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Microencapsulated oregano essential oil in grated Parmesan cheese conservation

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Abstract

The objective of this study was to produce particles containing oregano essential oil by spray drying applying whey protein isolate as wall material, and to evaluate its antimicrobial effect on inhibiting growth of fungi in grated Parmesan cheese. The microcapsules contained 18% (w/w) of oregano essential oil and a 90% microencapsulation efficiency was found. Carvacrol, cymene, γ-terpinene, thymol and β-caryophyllene were the main components identified in free oregano essential oil and in the oil extracted from the microcapsules. Application of microencapsulated oregano oil was effective inhibiting the growth of fungi and yeast during 45 days of grated cheese storage. Only the treatment containing 0.5% of microencapsulated oil still remained with undetectable counting, being considered the most effective treatment in the control of filamentous fungi and yeast growth in grated parmesan cheese. The study confirms the antimicrobial effect of oregano oil and the maintenance of the microcapsules antimicrobial activity over the storage time.

Introduction

Cheese is a dairy product known for its many varieties, range of textures and flavours, nutritional value and its diversity as an ingredient (Callaghan and Kerry, 2014). The Parmesan cheese, also known as Grana, Parmigiano Reggiano or Grana Padano, is originated from the river Pó valley, one of the most traditional places in cheese production in Italy and typically is subjected to the ripening process during one to three years for obtaining a soft and crispy texture (Sora *et al*., 2013). The Grana Padano and Parmigiano Reggiano cheeses are made by hand in specific North areas of this country and use raw and reduced-fat bovine milk without heat treatment (Langford *et al*., 2012). In Brazil, this type of cheese is subjected to ripening for at least six months, although the current Grana cheese type is industrialized within a 12 months ripening period (Sora *et al.*, 2013). The grated Parmesan cheese is a product ready for consumption drawn from the crumbling or grating of the mass of one or up to four varieties of low moisture cheeses apt to human consumption and may be partially dehydrated or not (Brazil, 1997; Pimentel *et al*., 2002), and typically consumed with sauces and pasta (Trombete *et al.*, 2014).

The lack of control and/or poor hygiene conditions during the production of cheese can contribute to the occurrence of microbial contamination. The cheese pH (5.0-6.5), salt concentration, water activity, time of ripening, storage temperature and the presence of co-factors allow a favorable environment for microbial growth (Custódio *et al*., 2007). The food deterioration due to the presence of bacterial and fungal contamination has been one of the major concern, besides causing considerable loss of food worldwide (Wang *et al*., 2009). Moreover, the conditions of production, distribution and marketing can make cheese improper for consumption because of contamination with deteriorative and pathogenic microorganisms as well as compromising the sensory characteristics of the cheese (Wolupeck *et al*., 2012).

The additives used in foods have antimicrobial and antioxidant properties and buffering capacity and are used as preservatives in different types of products to prevent their deterioration. The antimicrobial properties of the potassium sorbate are well known, being a frequently used additive conservation of a wide variety of foods (Knicky and Spörndly, 2011). The potassium sorbate is widely used to preserve processed foods such as soft drinks and fruit juice by inhibiting the growth of fungi and preventing deterioration (Gören *et al*., 2015). Furthermore, it is a preservative commonly used by Brazilian dairy

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industry.

Essential oils are generally extracted by distillation, cold pressing or maceration and their antimicrobial or biological activities are directly correlated to the presence of the bioactive volatile components (Calo *et al*., 2015). Oregano *(Origanum vulgare)* is an important herb rich in phenolic compounds with strong antioxidant and antimicrobial power, often used to extend the shelf life of foods (Figiel *et al*., 2010). These properties are mainly related to the presence of compounds such as carvacrol and thymol (Costa *et al.,* 2012). The processing of liquid essential oils into the solid powder may facilitate the use of these products in food product formulations, moreover, can allow better standardization of the composition of these products, based on the dosing process and more simplified handling, with consequent reduction of storage and packaging costs.

Microencapsulation is a technology that enables sensitive ingredients to be physically trapped in a homogeneous or heterogeneous polymer matrix aimed at their protection (Costa *et al*., 2012). The most common way to promote entrainment of components by microencapsulation is drying by atomization or spray drying, which turns a liquid solution or emulsion into dry particles by feeding atomization in a chamber by passing heated air (Soottitantawat *et al*., 2005). The choice of the wall material is an important step for a successful microencapsulation process.

