellar caseins (MCC) from milk serum using membranes of ~0.15 μm pore size; the composition of such permeate is similar to that of whey. Native whey proteins present in MF permeate can be recovered using ultrafiltration (UF) process. UF permeate (Steinhauer, Marx, Bogendörfer, & Kulozik, 2015) which contains mostly lactose and minerals, can subsequently be utilized for standardizing cheese milk adding it to cream, MCC. Therefore, microfiltration is considered an effective and efficient way of recovering native whey proteins from the milk before cheese making (Soodam and Guinee, 2018).

Zhu and Damodaran (2012) suggested that, in solution, norbixin might be found in the form of micelles dispersed in the aqueous medium (such as cheese whey), and thus may be associated with residual MFGM material present in the whey rather than being completely in a soluble state. Therefore, a MF process might physically remove norbixin micelles. In fact, the molecular weight cut-off of MF membranes (8 kDa) allows whey proteins (β-LG, α-LA and BSA), lactose, minerals, and water to pass through the membrane, while bacteria, fat, CN particles and fines, and large whey proteins (i.e., immunoglobulins) are retained in the retentate (Qiu et al., 2015).

Considering the above fact, Qiu et al. (2015) evaluated the efficacy of microfiltration for removal of norbixin from whey. The residual norbixin content of WPC80 produced from whey treated with MF (9.6 mg/kg of solids) and hydrogen peroxide (HP) treatment (9.4 mg/kg of solids) was significantly higher as compared with the lactoperoxidase (LP) treatment (1.2 mg/kg of solids). The reduction in norbixin content of WPC80 because of treatment of whey with MF and HP (~40%) and LP treatment (92.8%) was significantly lower as compared to that of the control whey (~17.25 mg norbixin per kg of solids). The residual content of norbixin in WPC80 powder produced from MF-treated whey indicates some level of norbixin binding to the other (than MFGM or fat) whey components, i.e., proteins or minerals. Further detailed studies are recommended to identify the type of interactions and to establish their consequences for protein functionality. Color, flavor and functionality of WPC80 obtained from MF-treated whey were compared with untreated whey and whey bleached with HP or lactoperoxidase (LP). MF treatment of fluid whey reduced yellowness in WPC more than HP or LP treatments and achieved improved clarity and lightness of whey may be due to removal of fat by the MF process. The MF-treated WPC, based on sensory analysis and volatile compound analysis, was characterized by an increase in sweet aromatic flavor and a lower concentration of lipid oxidation compounds as compared to that obtained from bleaching with HP and LP. On the other hand, the strong oxidizing nature of the LP enzyme destroys conjugation bonds and therefore, causes significant loss of norbixin, with concurrent lipid oxidation and off flavors (Jervis & Drake, 2013).

8. Structured delivery systems for colorant molecules

A variety of molecules and ions can bind to milk proteins with different degrees of affinity and specificity; in particular, hydrophobic molecules bind to milk proteins by several bonding mechanisms, such as hydrophobic interactions, van der Waals attraction forces and hydrogen bonds.

Among milk proteins, because of their relatively open structure, caseins are more prone to binding small components than whey proteins, and naturally bind calcium and calcium-phosphate nanoparticles. In fact, caseins are rich in proline residues, have distinct hydrophobic and hydrophilic domains, and therefore are present in rheomorphic open structures. These proteins assume favorable structural conformations in aqueous media that make the system thermodynamically stable; around 95% of the caseins are naturally self-assembled into casein micelles, which are spherical colloidal particles of 50-500 nm (average 150 nm) in diameter, dispersed in the serum phase of milk. The relatively open structure of the caseins makes various compounds to bind to accessible regions. Therefore, the casein micelle is an example of a natural nanovehicle for delivery of nutrients (Fox and McSweeney, 2003). For example, Semo et al. (2007) used casein micelles as a nanocarrier for delivery of hydrophobic nutraceuticals (vitamin D). Vitamin D was bound to soluble caseinate through a ligand, and casein micelles were reformed by reinstating the original salt balance of milk.

Table 4 presents various delivery methods used in the past to deliver colorant molecules. Casein micelles were used successfully for delivery of curcumin, a natural food colorant, (Sahu et al., 2008). Because of its hydrophobic nature, curcumin specifically binds to non-polar regions of the casein structure and is unaffected by either the presence or absence of colloidal calcium phosphate (Rahimi Yazdi & Corredig, 2012).

Nanoencapsulation of β-carotene in a casein–graft-dextran copolymer complex was demonstrated by Pan, Yao, and Jiang (2007) using hydrophobic interactions and a copolymer fabricated by Maillard reaction. The spherical core-shell nanocapsules ranged in size between 175-300 nm depending on pH and loading ratio (Pan et al., 2007). Conjugates obtained from casein and whey protein interactions were also used as effective encapsulating materials for delivery of fish oil in processed cheese of superior sensory quality compared to controls (Ye et al., 2009; Livney, 2010).

