ISSN: 2501 - 2568 (2016) Volume 1, Issue 2, 165 - 181

Journal of Food Bioengineering and Nanoprocessing
open access journal
www.nanobiofoods.com

received: 10.12.2015
revised: 15.02.2016
accepted: 20.04.2016
published: 02.06.2016

Review

Food antimicrobial agents using phenolic compounds, chitosan, and related nanoparticles

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ABSTRACT

Essential oils and several of their derivatives possess various advantages as food antimicrobials, due to their high and broad-spectrum antimicrobial activities, high mortality rates and low toxicities. Among the essential oils that have proven antimicrobial effectiveness are cinnamon, clove, and thyme essential oils, which contain high concentrations of phenolic compounds; their main antimicrobial effect has been attributed to cinnamaldehyde, eugenol, and thymol, respectively. The use of charged biopolymers to stabilize nanoparticles is a recent field for investigation in food science. Of these biopolymers, chitosan is of particular interest because of its antimicrobial activity that has been studied against a wide variety of microorganisms including fungi, algae, and bacteria, allowing its consideration as a feasible alternative to develop antimicrobial particles that can be applied to foods for protection against different microorganisms. Given the antimicrobial characteristics of chitosan and phenolic compounds, and taking into account that both are obtained from natural sources, combining their properties may result in a matrix with enhanced effects at low cost. This review will focus on formation and characterization of chitosan nanoparticles with phenolic compounds extracted from essential oils to evaluate their potential antimicrobial properties, using selected scattering methods for nanoparticle preparation.

Keywords: *Antimicrobial agents, essential oils, phenolic compounds, chitosan nanoparticles.*

INTRODUCTION

Food preservation can be defined as a set of applied treatments to prolong food shelf-life, maintaining the highest possible quality attributes, including color, texture, flavor, and nutritional value; while focusing on ensuring food safety. This definition involves a wide range of shelf-life periods; from short periods, when using domestic cooking methods and cold storage; to very long periods, in products resulting from industrial processes strictly controlled as in the case of canning, freezing, and dehydration (Mau *et al.*, 2001).

Current consumer trends indicate a preference for high-quality safe foods with natural-like aspects, which are minimally processed but have a longer shelf-life than their natural counterparts. Therefore, food preservation technologies are tasked with obtaining safe and long-lasting products without compromising their nutritional and sensory characteristics (Cooksey, 2000). When we buy unprocessed food (fruits, vegetables, etc.) at the grocery store it is possible that these foods may have been unintentionally contaminated or may contain some additives. These contaminants can be found in the food itself: toxins produced by some bacteria, byproducts from biochemical reactions, and environmental pollution resulting from the handling of them such as pesticides or fertilizers, among others. Furthermore, some additives could be incorporated intentionally to preserve and/or enhance food characteristics, an example would be antimicrobial agents (Wang *et al.*, 2011).

Conditions regarding the use of antimicrobial agents in food are strictly regulated in most countries of the world. Usually there are caps on the amount of an additive that can be added. Authorized concentrations of food antimicrobials, generally do not inactivate microorganisms, only prevent their proliferation. Therefore, they are useful only with raw materials of good microbiological quality (Vardar-Unlu *et al.*, 2003).

The main cause of food spoilage is the attack by different types of microorganisms (bacteria, yeasts, and molds). Microbial spoilage of food has obvious economic implications, both for manufacturers (deterioration of raw materials and finished products before they are marketed, loss of brand image, etc.) as well as for distributors and consumers (product deterioration before or after purchase, and before consumption). Food quality is affected by physical, chemical, biochemical and microbiological factors. Controlling such factors, the latter in particular, is essential for food preservation. Applying microbiological stress-factors is useful to achieve microbial inhibition and/or death. The main factors affecting microbial survival and growth can be classified as follows (Gutierrez *et al.*, 2009):

- Implicit and microbial factors: type and number of microorganisms present, growth rate and lag phase, possible synergic effects, among others.
- Intrinsic factors: those chemical and physical factors acting within the food such as nutrients, pH, water activity, redox potential, presence of preservatives and other antimicrobial substances, microstructure, etc.
- Extrinsic factors: temperature, relative humidity, partial oxygen pressure, among others.

Traditional preservation methods such as freezing, pasteurization, sterilization, and/or drying are based on manipulating one or more of these factors. Today, many methods combine two or more factors that interact additively or synergistically controlling the microbial population, avoiding severe modification of quality factors when a single conservation factor is applied. This improves the sensory and nutritional quality of the food; allowing the product to be less processed, healthier, with fewer additives and ready to be prepared and served, and/or more similar to its fresh counterpart. This combination of factors has been called “hurdle” or “combined methods technology” (Leitsner & Gould, 2002; Alzamora, *et al.*, 2000; Alzamora & Salvatori, 2006; Yang *et al.*, 2010).

FOOD ANTIMICROBIALS

Preservatives are added to food in order to control the growth of microorganisms (bacteria and fungi). Although the most utilized are synthetic preservatives, there are a number of natural products obtained from plants that can be used as food preservatives. Food antimicrobials remain among the most important food additives, they can be synthetic compounds (intentionally added to foods) or from natural origin (López-Malo *et al.*, 2005).