Essential oils can easily be released with the assistance of microencapsulation technologies. The method is cost-effective, fast and effective, and leaves minimal residue when applied to food. The development of delivery systems at a specific site of action is a current challenge in the food area. The objective of this study was to produce particles containing oregano essential oil using the spray drying technique applying WPI as a wall material, and to evaluate its antimicrobial effect on inhibiting the growth of fungi in grated Parmesan cheese.

Materials and Methods

Oregano essential oil was purchased from Ferquima (Vargem Grande Paulista-SP, Brazil). WPI (minimum of 90% protein content) was obtained from Alibra Ingredientes Ltda (Campinas, Brazil). The Parmesan was kindly donated by Nutrileite Indústria e Comércio Ltda (Matutina-MG, Brazil).

Preparation of emulsions

The wall material (WPI) was dissolved in distilled water with stirring. The solution was prepared one day before being emulsified and it was kept overnight at room temperature to ensure full saturation of the polymer molecules. Then, oregano essential oil (core material) was gradually added to the wall material solution with stirring at 3500 rpm over 10min, using a rotor–stator blender (Ultra-Turrax IKA T18 basic, Wilmington, USA), to form an emulsion. The emulsion was used as the feed liquid for the spray-drying process. The wall material concentration was 20% (Fernandes, Borges, Botrel *et al*., 2013; Fernandes, Borges and Botrel 2013) and the amount of oregano essential oil used was 25% of the mass of the wall materials (Jafari *et al*., 2008; Fernandes, Marques, Borges *et al.*, 2014). These conditions were determined through previous studies.

Microencapsulation by spray drying

A spray-dryer model MSD 1.0 (Labmaq do Brasil, Ribeirão Preto, Brazil) was used. The spraying system was equipped with a two-fluid nozzle atomiser. The following operational conditions were used, as described in previous studies: inlet temperature of 170°C and feed rate of 0.9 Lh^{-1} (Fernandes, Borges, Botrel *et al.,* 2013; Fernandes, Borges and Botrel 2013). The constant process parameters included atomizing air flow 40 $Lmin^{-1}$ and outlet temperature at 93°C. The dried resulted powder was collected from the cyclone and stored in opaque airtight containers at 4°C until further analysis.

Characterisation of the microcapsules

Oil retention

The total oil content of the spray-dried microencapsulated product was determined by distilling 10 g of the microencapsulated powder for 3h in a Clevenger-type apparatus (Jafari *et al.*, 2007). Ethyl ether was used to successively extract the essential oil from the water phase three times. After evaporating the solvent at room temperature (25°C for 24h), the resulting oil was weighed and the percentage of total oil in the particles was calculated. The oil retention was defined as the ratio of the total oil in the final powder to that of the initial oil load (dry basis) and was calculated as follows (Equation (1)):

$$\text{Oil retention (\%)} = (\text{total encapsulated oil (\%)})/(\text{initial oil load (\%)}) \times 100 \quad (1)$$

Gas chromatography–mass spectrometry

After dilution in diethyl ether (1µL in 1000 µL),

the free oregano essential oil and the sample of oil retained after total oil determination (as described above) were analyzed with a ShimadzuCG-MS-QP2010 Plus gas chromatograph mass spectrometer and an Equity-5 capillary column (95% polydimethylsiloxane, 5% phenyl, 30.00m length, 0.25mm i.d.; 0.25µm film thickness, Supelco, St.Louis, MO, USA). The relevant working conditions consisted of the following: the injector temperature was 220°C; the oven temperature was set by starting at 40°C for 1min, followed by a programmed increase from 40 to 180°C at 4°Cmin^{-1}, then a further increase to 250°C at 30°Cmin^{-1}, where it was held for 2min; carrier gas helium was at a linear velocity of 40cms^{-1}; the split ratio was 1:20; ionization was EI 70eV; and the acquisition parameters were scanned at a m/z of 45–500 (Fernandes, Marques, Borges *et al.*, 2014; Fernandes, Borges, Botrel *et al*., 2014). The compounds were identified by comparisons with spectra existing in the literature (Wiley 8 and FFNSC 1.2), and the analyses were repeated at least two times. The components profile was expressed as percentage according to the relative peak area.