Zhang and Zhong (2013b) produced bixin powder by spray-drying of a mixture of sodium caseinate and bixin dissolved in aqueous ethanol solutions. The physicochemical properties of the bixin powder were characterized, including encapsulation stability, particle size, color indices, FTIR spectra, and fluorescence. Encapsulation improved the stability of bixin in acidic conditions. However, b* values (yellowness) after reconstitution were found to be lower than that of the control. Samples showed precipitation at pH values close to the isoelectric point of casein (pH 4.6), thus soluble soybean polysaccharide (SSPS) was added to stabilize the dispersion (Liu, Nakamura, and Corredig, 2006) as, at a pH near its pI, SSPS absorbed onto casein particles and stabilized them against aggregation (Zhang and Zhong, 2013b).

Ravanfar, Celli and Abbaspourrad (2018) developed a novel core-shell-structured, enzyme-triggered microcapsule that selectively carries bixin molecules to the cheese matrix, resulting in colorless whey. The core of the microcapsule consists of κ-carrageenan gel matrix that can physically entrap the bixin molecule (Figure 6). The core is coated with a double-layered shell made with an inner solid lipid layer (composed of beeswax, palmitic acid and lecithin) and an outer casein (sodium caseinate)-poloxamer 338 layer. The outer casein-polymer layer provides structural integrity and stability during transport to the site of action and can be tailored to ensure selective binding of the microcapsule to the casein fraction during cheese-making. The presence of lipase in the system triggers release of the colorant molecules from the core of microcapsule after hydrolysis of ester bonds in the lipid layer during ripening. The efficacy of the colorant delivery was tested during cheese-making from non-renneted but acidified cheese milk (pH 4.6) and after ripening of the cheese at 26°C for 14 days. The results indicated that the most of the colorant was retained in the curd portion and that the whey was almost colorless. This approach may have significant advantages over the existing bleaching method of decolorizing whey, provided the method is applicable to standard cheese making systems comprising of starter inoculation, rennet coagulation and subsequent ripening of the cheese.

However, it is likely that non-dairy ingredients, including beeswax, palmitic acid, lecithin, κ-carrageenan, and poloxamer 338 polymer molecules, will enter into the whey stream and to any subsequent products to which the concentrated whey products will be added. In particular, this may be an issue for whey products used in the manufacture of infant milk formula. These additional non-dairy ingredients need to be declared on the label to meet the legal requirements of the market. Regulatory approval for the use of these ingredients for the manufacture of Cheddar cheese may be required in the longer term.

In a similar approach, Celli, Ravanfar, Kaliappan, Kapoor and Abbaspourrad (2018) entrapped norbixin into casein-chitosan (oppositely charged biopolymer) complexes prepared based on electrostatic interactions. The affinity of these complexes is expected to be strong towards the cheese curd. When negatively charged casein molecules come into contact with positively charged annatto-containing chitosan solutions, electrostatic complexes form. Relatively low encapsulation efficiency (~38.2%) of annatto molecules suggests either insufficient concentration of chitosan or the possibility of electrostatic repulsion between casein and chitosan, as the former may become positively charged below its pI. The delivery system was applied to acid-coagulated milk. Whey powder obtained from the acid-coagulated milk with casein-chitosan-complex-treated annatto samples exhibited improved color characteristics compared to that obtained with annatto powder, suggesting that this could considered as another approach for elimination of colorant transfer to the whey. More research is needed to ascertain the efficacy of this method for retention of color molecule in rennetted cheese curds and its impact on cheese ripening behavior. Labeling requirements must also be considered while designing further formulations.

9. Conclusions and future perspectives

Carry-over (~ 20%) of colorant molecules (such as norbixin) into whey during cheese making is an issue for whey products, in particular those destined for infant milk formula applications. Considerable attempts have been made in the past to find sustainable solutions for producing clear whey streams without compromising with final cheese quality. Most of these solutions are based upon either bleaching of the color present in the whey, developing alternative colorant formulations which bind more strongly with the cheese matrix, leaving only traces in whey streams, or recovering whey components from the milk using microfiltration before cheese making.

Use of alternative colorant solutions such as β-carotene, paprika, and saffron is a potentially viable option. Similarly, the development of structured delivery systems for specific delivery of regular annatto fractions or alternative colorants to the cheese curd is an area which has been actively pursued in the recent past. However, the effect of these delivery systems on cheese characteristics would benefit from further published studies to facilitate the uptake of these novel technologies in the near future. Application of microfiltration of milk will generate additional liquid streams with the need for further processing; however, it may also offer opportunities to develop novel dairy ingredients from these intermediate streams. A thorough study is required to explore potential use of microfiltration for elimination of color transfer to the whey and to present a detailed business case scenario.