Natural antimicrobial systems present in plants, animals, or microorganisms are gaining popularity for their potential usage in minimally processed foods. Natural antimicrobial systems can be classified by origin:

- Animal Origin: includes some proteins, lytic enzymes such as lysozyme, hydrolases such as lipases and proteases, and polysaccharides such as chitosan.
- Plant Origin: includes essential oils, extracts and phenolic compounds from bark, stems, leaves, flowers, organic acids in fruits, and phytoalexins produced in plants.
- Microbial Origin: it includes compounds produced by microorganisms, such as some bacteriocins.

Many foods contain natural compounds with antimicrobial activity; these compounds may play a significant role in prolonging food shelf-life. Many of them have been studied for their potential as direct antimicrobials added to food. Their usage has been gaining popularity throughout the globe, natural antimicrobials that reinforce food safety and preservation by inhibiting bacteria, fungi, and viruses are being deeply evaluated and studied. Foods such as celery, thyme, oregano, clove, bay, almond, coffee, and cranberry contain natural antimicrobials with the ability to inhibit the growth of several microorganisms (López-Malo *et al.*, 2005).

The antimicrobial activity of essential oils and extracts from various types of plants as well as several plant parts have been subject of many studies, with the aim of taking advantage of the fact that such compounds have been traditionally used as flavoring agents in some foods. Many papers concur that more than 80 plant products contain high levels of potential antimicrobial agents such as cloves, garlic, onion, sage, rosemary, cilantro, parsley, oregano, mustard, and vanilla among others. The FDA considers antimicrobial agents of natural origin as GRAS (Generally Recognized as Safe) substances, including plant products such as essential oils, oleoresins, and natural extracts including distillates for use as antimicrobial agents (Yang *et al.*, 2010).

The use of food additives of natural origin involves the isolation, purification, stabilization, and incorporation of these compounds to foods with antimicrobial purposes, without adversely affecting sensory and nutritional characteristics and guaranteeing consumer health. This must be achieved while keeping formulation, processing and/or marketing costs in mind (Karaosmanoglu *et al.*, 2010).

Essential oils and phenolic compounds

The antimicrobial compounds present in plants are contained mainly in the leaves, flowers, buds, bulbs, rhizomes, fruits and other plant parts. These components can be lethal to microbial cells or may simply inhibit the production of a metabolite preventing microbial growth (López-Malo *et al.*, 2005). It is well established that the active components of plants, herbs, and spices are phenolic compounds. Bauer *et al.* (2001) noted that essential oils may have one major component that constitutes around 85% of its chemical makeup, while other components may be present only in trace amounts.

Phenolics are chemicals that are commonly found in fruits, vegetables, herbs, spices, etc. Phenolic compounds are one of the most important classes of secondary metabolites in plants, mostly deriving from phenylalanine and to a lesser extent from tyrosine. These compounds are a large group of substances in plants with different chemical structures and metabolic activities. There are over 8000 identified phenolics (Shahidi *et al.*, 1996). These compounds can be chemically defined as substances possessing an aromatic ring with one or more hydroxyl groups, including functional derivatives thereof. These can be classified into the following groups:

- Simple phenols and phenolic acids (p-cresol, 3-ethylphenol, gallic, vanillic, p-hydroxybenzoic acid, and aldehydes such as vanillin).
- Hydroxycinnamic acid derivatives (coumaric, caffeic, ferulic, and sinapic).
- Flavonoids (catechins, proanthocyanidins, anthocyanins, flavones, flavonoids and their glycosylated counterparts).
- Tannins (gallotannins, ellagitannins, among others).

Simple phenols are commonplace in all plant species. Similarly, phenolic acids are also abundant in plants and ferns. Cinnamic acids are seldom present as free compounds, as a rule they are generally present as derivatives. Flavonoids are a subclass of polyphenols that are characterized by structures C_6-C_3-C_6 and two or more aromatic rings, each having at least one aromatic hydroxyl bonded to a carbon (Shahidi & Naczk, 2003). Tannins are water-soluble phenolic compounds, containing a large number of hydroxyl groups including functional groups being capable of binding to proteins and other macromolecules (Martínez *et al.*, 2000).

Phenolic compounds are related to the sensorial quality of processed or fresh foods of vegetable origin. Currently, this group of phytochemicals is recognized of great nutritional value for their contribution to the maintenance of human health (Clifford, 1992). Some polyphenols are considered important in food; these compounds are gallic, ferulic, synaptic, caffeic, p-coumaric acids and their derivatives as well as flavonoids and their glycosides.

Essential oils are characterized by a complex mixture of several compounds of volatile aromas belonging to different classes: phenols, hydrocarbons (terpene compounds), alcohols, aldehydes, ketones, esters, and ethers. Among the main compounds present in essential oils are: cinnamaldehyde (cinnamon), eugenol (clove), carvacrol (oregano), cineol (eucalyptus), and thymol (thyme), among many others (López *et al*., 2007).

Antimicrobial activity

It has been reported that some phenolic compounds have a broad antimicrobial spectrum. Phenolic compounds such as caffeic acid, chlorogenic, p-coumaric, ferulic and quinic are present in parts of plants which are used as spices. The antimicrobial activity of these and other hydroxycinnamic and cinnamic acids can delay microbial contamination as well as spoilage of fresh produce. Gram-positive and Gram-negative bacteria, molds and yeasts commonly found as deteriorative organisms are sensitive to hydroxycinnamic acid derivatives. Caffeic, ferulic and p-coumaric acids, for example, inhibit *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*. Other phenolic compounds that have exhibited antimicrobial activities are tannins and tannic acid. The latter for example inhibits *Listeria monocytogenes*, *E. coli*, *Salmonella* Enteritidis, *S. aureus*, *Aeromonas hydrophila*, and *Streptococcus faecalis* (Beuchat, 2001).