Application of particles in the grated Parmesan cheese and evaluation of the antifungal activity of the microencapsulated essential oil

To evaluate the inhibition of filamentous fungi and yeast growth during the storage time four treatments were studied: 1) control (without addition of oregano essential oil), 2) treatment with addition of 0.1% of potassium sorbate (w/w), 3) treatment with addition of 0.1% microencapsulated essential oil (w/w), and 4) treatment with addition of 0.5% microencapsulated essential oil (w/w). The amount of oil was calculated based on the results obtained in oil retention analysis. Potassium sorbate and powders containing oregano oil were added to the microencapsulated formulation homogeneously mixing them to grated cheese, previously dried in a stove. The different treatments were placed in polypropylene (PP) bags containing 50 g of product, sealed and stored in chamber at 25°C. The experiment was conducted in three replications. Microbiological analysis for filamentous fungi and yeast counting were performed according to methodology recommended by Downes and Ito (2001). Samples of 25 g grated cheese from each treatment were placed in 225 mL of 0.1% peptone water and homogenized for 2 minutes. Dilutions of 10^{-1} to 10^{-4} from each test containing 9 mL of peptone water 0.1%, for appropriate platings were carried out. The counting of filamentous fungi and yeast was performed by sowing 0.1mL from the dilutions in petri dishes containing approximately 20mL of solidified PDA culture medium, acidified with 10% tartaric acid. The inoculum was spread and the dishes were incubated in a stove at 25°C for 3 days. The filamentous fungi and yeast counting were held from 15 to 15 days during 45 days of storage period at 25°C.

Determination of moisture content of grated Parmesan cheese

The moisture content of the cheeses, in each treatment and during storage at 25°C, was determined according to the method described by AOAC (2007) The weight loss after drying at 105°C was obtained, and the moisture content (%) was calculated. The analysis was performed in triplicate and was expressed as average value.

Statistical analysis

The evaluation of the total filamentous fungi and yeast and the moisture content determinations in the cheese were conducted using a completely randomized design (CRD) with three replications. Analysis of variance was performed to evaluate the difference of the major components between free oil and encapsulated oil. Difference between treatments were evaluated applying Tukey test, at 5% of probability. Statistical analyses were performed using the R® software (ver. 2.14.2, R Development Core Team).

Results and Discussion

The microencapsulation efficiency, described as the oil retention is one of the most important parameters when studying essential oil microencapsulation processes. This is the real value that the microencapsulated material was retained in the encapsulating matrix, disregarding the amount of lost material during drying. In this study, the microcapsules contained 18% (w/w) of oregano essential oil. Considering the initial amount of oil 20% (w/w), compared to the total solids in the emulsion feeding, a 90% microencapsulation efficiency was found. When compared with data in the literature, the obtained value shows that the process was efficient and the retention of oil compounds was considerable. In the microencapsulated oregano essential oil evaluation by spray drying, Botrel *et al.* (2012) found the maximum value of 33.9% for retention when used modified starch, maltodextrin and Arabic gum as encapsulants. Teodoro *et al.* (2014) found the value of 50% efficiency to the rosemary essential oil microencapsulated in modified starch matrix and maltodextrin. Baranauskiené *et*

al. (2006) applied concentrate whey protein and reduced-fat milk powder as encapsulants matrices of oregano essential oil, and achieved the value of 71.8% and 80.2% of microencapsulation efficiency, respectively for the wall material used. The nature of the wall material is a decisive factor in the retention of volatile constituents. Based on the properties of the wall material, the microencapsulated product shows a better stability in the presence of environmental factors such as light, heat and oxygen, it has reduced volatility and aroma may also have undesirable flavor and aroma suppressed (Soottitantawat *et al.*, 2005; Jafari *et al*., 2008; Teodoro *et al*., 2014; Botrel *et al.,* 2015). A good part of application of microencapsulation process for food products aims to protect and isolate volatile components, which will be responsible for providing aroma to the products and also act beneficially in food by the antioxidant and/or preservative action.