Diffusion of color into the curd particles post-whey-withdrawal can be a most practical and novel approach to ensure no contact of the colorant with the whey. However, it is unclear what factors affect colorant diffusion in cheese matrices, as few studies have been conducted on this aspect. Inward diffusion of small solutes such as colorant molecules in the cheese matrices should be studied under diverse physico-chemical environments. The impact of colorant diffusion on cheese quality, texture and sensory characteristics should be investigated.

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11. Author's Contributions

Prateek Sharma and Annalisa Segat undertook the literature search, wrote the manuscript drafts, circulated and discussed the previous findings with co-authors. Alan Kelly and Diarmuid Sheehan read the manuscripts thoroughly, and provided critical inputs to improve the quality of work.

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Table 1a Permitted color and maximum levels in different food applications

Foodstuffs	Permitted color	Maximum level*
Red Leicester cheese	E 160b Annatto, Bixin, norbixin	50 mg/kg
Red marbled cheese	E 120 Cochineal, Carminic acid, Carmines	125 mg/kg
	E 163 Anthocyanins	*Quantum satis**
Mimolette cheese	E 160b Annatto, Bixin, norbixin	35 mg/kg
Ripened Orange, Yellow and broken-white cheese: unflavored processed cheese	E 160a Carotenes	*Quantum satis*
	E 160c Paprika extract	*Quantum satis*
	E 160b Annatto, Bixin, norbixin	15 mg/kg
Morbier cheese	E 153 Vegetable carbon	*Quantum satis*
Sage Derby cheese	E 140 Chlorophylls, chlorophyllis	*Quantum satis*
	E 141 Copper complexes of chlorophylls and chlorophyllins	
Butter (including reduced-fat butter and concentrated butter)	E 160a Carotenes	*Quantum satis*[1]
Margarine	E 160a Carotenes	*Quantum satis*
	E 100 Curcumin	*Quantum satis*
	E 160b Annatto, Bixin, Norbixin	10 mg/kg

Source: Council Directive 94/36/EC; Komisyonu, 1994

* In the Annexes to this Directive *'quantum satis'* means that no maximum level is specified. However, coloring matters shall be used according to good manufacturing practice at a level not higher than is necessary to achieve the intended purpose and provided that they do not mislead the customer.

Table 1b Potential food colorants which may be considered for application in cheese

Foodstuffs	Colorant
Ripened cheeses	Lutein from *Tagetes erecta*, Caramel II, sulphite caramel, Curcumin, Zeaxanthin, synthetic
Ripened Cheese, includes rind	Caramel IV – sulfite ammonia caramel, Carmines, Carotenoids, Paprika extract, Riboflavins, Chlorophylls and chlorophyllins, copper complexes,

Table 2 Chemical and physical properties of annatto (norbixin and bixin) and β-carotene

	Norbixin	**Bixin**	**β-carotene**
Chemical name	*cis*-Norbixin: 6,6'-Diapo-Ψ,Ψ-carotenedioic acid *cis*-Norbixin dipotassium salt: Dipotassium 6,6'-diapo-Ψ,Ψ-carotenedioate *cis*-Norbixin disodium salt: Disodium 6,6'-diapo-Ψ,Ψ-carotenedioate*	*cis*-Bixin: Methyl (9-*cis*)-hydrogen-6,6'-diapo-Ψ,Ψ-carotenedioate*	β-carotene
Molecular weight	380.47672 g/mol (acid) 456.7 (dipotassium salt), 424.5 (disodium salt)	394.511 g/mol	536.888 g/mol
Molecular formula	$C_{24}H_{28}O_4$ $C_{24}H_{26}K_2O_4$, $C_{24}H_{26}Na_2O_4$	$C_{25}H_{30}O_4$	$C_{40}H_{56}$
Color	Yellow-red solutions or powder/Extract	Orange crystals or Dark red-brown to red-purple powder*	Red to brownish-red crystals or crystalline powder
Solubility	Soluble in alcohol, ether, oil In water: 5.23*10-13	Insoluble in water, slightly soluble in ethanol Soluble in oil	Soluble in benzene, chloroform, carbon disulfide;

	mg/L at 25 °C pKa-4.8		Moderately soluble in ether, petroleum ether, oils; very sparingly sol in methanol and ethanol; practically insoluble in water, acids, alkalies. 0.6 mg/mL in water; 2 mg/L in ethanol; Soluble in acetone and vegetable oils
UV/VIS absorption	Sample in 0.5% potassium hydroxide solution shows absorbance maxima at about 453 nm and 482 nm*.	The sample in acetone shows absorbance maxima at about 425, 457 and 487 nm; at 469 nm in chloroform and at 456 nm in ethanol. (Chapman, Thompson & Slade, 1980)	Max absorption (chloroform): 497, 466 nm; (benzene): 278, 364, 463, 494nm; (ethanol): 456, 484 nm; (cyclohexane) 453 nm to 456 nm
Fluorescence	$\lambda_{excitation}$ 381 nm[$] $\lambda_{emission}$ 474 nm[$]	$\lambda_{excitation}$ 360 nm[$] $\lambda_{emission}$ 570 nm[$]	-
Vapor pressure	$1.75X10^{-11}$ mm Hg at	-	$1.8X10^{-11}$ mm Hg at