Phenolic compounds such as flavonols, typically present in fruit and green tea, have antibacterial activity. Puupponen-Pimiä *et al.* (2001) demonstrated that myricetin inhibited the growth of lactic acid bacteria derived from the microflora found in a human gastrointestinal tract, while extracts prepared directly from strawberries and raspberries were strong inhibitors of *Salmonella* spp. and *E. coli*.

Several studies have reported that some essential oils from clove, cinnamon, mustard, oregano, rosemary, and thyme are those with a more powerful antimicrobial effect. However, an *in vitro* standardized laboratory procedure has yet to be established. Normally, these tests involve evaluating the minimum inhibitory concentration (MIC) which prevents the growth of microorganisms. Being fat soluble essential oils, two-fold serial dilutions of essential oil in broth with an emulsifying agent or emulsifier are typically performed (Engels *et al*., 2009).

Studies with extracts of cinnamon, thyme, clove, and oregano have demonstrated activity against *Clostridium perfringens*. Lanciotti *et al.* (2004) indicated that the application of essential oils of mandarin, lemon and lime, increased the shelf-life of fruit salad and reduced microbial load, without altering the sensory characteristics of the product. In other studies, the activity of essential oils against *Salmonella* spp. was demonstrated. From these works, essential oils with the greatest activity against *S*. Typhimurium were extracted from cinnamon, cloves, oregano, and thyme. For *S*. Enteritidis the best results were obtained with oils from mustard, cloves, thyme, oregano and cinnamon (Henares & Morales, 2008).

Considering the wide variety of chemical compounds present in essential oils, it is likely that their antimicrobial activity is not attributable to a specific mechanism, but the combined action of several of them, on different cell sites (Vardar-Unlu *et al*., 2003). There are many factors that affect the antimicrobial activity of spices and essential oils. The degree of inhibition depends on the type of microorganism, the type of substrate, variations in the composition of the plant, spice or fruit by differences in areas of cultivation, climate and many other factors (López *et al*., 2007).

The targets within microbial cells of antimicrobials include the cell wall, cell membrane, metabolic enzymes, protein synthesis, and genetic structure (Davidson & Branen, 1993). Since all these processes and structures are essential for survival, damage to any of them can inactivate the microbial cell. The exact mechanism of action of each of the antimicrobial agents is not entirely known, and some of the general mechanisms of action for different groups of antimicrobial agents are assumed.

Davidson (1996) mentions that the reasons for this lack of knowledge is that researchers need to focus on a single point within the microbial cell, e.g. a specific enzyme or cell membrane without determining the effects on other functions of the cell. However, it is difficult to define a target or study point, since many factors can influence the microbial response, including those that disturb the functions of the cell membrane can cause loss of intracellular contents, interference with active transport or metabolic enzymes, or may drain the cell of energy in the form of ATP (Eklund, 1989; Davidson, 1996).

The mode of action of phenolic compounds has not been determined; they can inactivate essential enzymes, react with cell membrane or alter the function of the genetic material, and it has been observed that food composition (fat, protein, salt concentration, etc.), pH, and temperature affect the antimicrobial activity of these compounds.

In the case of susceptible Gram negative bacteria as well as Gram positive, essential oils are inserted through the cellular and mitochondrial membrane, altering its structure and making them more permeable. This is followed by leakage of ions and other cellular contents, more or less intensively, which can lead to cell death (Kubo *et al*., 1995).

The dose required for an antimicrobial effect to take place is relatively small, on the order of 100 to 200 ppm, depending on the essential oil. Generally, essential oils that possess significant antimicrobial properties contain a high percentage of phenolic compounds such as carvacrol, thymol, or eugenol. Carvacrol (major component of oregano) and thymol (from thyme) are able to disintegrate the outer membrane of Gram negative bacteria. Phenolic derivatives such as carvacrol and eugenol from clove and thyme cause the disintegration of the membrane *E. coli* and *S.* Typhimurium. Eugenol (major component of clove oil), and cinnamaldehyde (from cinnamon) act by inhibiting the production of intracellular enzymes, such as amylases and proteases, thereby causing deterioration of the wall and cellular lysis (Engels *et al*., 2009).

Chitosan

Several biodegradable polymers (such as chitosan) due to their antimicrobial properties, functionality, and extensibility are currently studied. In addition, its polycationic character confers to chitosan high affinity for associating macromolecules which could protect foods against enzymatic and hydrolytic degradation (Keawchaoon & Yoksan, 2011).

Over the past decade chitosan has been widely utilized in the development of potentially innovative systems for the food industry (Choi *et al*., 2011; Gómez-Estaca *et al*., 2010). Chitosan is an amino-polysaccharide obtained at industrial scale by thermos-alkaline process of N-deacetylation of chitin isolated from crustacean wastes.