For efficient microencapsulation, the wall material must have adequate properties, being the emulsification capacity an important factor. The emulsifying properties is an essential requirement for an efficient retention of volatile in the sense that the acquired stability of the formed emulsion leads to a higher microencapsulation efficiency and, therefore, a smaller loss of volatiles (Fernandes, Borges, Botrel *et al*., 2014). WPI is the main source of globular proteins used in the food industry due to its emulsifying properties (Bernard *et al.*, 2011) as well as presenting optimal nutritional quality and inherent functional properties that meet the demands of encapsulation (Ezhilarasi *et al.*, 2013). During the emulsification stage, these proteins change their conformation and position themselves in the oil-water interface and contribute to the repulsive forces that make the emulsions more stable (Jafari *et al*., 2008).

It was found with this work that WPI was considered an effective WPI wall material in the process of oregano essential oil microencapsulation by spray drying, being a material with potential for development of new food formulations. Also, when it refers to dairy products, where the addition of some ingredients is controlled by regulation, such as starch for example, WPI becomes even more attractive for this sector, for example in cheese production.

The profile of the main components found in oregano essential oil was evaluated for free oil and for the oil extracted from the microcapsules. Figure 1 illustrates the variation of the percentage of the peak area found for the major components of the identified oregano essential oil. Some components were not included because their maximum values represented

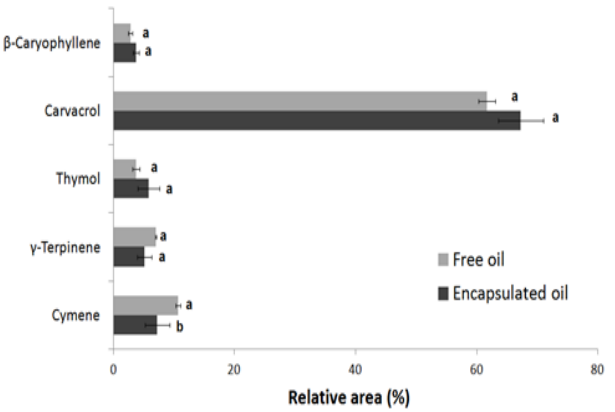

Figure 1. Variations in the profile of the major components of the oregano essential oil, expressed as the relative peak area (%) for the main components. Means followed by the same letter do not differ significantly at 5% probability for each component.

less than 1% of the total peaks area.

Silva *et al.* (2010) also found carvacrol as the main component in five different types of oregano essential oil, with levels of this component of 74.5%, 67.7%, 72.6%, 93.4% and 61.7% comparing to the total oil composition. In the study of Figiel *et al.* (2010) the main components found in fresh oregano were carvacrol and thymol. When the microencapsulation of aroma compounds is studied, it is important to evaluate the changes caused during the process, especially during drying due to the contact with high temperatures, because it is useful for the preparation of products and in directing the application of these ingredients. The composition of free and microencapsulated oregano oil was similar, presenting all components with no indication of components loss. However, some variations in relative percentages of certain individual compounds were observed.

The γ-terpinene and cymene components presented a greater reduction in their contribution plots (relative area) of the total oil composition, compared to free oil and also the oil extracted from microcapsules. However, only for cymene the free oil and the oil extracted did significantly differ ($p<0.05$). This indicates that this component has probably suffered most volatilization or chemical changes when subjected to 170°C of drying temperature, compared to the other four components (γ-terpinene, carvacrol, thymol and β-caryophyllene). These last remained broadly unchanged, what was evidenced by the increase in their relative areas depending on the sum of the balance of the areas of the main compounds.

In the production of essential rosemary microencapsulated oil by spray drying, Fernandes,

Table 1. Counting values (log CFU.g^{-1}) for filamentous fungi and yeast during storage time at 25°C for the studied treatments.

Time (days)	Control	Sorbate (0.1 %)	Microencapsulated oil (0.1 %)	Microencapsulated oil (0.5 %)
0	ND	ND	ND	ND
15	ND	ND	ND	ND
30	4.82±0.19^{a}	ND	4.49±0.13^{a}	ND
45	5.74±0.17^{a,b}	4.88±0.11^{b}	5.81±0.13^{a}	ND

ND: not detected.
^{a,b}Means followed by the same letter in the same line do not differ significantly at 5% probability.