	25 °C	25 °C (est)

*Source: PubChem, n.d., * Joint FAO/WHO, 2007, [$]Santos et al., 2014*

Table 3 Methods of analysis of annatto

Samples	Method	Analyte (s)	References
Cheese and Margarine	Two-dimensional thin layer chromatography	Bixin, norbixin and beta-carotene	Montag, 1962
Cheese, margarine and hard candy	Solvent extraction followed by high performance liquid chromatography (HPLC)	Bixin and norbixin	Lancaster and Lawrence, 1995
Sodium caseinate dispersions	UV–vis spectrophotometer	Bixin	Zhang and Zhong, 2013a,b
Cheese, butter and ice-cream	Solid phase extraction (SPE) followed by HPLC and spectrophotometry	Bixin and norbixin	Bareth, Strohmar and Kitzelmann, 2002
Cheese	Derivative spectroscopy and HPLC	Bixin and norbixin	Luf and Brandl, 1988
Annatto solutions and Bread	Fluorescence spectroscopy and photothermal	Bixin and norbixin	Santos et al., 2014
Cheese containing color loaded micro-capsules	UV–vis spectrophotometer and FTIR spectroscopy	Bixin	Ravanfar, Celli and Abbaspourrad, 2018
Cheese and whey	SPE + HPLC	Bixin and Norbixin	Campbell et al., 2012; Smith et al., 2014
Freeze dried whey fractions	UV–vis spectrophotometer	Norbixin	Zhu and Damodaran, 2012
WPC 80	HPLC	Norbixin	Campbell et al., 2014; Qiu et al., 2015; Carter et al., 2017

WPI, Sodium Caseinate,	Fluorescence quenching, FTIR spectroscopy	Norbixin	Zhang and Zhong, 2013a,b
Casein-chitosan complexes	UV–vis spectrophotometer, FTIR	Norbixin	Celli et al., 2018
Cheddar cheese	Quantum yield	Norbixin, β-carotene	Petersen, Wiking and Stapelfeldt, 1999
Cheddar cheese	Thin layer chromatography	Bixin and norbixin	Govindarajan and Morris, 1973
Cheese	Confocal raman microscopy	β-carotene, paprika	Burdikova et al., 2015; Smith et al., 2017
Food coloring formulation	Mass spectrometry with: RP-HPLC; fast atom bombardment (FAB); matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), etc.	Bixin and norbixin, β-carotene	Scotter et al., 1994 and 1995; Kelly et al., 1996; Guaratini et al., 2004; Vetter and Meister, 1985; Felicissimo et al., 2004; Bittencourt et al., 2005; Breithaupt, 2004; Galindo-Cuspinera and Rankin, 2005; Noppe at al., 2009
Colorant solutions	^{1}H and ^{13}C NMR; X-ray crystallography	Bixin family of apocarotenoids	Kelly et al., 1996; Scotter et al., 1994.

Table 4 Delivery methods used for colorant delivery into cheese matrices.

Colorant molecule	Mode of delivery	Type of vehicle	Considerations	References
Curcumin	Nanoencapsulation	Casein micelle	Stability of molecule in aqueous environment.	Sahu et al., 2008
β-carotene	Nanoencapsulation by hydrophic interactions	Maillard reaction casein–graft-dextran copolymer based spherical core-shell nanocapsules.	Enzyme (pepsin/trypsin) triggered release in liquid system.	Pan et al., 2007
Bixin	Physical entrapment	Sodium caseinate; soluble soybean polysaccharide	Reduced color intensity; off-flavor and aggregation issues.	Zhang and Zhong, 2013b; Liu, Nakamura, & Corredig, 2006
Bixin	Microencapsulation, lipase induced delivery	Core-shell-structured, enzyme-triggered microcapsule	Use of non-conventional additives e.g. poloxamer 338, beeswax, palmitic acid and lecithin.	Ravanfar, Celli and Abbaspourrad, 2018
Norbixin	Electrostatic interactions	Casein-chitosan (oppositely charged biopolymer) complexes	Unknown impact on cheese flavor and texture. Additional labeling requirement for chitosan.	Celli, Ravanfar, Kaliappan, Kapoor and Abbaspourrad. 2018
β-carotene	Emulsion and entrapment	Sodium caseinate, emulsifier and hydrocolloid	Not available	Moeller et al., 2012
β-carotene and paprika	Complex formation and emulsification	Caseinate and oil phase.	Not available	Sexton et al., 2010
Bixin	Solvent-mediated pressure treatment	Complexion of bixin with casein micelle	Residues of solvent phase and bixin in whey	Celli, Lawrence, P., Ravanfar & Abbaspourrad, 2019