Chemically, the term “chitosan” defines a family of linear hetero-polysaccharides that are composed of 2-amino-2-deoxy-n-D-glucose (D units) and 2-acetamido-2-deoxy -D-glucose (units A) joined in (1 & 4). The units of type "A" are often present in a lower proportion compared to units of type "D". The molar ratio of units "A" to the total (A + D), is known as the degree of acetylation expressed as a percentage (GA) or as a fraction (AF). Along with the degree of polymerization, GA is a fundamental parameter which directly determines physicochemical and biological properties of chitosan (Gómez-Estaca *et al*., 2010). In addition to the net molar proportion of units "A", its distribution in the chain determines many properties of chitosan and varies according to the preparation protocol. In samples of chitosan homogenously deacetylated, with an AF ranging from 0.04 to 0.49, 1H NMR has shown that the frequency distribution corresponds to a random pattern of dyads. Meanwhile chitosan samples falling within the same range of AF produced under heterogeneous conditions, seem to have a pattern slightly closer to that of a distribution block (Jang & Lee, 2008).

From the physicochemical point of view, water-soluble chitosan is a biopolymer that can form films, hydrogels, porous scaffolds, fibers and micro and nanoparticles, in mildly acidic conditions. Chitosan inhibits the growth of a variety of fungi (molds and yeasts) as well as bacteria. In dilute acid solutions of chitosan, positive charges interact with the negatively charged residues of the microbial cell surface, presumably competing for Ca^{2+} in the electronegative sites of the membrane, but without conferring dimensional stability, comprising and therefore weakening the integrity of the membrane (Du *et al.*, 2004). Table 1 describes some reports on the antimicrobial activity of chitosan against related microorganisms that usually cause infections in plants.

Table 1. Some studies on the biocidal activity of chitosan against selected phyto-pathogens

Mold	**Results**
Botryitis cinerea	Chitosan (50 ppm) promotes the death of the disease known as "gray mold" in cucumber.
Collectotrichum gloeosporioides	It was found that *in vitro* treatments with chitosan (2 and 3%) have fungicidal effects.
Fusarium solani	It was shown that the non-acetylated heptamer of chitosan has a high fungicidal activity
Phytophthora capsici	It was demonstrated that oligo-chitosan can penetrate the membrane of the pathogen and bind to DNA and/or RNA
Pythium debaryanum	Treatment with chitosan promotes seed germination and growth of lettuce in infected media

Adapted from Keawchaoon & Yoksan (2011)

The use of chitosan for coating fruits and vegetables has been proposed and tested for over 25 years, due to its bactericidal and fungicidal properties, ability to form films, and low toxicity in humans. In principle, the ability of chitosan to form films favors the preservation of the covered products due to the modification of the internal atmosphere and reduced transpiration losses (Maruno & Rocha, 2010). A summary of some studied fruits protected by chitosan coatings are presented in Table 2. In most of the studied systems, preservation of quality in chitosan-coated products was positively affected. For example, some authors have found that the coating of strawberries with chitosan solutions has remarkably beneficial effects in the preservation of the fruit (Rhim *et al.*, 2006). The main observations in these systems include:

- Decreased transpiration losses; slowly decreasing respiration, although initially it increased attributed to stress caused by the aqueous solution of lactic acid / sodium lactate used to dissolve the chitosan.
- A better texture is preserved over time; fruits treated with chitosan were firmer than those not treated.
- Apart from a slightly bitter initial taste, which quickly disappears and is not seen in subsequent storage, the presence of

Table 2. Some studied fruits coated with chitosan

Product	**Application**	**Observed results**
Strawberry	Fruits sprayed with 1.2% aqueous solutions of chitosan.	Induction of defense mechanisms (increased activity for chitinase and β-1,3-chitosanase)
Guava	Halves coated with films formed from an aqueous solution of chitosan.	Conservation of properties of sliced fruit.
Papaya	Fruits covered with films formed from 1% aqueous solutions of chitosan.	Fruit coating for protection of anthracnose. Inhibition was observed in the growth of mycelia and germination of spores.
Mandarin	Fruit introduced in an aqueous solution of chitosan and allowed to dry.	Control of fungal growth: *Penicillium digitatum* and *Penicillium italicum*.
Mango	Slices coated with chitosan films (obtained by air drying solutions of chitosan in 5% acetic acid).	Conservation of the properties of sliced fruit for longer time.
Cantaloupe	Fruit covered with films obtained from aqueous solutions of chitosan.	Extension of storage time.
Tomato	Fruit covered with films obtained from aqueous solutions of chitosan.	Extension of storage time.

Adapted from Maruno & Rocha (2010).

chitosan caused no appreciable differences between the sensory assessments of fruits treated with chitosan and untreated fruits.

- The fruit's microbiological load over time always remained lower in those treated with chitosan systems.
- Several countries have approved the use of chitosan as an additive for clarification of fruit juices.

NANOPARTICLES IN FOOD

Nanoparticles have their origin in 1982, this technological development allowed a real basis for the subsequent evolution of manipulation of matter at very small scale, between 1 and 100 nanometers (10^{-6} m and 10^{-9} m, Weiss *et al.*, 2006). The development of nanoparticles from the second half of the twentieth century until today has been of great importance. The ability to manipulate matter at the atomic scale, coupled with the discovery of new properties and functions at that level, generates a huge range of alternatives in creating devices, materials and systems, in every industry.