Marques, Borges *et al.* (2014) founded no significant changes in the major components of essential oil profile, utilizing inlet air temperature of 190°C and maltodextrin and modified starch as wall material. The maintenance of the oil components in the microcapsule, even after being subjected to the spray drying process, is an indication that their bioactive properties were also maintained.

The essential oregano oil has been widely studied due to its antimicrobial and antioxidant activities. These attributes justify the development of several products using the oregano essential oil as a natural preservative and that meets the current consumer demand for healthier food products. There is an urgent issue to identify alternative and antimicrobial agents, particularly those of natural origin (Bhargava *et al*., 2015). Additionally, oregano has a flavoring capacity, being widely used in the culinary around the world.

Several studies about essential oil suggested that their antimicrobial activity can be attributed to its ability to penetrate into microbial membranes of the cell and to exhibit inhibitory activity on the functional properties of the cell. The mechanisms of action may be related to the ability of phenolic compounds to alter the permeability of microbial cell damage in cytoplasmic membranes and their interference in the cell energy system generation (Smith-Palmer *et al.*, 1998; Burt, 2004; Fisher and Phillips, 2009; Friedly *et al*., 2009; Botrel *et al.*, 2010; Guinoiseau *et al*., 2010; Li *et al*., 2011; Bajpai *et al*., 2012; Botrel *et al.*, 2015; Calo *et al*., 2015).

In the study of antifungal activity of essential oils against filamentous fungi Tullio *et al.* (2007) reported that carvacrol was one of the major contributor to the bioactivity and it proved to be the overall best inhibitor and Vale-Silva *et al*. (2012) showed carvacrol with the highest potency among all the tested strains. Mota *et al.* (2012) related that thymol has strong antifungal activity but cymene did not show significant inhibition of species of yeasts and filamentous fungi. Moreover, γ-terpinene have been shown to possess antifungal properties (Couto *et al.*, 2015) and β-caryophyllene also displayed relatively good activity (Skočibušić and Bezić, 2004).

The antifungal activity of the oregano essential oil was evaluated by comparing the counting of filamentous fungus and yeast in grated parmesan cheese for the studies treatments. No filamentous fungi and yeast counting was observed for the initial times of the experiment, considering the dilutions used (Table 1). The cheese process goes through a drying process prior to achieve a moisture content around 20%, which can contribute to microorganisms reduction. Furthermore, the parmesan cheese is a product matured for several months and the contamination of the grated cheese is mainly because of the handling steps during the shredding and packing process. The possible contamination on grated cheese is usually detected during storage time because of microorganisms growth and development.

It was found that on day 30 of storage at 25°C, the treatments control and microencapsulated essential oil at 0.1% presented positive total fungal counting. Thus, it is possible to conclude that 0.1% of microencapsulated oil content did not reduce the microbial growth. On day 30 of storage the treatments sorbate 0.1% and microencapsulated oil 0.5% still presented no detectable counts. On the other hand, on day 45 of storage, only the treatment containing 0.5% of microencapsulated oil still remained with undetectable counting, being considered the most effective treatment in the control of filamentous fungi and yeast growth in grated parmesan cheese.

The results confirm the antimicrobial action of oregano essential oil and even after the microencapsulation process the antimicrobial effect was maintained. The release of the active constituents of microencapsulated oregano oil over time is responsible for its antimicrobial effect. The use of this type of product in powder form gives a better distribution of oil on the grated cheese package and ensures a more distributed antimicrobial action. The use of oil in its original form is hampered by

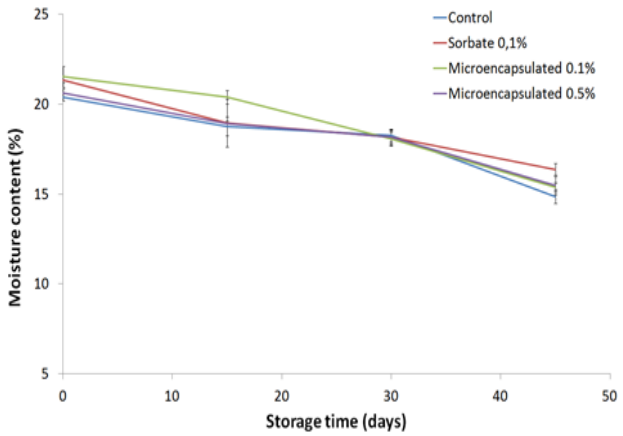

Figure 2. Moisture content (%) of grated cheeses subjected to different treatments during 45 days of storage