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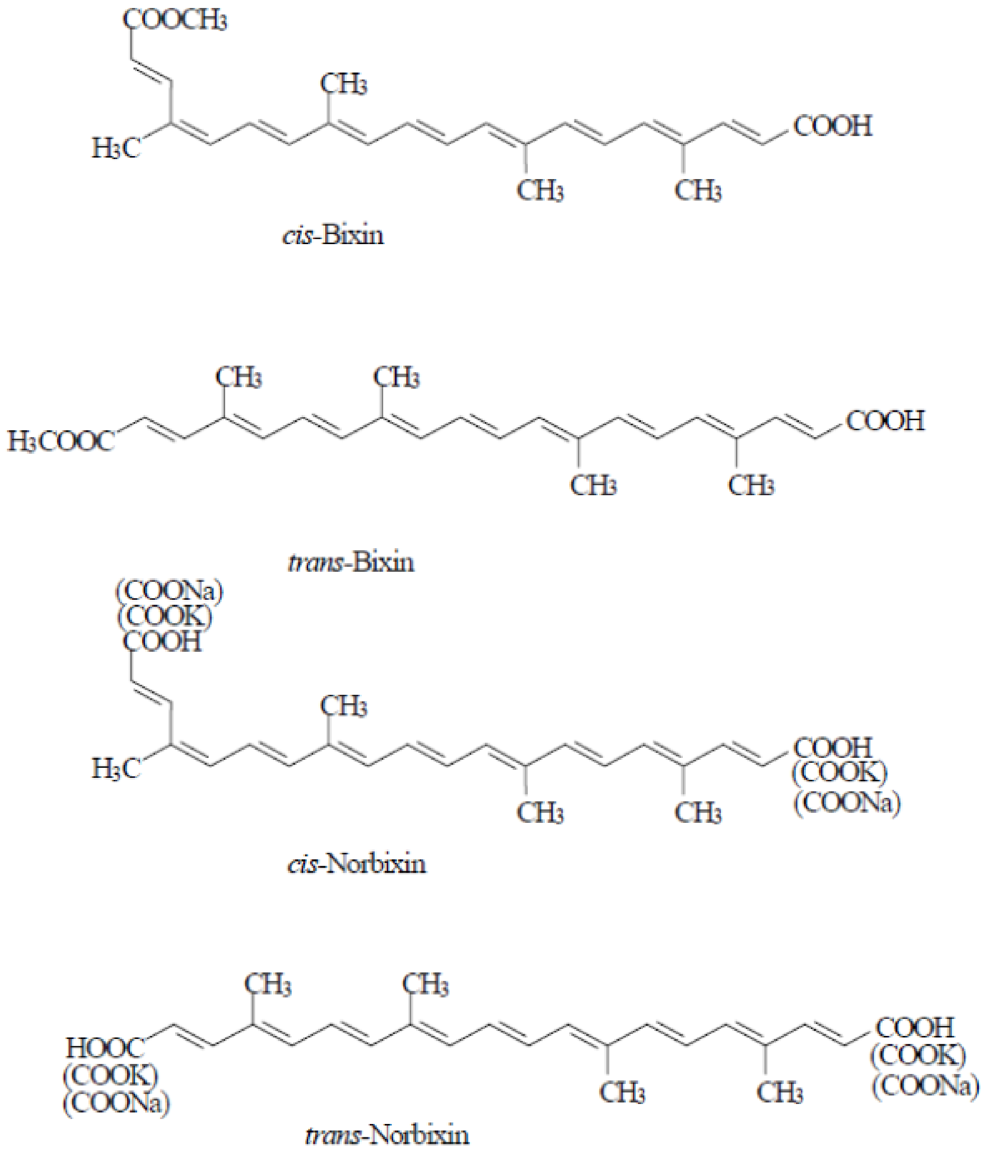