Nanostructured materials and devices have different properties than their counterparts in a larger scale. Some of these properties make new products possible, such as nanostructured plastics with the ability of conducting electrons rather than acting as insulators (Roco, 2004). These nanostructured materials can be obtained in two ways: a so-called "top down", in which the nanostructures are carved on a block of material, and another called "bottom up", in which nanostructured materials are constructed from nanoparticles. Technically "top down" methods are similar to current production techniques of electronic microprocessors. In the same way, "bottom up" techniques are based on processes like those used in materials technology, and can lead to powders, compact objects or thin layers with changed properties compared to the same materials obtained by conventional technologies (Dutta & Hofmann, 2004).

Nanotechnology begins to find applications in the field of functional foods through modified engineering of biological molecules to provide many different functions. This has opened a whole new scope of research and development. The term “nano-food” is used when nano-technological techniques or tools or nanoparticles are used during food cultivation, production, processing, or packaging (Paul *et al.*, 2003; Chaudhry *et al.*, 2008).

There is yet much development to be made of nanoparticles in the food area; it has possible applications related to food quality, food safety, development of new products and packaging, among others. It provides functional properties, for example, a food of low-sodium content with a salty taste (Takhistoy *et al.*, 2006).

One of the first commercial applications of nanotechnology in the food sector was in packaging. It is estimated that there are currently between 400 and 500 nano products being marketed, and it is expected that in 2017 nanotechnology will be used in the manufacture of 25% of all food packaging materials (Scrinis & Lyons, 2007).

One of the main purposes of nano packaging is to achieve a longer shelf-life by improving the barrier functions of the material used, to reduce the exchange of gases, moisture and exposure to ultraviolet radiation (Chaudhry *et al.*, 2008). There are other packaging and food contact materials that, unlike packaging materials that release chemicals under certain circumstances have incorporated antimicrobial nanomaterials so that the container itself acts as an antimicrobial agent. These products generally use silver nanoparticles, although some use zinc oxide nanoparticles or chlorine dioxide. It is also envisaged that in the near future nanoparticles of magnesium oxide, copper oxide and carbon nanotubes will be used as antimicrobial materials for food packaging (Bowman & Hodge, 2006). Nanoparticles can also improve food processes that use enzymes to produce health benefits and better nutrition. Research and development of nano-scale science has made great advances, from food packaging with less risk of migration of toxins in food, to techniques in the detection of pathogens.

The application of nanotechnology in the field of food will allow the development of healthier, more resistant, and longer-lasting foods. Nanotechnology is becoming increasingly important and because of its numerous and diverse applications in this field it will continue to be subject for innovation.

With regards to foods, research is current on the development of nanoparticles with features that ensure greater protection of foods against microorganisms. Among the research being undertaken, researchers have focused on the use of essential oils or some phenolic compounds and polymers such as chitosan, given the antimicrobial properties known individually for each, and the feasibility in their theoretical reactivity, added to the fact both substances are obtained from natural sources of low cost, thus an enhanced effect that incorporates the properties that characterize each component can be obtained.

Chitosan nanoparticles

Different methods have been used to produce chitosan particulate systems, which Dash *et al.* (2011) comprehensively reviewed. The preferred method will rely on factors such as particle size condition, thermal and chemical stability of the active agents, reproducibility of the kinetic release profiles, stability of the final product, residual toxicity related with the final products, the nature of the active molecule, and even the type of the delivery device (Agnihotri *et al.*, 2004).

Chitosan nanoparticles were first described in 1994 when Ohya *et al.* (1994) proposed the release of 5-fluorouracil, an anticancer drug, hauled through emulsification and nanoparticles obtained by crosslinking. Since then, these systems have been extensively studied.

Polymeric nanoparticles are usually classified as nanocapsules or nanospheres. Nanocapsules have two compartments; a polymeric wall and a core, which is commonly oily. Nanospheres are complex matrix systems. The antimicrobial essential oil may be conjugated with the polymer (in the matrix or wall) or in the oily core. When considering their antimicrobial activity in medicine and/or as preservatives for food and cosmetics, these particles must be constituted of biocompatible materials. Biocompatible polymers may be of synthetic origin such as poly (DL-lactide-co-glycolide) (PLGA) or from natural origin such as chitosan (Reis *et al.*, 2006).

Chitosan nanoparticles have been of considerable interest, due to their unique spectrum of properties, such as biocompatibility, biodegradability, metal complexation, and antibacterial activity. The development of chitosan nanoparticles has been achieved through preparation methods that involve manipulation in an aqueous medium, thus avoiding using organic solvents. Additionally, many researchers have developed new formulations of chitosan nanoparticles including materials with the capacity to form secondary matrices (Du *et al.*, 2004; 2009; Sarmento *et al.*, 2006 a; b; Grenha *et al.*, 2010).

For the formation of chitosan nanoparticles several investigations using low energy methods have been developed, due to being simple methods, and the fact that they do not expose bioactive compounds to extremes of temperature or light, which can cause changes. Nanoparticles of chitosan have been synthesized and are used mainly as a vehicle for different types of drugs (Du *et al.*, 2009).