its oily characteristics. Moreover a greater and faster volatilization of oil components may occur when the oil is used in a free form, decreasing its antimicrobial effect throughout the storage time. The controlled release can be defined as a method in which one or more active ingredients or agents remain available in a desired location, and specific time and rate. For encapsulating systems applied to volatile compounds, the release depends on several interdependent processes such as diffusion of the component through the matrix, type and geometry of the particle, the transfer of the matrix for the environment and degradation/dissolution of the wall material (Pothakamury and Barbosa-Canovas, 1995; Madene *et al*., 2006).

The preservation of organic cottage cheese quality using oregano essential oil was evaluated by Asensio *et al.* (2015). These authors concluded that the cottage cheese with oregano presented a lower degree of chemical deterioration during storage and that the use of oregano could be an alternative to reduce the organic acid production, associated with the microbial activity reduction. Guarda *et a*l. (2011) demonstrated the possibility of using microencapsulated thymol and carvacrol in a polymer matrix (Arabic gum) for fresh food storage. In this study the antimicrobial capacity was not altered by microencapsulation, but the release rate of antimicrobial agents was lower and more controlled when compared to films in which the antimicrobial agents are incorporated directly into the matrix.

Curcumin microencapsulated by spray drying was evaluated in the food pathogens control (Wang *et al*., 2009). The results of in vitro assays, the antibacterial and antifungal activity and the minimum inhibitory concentrations for curcumin microcapsules indicated that this product was more effective on fungi control when compared to bacteria control.

The addition of oregano and rosemary essential oils increase the fermentative and oxidative fermentative stability of flavored cheese prepared with cream cheese basis, preventing lipid oxidation and the development of rancid and fermented flavors (Olmedo *et al.*, 2013). As a consequence, these essential oils increased the product shelf life. Industry should consider adding essential oils as an alternative to natural and antioxidant products aiming the preservation of quality parameters in these types of product. Because of the strong aroma of the essential oils, such as rosemary and oregano oil, the microencapsulation allows a higher consumer acceptance, since it enables the masking of the sensory attributes of these food ingredients in the formulation.

Teodoro *et al.* (2014) studied the addition of 1.5% of rosemary microencapsulated essential oil in fresh mass and it resulted in an increase of antimicrobial activity period when compared to the essential oil added in a free form. In this study the fungi counting during 12 days of storage at 25°C was evaluated. In a study held by Azevedo *et al.* (2012) which objective was to evaluate the effect of microcapsules of oregano essential oil addition in Quark cheese at different concentrations, observed that cheese formulations up to 0.45% of microcapsules of oregano essential oil was the maximum concentration to increase the consumer acceptance. The classification obtained was between the terms "liked slightly" and "liked moderately", with positive acceptance, turning into a potential application of this type of product.

The transformation of a liquid product in powder, i.e., of the essential oil in a free form to its microencapsulated form, besides being interesting by factors previously mentioned, allows the introduction of a particulate in a range of food matrices, such as the grated cheese. The microencapsulation process, depending on the process and of the wall material used, allows the gradual volatilization of the encapsulated, which contributes to a prolonged effect of the bioactive components, such as the antimicrobial and antioxidant effects.

Cheese moisture content

Figure 2 shows the moisture content of the packaged cheese samples throughout the storage time. The observed variation in the water content can be explained in terms of moisture loss by the product to the environment and through the package during the storage time. The treatments did not significantly affect ($p>0.05$) the moisture content of the cheese except on day 15. This result shows that moisture contents between treatments remained very close and showed the same behavior over time and

cannot be considered as a determining factor in the microbiological counting. The Brazilian regulation establishes that the moisture content of low-moisture grated cheese should be lower than 20% (Brazil, 1997).

Conclusion

The application of microencapsulated oregano oil by spray drying in WPI matrix was effective inhibiting the growth of fungi and yeast during 45 days of grated cheese storage. The study confirms the antimicrobial effect of oregano oil and the maintenance of this antimicrobial action of the microencapsulated oil over storage time. The oregano essential oil, in the microencapsulated form, can be used by food industry as a potential substitute synthetic additive and presents the convenience for using and handling of a powdered product.

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