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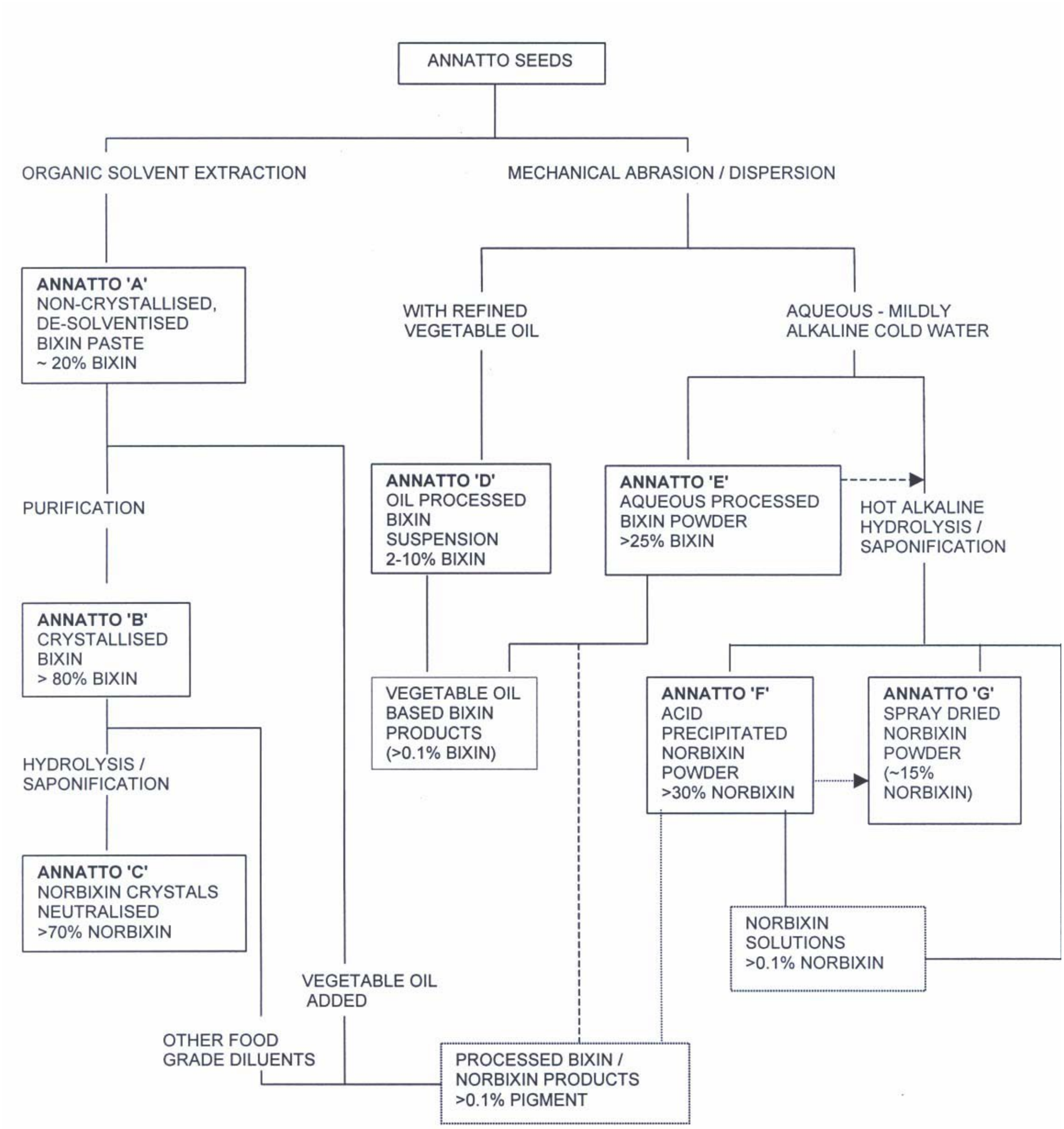

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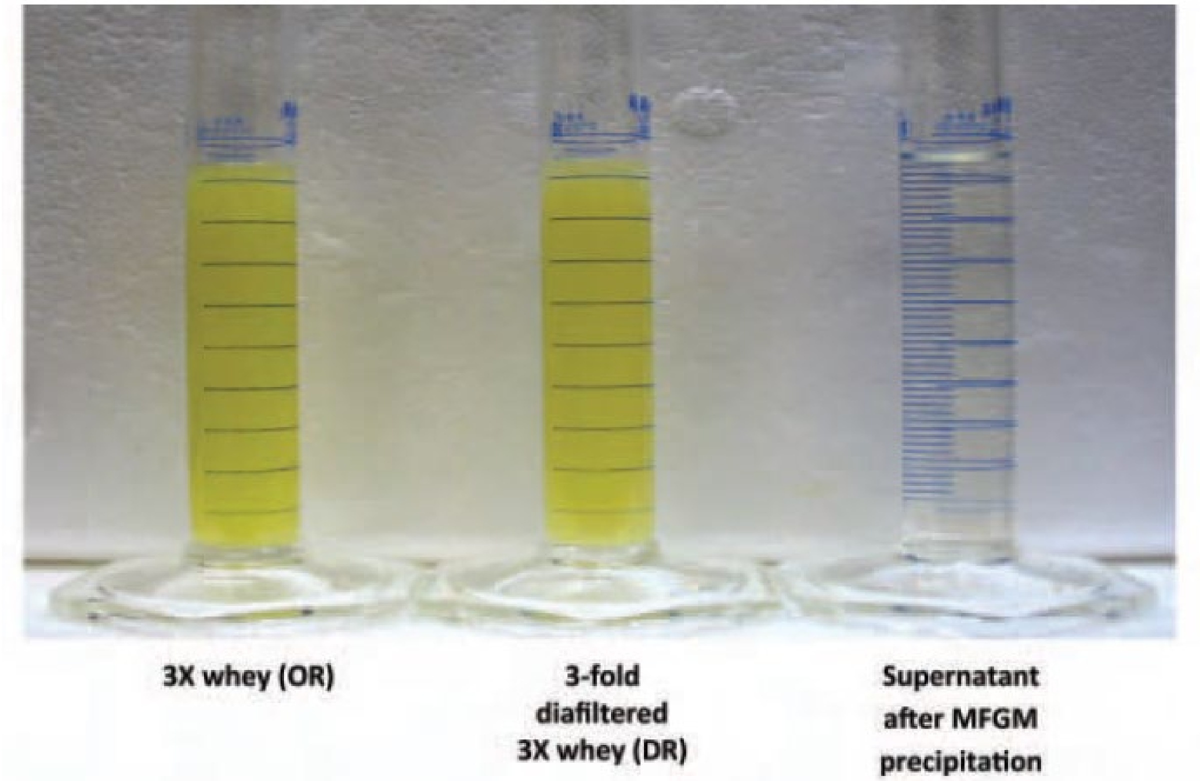