When applying active agent immobilization technologies, chitosan has received a lot of interest for encapsulation of active compounds in order to improve the efficiency of active agents and reduce the costs of production. Chitosan nanoparticles have been prepared to improve their antimicrobial activity (Qi *et al.*, 2004). Du *et al.* (2009) conducted studies on the preparation of chitosan nanoparticles loaded with metal ions such as Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+} through ionotropic gelation method, in order to evaluate their antibacterial activity against various pathogens including *E. coli*, *Salmonella* Choleraesuis and *Staphylococcus aureus*; results showed that the antibacterial activity of chitosan nanoparticles with metal ions was significantly higher than that of the lone metal ions; similarly, it was observed that Gram negative bacteria were more sensitive than

Gram positive bacteria and the antibacterial activity was determined to be directly proportional to the zeta potential.

Another development in this area has been the formation of chitosan nanogels, in order to encapsulate bioactive substances and improve their physical properties, stability, and releasing capacity of the compounds. These nanogels are cross-liked polymer particles of submicron size with unique properties like high water content, three-dimensional structure, biocompatibility, and the most important, its swelling degree which permits drug release by changing external environmental conditions to the nanogel such as temperature and/or pH, among other possible factors/conditions.

Abreu *et al.* (2012) developed a composite of chitosan and cashew gum (as the encapsulating agent) for oil matrix *Lippia sidoides* (larvicide), in order to increase its larvicidal activity. Cashew gum is an exudate from the *Anarcardium occidentale* tree that has similar properties to those of Arabic gum, whereby their structures have a main chain of galactose units, while including branches of arabinose, glucose, and rhamnose. The concentration of rubber and cashew oils had an influence on the properties of the developed nanogels, it was possible to obtain nanoparticles in an average range of 335-558 nm. The release profiles *in vitro* showed that the nanoparticles had a slower, sustained release, and were found to have enhanced larvicidal activity compared with *L. sidoides* pure oil.

Another application that has had great impact is the application of chitosan in conservation, storage, and quality maintenance of shrimp. White shrimp is a food that has great potential due to the exceptional advantages such as rapid growth, relatively low requirements in protein, and a good adaptation to several environmental conditions; however, white shrimps are highly perishable, with a post-capture lifetime of a few hours; as a result, yearly deterioration of shrimp worldwide leads to significant economic losses. Wang *et al.* (2015) conducted a study to assess the effectiveness of chitosan coating on shrimp in different aspects such as physical, chemical, and microbial changes during storage for 10 days at 4°C. A positive effect was observed when a coating of chitosan was applied to shrimps, showing an improvement of the quality of the shrimp compared to a coating of carboxymethyl chitosan; furthermore, the chitosan coating significantly increased the hardness, elasticity, and shrimp shelf-life. Chitosan coating could represent a natural conservation method and provide potential benefits for shrimp quality. Table 3 summarizes selected works that tested chitosan nanoparticles with distinctive purposes related to foods.

In the last decade different chitosan compounds have been developed for use in the field of agriculture, where pesticides may be trapped in a polymer matrix, maximizing its effect at low concentrations.

Phenolics' nanoparticles

Polymeric nanoparticles have attracted the attention of many researchers in recent years, who have investigated the effects of binding these nanoparticles with phenolic compounds to potentiate their action. Nanoparticles can be produced using pure phenolic compounds (as a single molecule) or with extracts rich in different phenolic compounds usually obtained from fruits, vegetables, tea and infusions, among other possible sources. Binding the core with phenolic compounds allows for the protection of bioactive compounds from severe conditions of light, heat, and oxygen, in addition to serving as a carrier for control and release of the active compound.

The association of essential oils and or phenolic compounds with polymeric nanoparticles presents several advantages such as their controlled release (Li *et al.*, 2012; Gomes *et al.*, 2011; Keawchaoon & Yoksan, 2011; Hosseini *et al.*, 2013), enhanced apparent water solubility (Wu *et al.*, 2012), reduced cytotoxicity (Chen *et al.*, 2009), and equal or enhanced antimicrobial activity (Iannitelli *et al.*, 2011).

Table 3. Diverse activities of selected chitosan nanoparticle formulations.

Formulation	Preparation method	Particle Size (nm)	Objective	Relevant findings	Reference
Chitosan nanoparticles loaded with metal ions	Ionotropic gelation	53-210	*E. coli*, *Salmonella* Choleraesuis, and *Staphylococcus aureus*	Antibacterial activity of nanoparticles was significantly higher than those of the lone metal ions	Du *et al.* (2009)
Chitosan nanoparticles loaded with *Lippia sidoides* essential oil	Coacervation by spray drying	335–558	*Stegomyia aegypti*	Larvicidal activity	Abreu *et al.* (2012)
Chitosan nanoparticles	Ionotropic gelation	82.6	Shrimp shelf-life	Increased hardness, elasticity, and shelf-life of shrimps	Wang *et al.* (2015)
Ascorbyl palmitate loaded chitosan nanoparticles	Ionotropic gelation	151-228	Enzyme activity	Inhibition of polyphenol oxidase	Kim *et al.* (2013)

A phenolic compound of interest is eugenol, known for its great antimicrobial activity as well as antioxidant properties; however, it is a hydrophobic molecule highly volatile, unstable, and sensitive to oxygen, light, and heat during processing, which limits its use within the food industry; consequently, alternatives have been sought for use. Fatouh *et al.* (2012) used essential oil of clove (and its major phenolic constituent eugenol) to form nanoparticles in a microemulsion system based on water in order to evaluate its antioxidant and antimicrobial activities. This technique offers numerous benefits, including enhanced stability, protection against oxidation, and retention of volatile ingredients. Their results showed that the microemulsion improved evaluated activities of both the essential oil and eugenol compared to their counterparts separately. The results of this study could have potential applications in water-based disinfectants, food flavoring and conservation, as well as for nutraceutical drinks.

Yang *et al.* (2009) tested nanoparticles coated with polyethylene glycol with encapsulated garlic essential oil to evaluate their insecticidal activity against the brown flour beetle (*Tribolium castaneum*). The nanoparticles were prepared using the melt dispersion method. Their results showed that control against *T. castaneum* remained higher than 80% after five months, due to the slow and sustained release of the active components of the nanoparticles. This contrasted the effective control of the lone essential oil of garlic at a similar concentration which was only 11%. This indicates that it is feasible to use nanoparticles with polyethylene glycol coating and encapsulation of essential oil of garlic to control such pests.

Gomes *et al.* (2011) synthesized nanoparticles of poly (DL-lactide-co-glycolide) (PLGA) with trans-cinnamaldehyde and eugenol. By characterizing these new systems, they could understand the mechanism of controlled release and antimicrobial efficiency for inhibiting the growth of *Salmonella* spp. and *Listeria* spp.

Polymeric nano-capsules containing essential oils in their core usually present a two phase release profile; a burst release followed by a prolonged release phase (Li *et al*., 2012; Gomes *et al*., 2011). Kailaku *et al.* (2014) revealed that the creation of chitosan nanoparticles coupled with the phenolic compound of catechin extracted from tea, helps prolong the release of this compound in a specific medium.

Other phenolic compound of interest that has been already studied is thymol. Thymol, the most abundant component of thyme (*Thymus vulgaris*), is formed via *p*-cymene from ɤ-terpinene. Its antimicrobial properties were investigated by *in vitro* and *in vivo* assays, demonstrating that it is effective against natural spoilage bacteria and against foodborne pathogens, for example, *Salmonella* Typhimurium, *E. coli*, *Listeria monocytogenes* (Altieri *et al*., 2005; Valero *et al*., 2006; Falcone *et al*., 2005; Zhou *et al*., 2007). Thymol cumulative release was dependent on the encapsulated concentration; in low concentrations (10% and 20%) thymol released a lower proportion of the total essential oil load than when in higher concentrations (30% and 40%) were utilized.

Polymeric nanocapsules can be easily incorporated in creams and gels (Lboutounne *et al*., 2002), which facilitates the use of essential oils as cosmetic and pharmaceutical preservatives.

There are recent studies that have developed chitosan micro- and nano-capsules for the bioactive compound carvacrol. Carvacrol is the major component of herbs such as oregano, thyme, marjoram and savory. It has been used as a flavoring, antioxidant, and antimicrobial agent. Unfortunately carvacrol is a volatile compound that evaporates easily and is decomposed by heat, light, or oxygen losing its properties when subjected to food processing. Encapsulation is an effective way to prevent the decomposition of this compound. Keawchaoon & Yoksan (2011) developed chitosan nanoparticles encapsulating carvacrol, obtaining particles with an average size of 40 to 80 nm with spherical form and a positive surface charge; zeta potential values ranged from 25 to 29 mV, decreasing the potential with larger amounts of initial carvacrol content. The chitosan-carvacrol capsules showed greater antimicrobial activity against *S. aureus*, *B. cereus* and *E. coli* than their respective parent compounds acting individually.

Nanoencapsulation of bioactive compounds represents a viable and efficient approach to increase the physical stability of active substances, protecting them from interactions with food ingredients, and because of their sub-cellular size, increasing their bioactivity. In the case of antimicrobials, encapsulation can increase the concentration of the bioactive compounds in food areas where microorganisms are preferably located, for example water-rich phases or liquid-solid interfaces (Weiss *et al*., 2009). Most published investigations comparing antimicrobial activity of free and encapsulated essential oil find that encapsulation does not reduces inhibitory doses while improving other essential oils' characteristics such as control release and cytotoxicity (Keawchaoon & Yoksan, 2011). Considering mammalian use of polymeric nanoparticles loaded with antimicrobial essential oil, an important advantage is the reduction of essential oil cytotoxicities.

Among variety of methods developed to prepare chitosan nanoparticles, ionic gelation technique have attracted considerable attention due to this process being non-toxic, organic solvent free, convenient, and controllable (Agnihotri *et al*., 2004). Ionic gelation technique is based on the electrostatic interaction between the positively charged primary amino groups of chitosan and the negatively charged groups of polyanions, such as sodium tripolyphosphate (TPP) (Calvo *et al*., 1997; Dyer *et al*., 2002; Yang *et al*., 2011). The chitosan-TPP nanoparticle, composed of food-safe ingredients, has shown its capacity for the encapsulation and delivery of polyphenolic compounds (Bao *et al*., 2009; Dudhani & Kosaraju, 2010). Keawchaoon & Yoksan (2011) revealed that encapsulation of essential oil-derived bioactive compounds such as carvacrol into chitosan-tripolyphosphate particles could extend their shelf life and retain several functional properties of phenolic compounds such as carvacrol, eugenol, and thymol. Table 4 summarizes some works