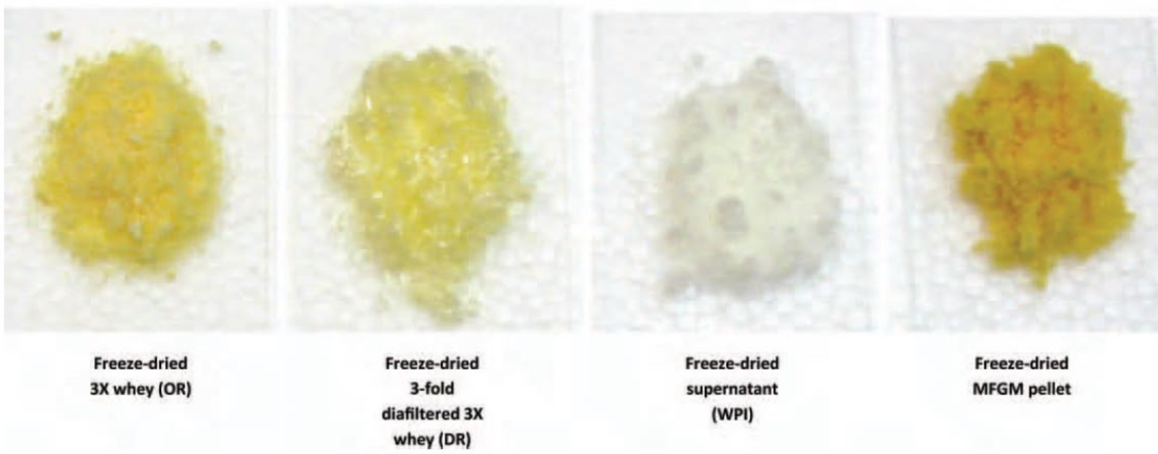

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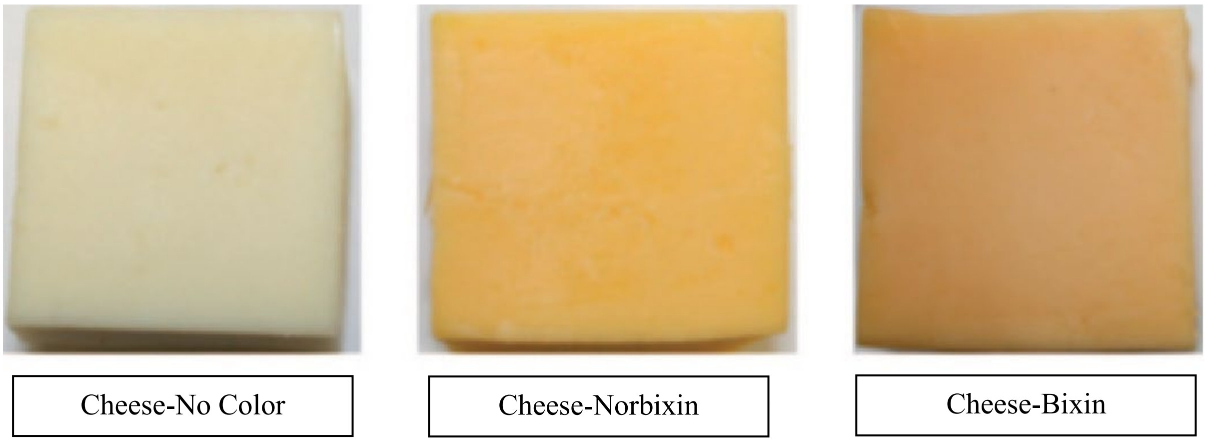

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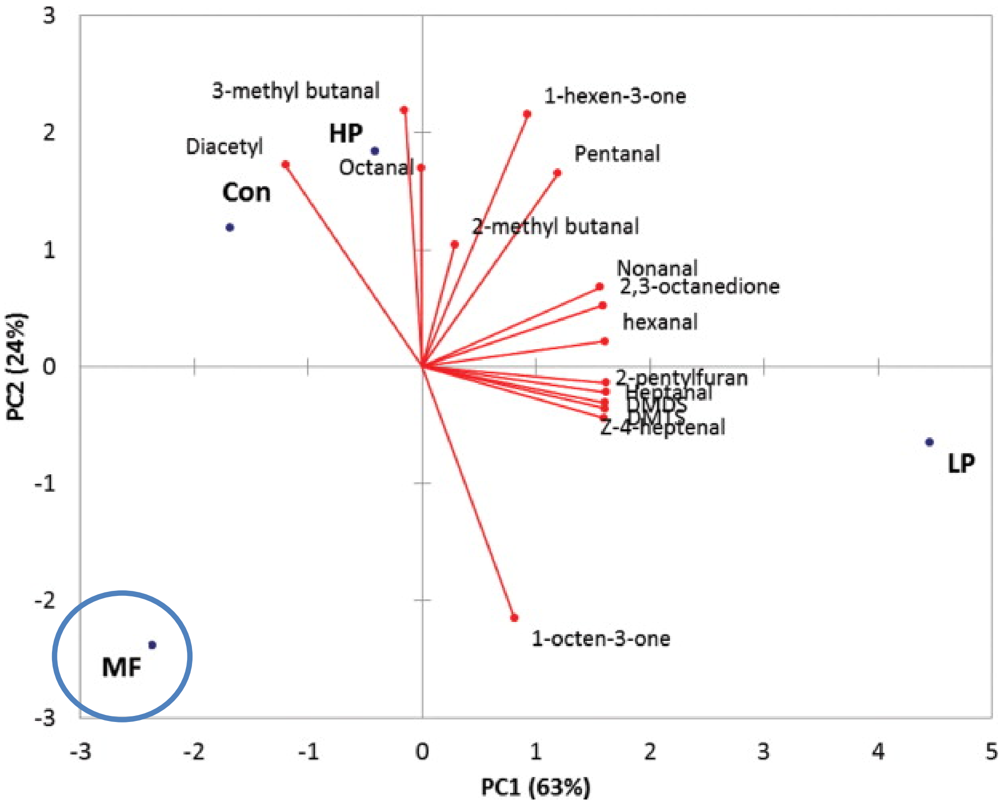

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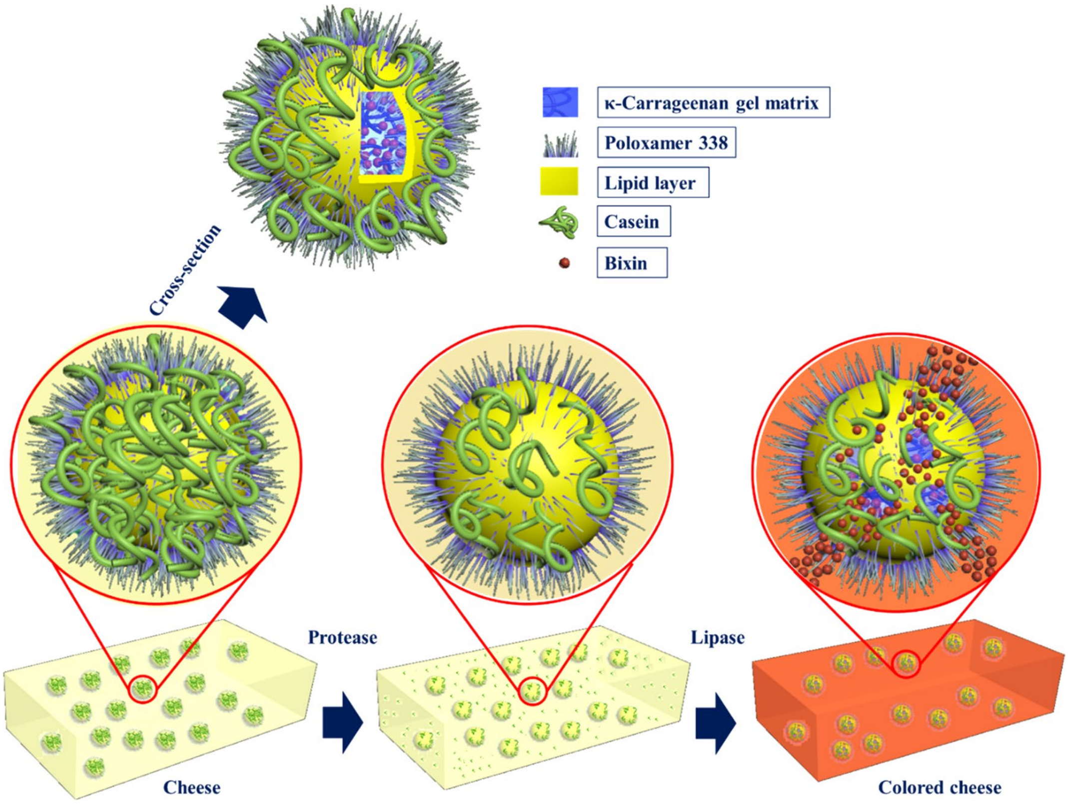

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