Table 4. Activity of different phenolic compounds loaded as nanoparticles in selected polymers

Formulation	Phenolic Compound	Target Microorganism	Application	Reference
Nanoparticles of poly DL-lactide-co-glycolide (PLGA)	trans-cinnamaldehyde and eugenol	*Salmonella* spp. and *Listeria* spp.	Mechanism of controlled release and antimicrobial efficiency for inhibiting bacterial growth	Gomes *et al.* (2011)
Chitosan nanoparticles	Catechin	---------	Prolong the release of studied compound in a specific medium	Kailaku *et al.* (2014)
Zein-thymol nanoparticles stabilized with sodium caseinate and chitosan	Thymol	*Salmonella* Typhymurium, *E. coli*, *Listeria monocytogenes*	Antimicrobial activity	Zhang *et al.* (2014)
Chitosan nanoparticle	Carvacrol	*S. aureus*, *B. cereus* and *E. coli*	Antimicrobial activity	Keawchaoon & Yoksan (2011)
Carum copticum essential oil loaded chitosan nanoparticles	Thymol and Carvacrol	*S. aureus*, *S. epidermidis*, *Bacillus cereus*, *E. coli*, *S.* Typhimurium, *P. vulgaris*	Antioxidant and Antimicrobial activity	Esmaeili & Asgari (2015)
Encapsulation of oregano essential oil in chitosan nanoparticles	Thymol and Carvacrol	-------------	Nutraceutical and Food applications	Hosseini *et al.* (2013)

described in the literature related to phenolics loaded as nanoparticles.

The formation of nanoparticles and nano-capsules of phenolic compounds has great potential to improve the effectiveness and efficiency of their release in food systems. By characterizing these new systems, we can understand the mechanism of controlled release and antimicrobial efficiency. Corresponding knowledge will enable food manufacturers to design new food systems for several applications, including packaging and processing systems capable of ensuring food safety to consumers.

CHITOSAN NANOPARTICLES WITH PHENOLIC COMPOUNDS

Currently, there are several methods for nanoparticle formation, which mainly fall into two categories. The first category includes methods that require employing and utilizing considerable amounts of mechanical energy, for example: high pressure homogenization, ultrasound, high-speed stirring, spray drying, and freeze-drying. These methods have been used for a great number of polymers.

Chitosan nanoparticles loaded with phenolic compounds prepared according to the method of self-assembly (Keawchaoon & Yoksan, 2011; Du *et al*., 2004; 2009) presented similar particle distribution patterns as can be observed in Figure 1. Dynamic Light Scattering (DLS) is frequently utilized to measure nanoparticle diameters, distribution as well as Z-potential. Chitosan nanoparticles had a zeta potential value of 43.21 mV, implying a positively charged particle surface. The zeta potential was reduced to values ranging from 24.5 to 29.9 mV for the chitosan-phenolic nanoparticles. This may reflects that loading with phenolics reduce the positive surface charge and decreases the stability of the dispersion of particles in water. The reduction in zeta potential may be the result of phenolics coating the surface of the nanoparticles.

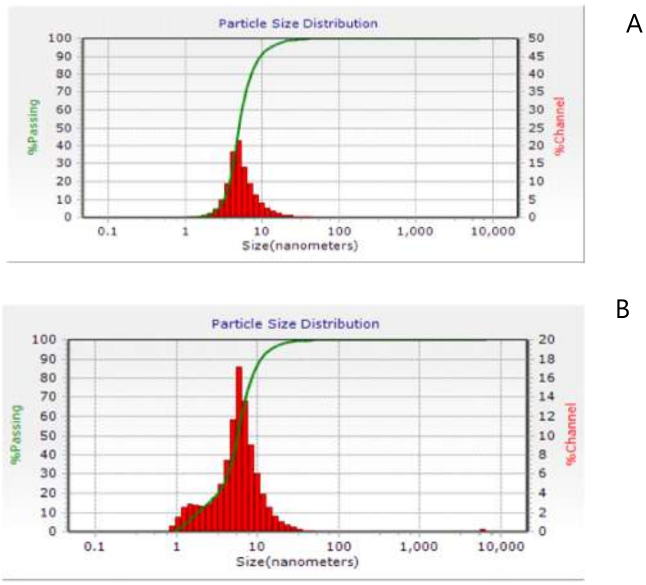

Figure 1. Determination of particle size distribution by Dynamic Light Scattering (DLS), (A) chitosan and (B) chitosan-phenolic

The second category includes methods that require less energy, as they make use of chemical energy stored in the system, which are also cheaper than the aforementioned methods. Such methods have been extensively researched and developed, and in recent years there has been a growing interest in these methods, evidenced by the large number of existing studies. Among these methods are: spontaneous emulsification, polyelectrolyte complexation, and ionotropic gelation.

Spontaneous emulsification, forms an emulsion with minimal agitation, this implies a high level of thermodynamic stability which is highly desirable. By spontaneous emulsification, nano-emulsions are obtained through the dilution process in a continuous phase, with subsequent diffusion of surfactant molecules and/or solvent from the dispersed phase to the continuous one, without changes in the tensoactive curvature (Bilbao-Sainz et al., 2010). Using phase-inversion emulsification, nanoemulsions are also obtained in a process where a change occurs in the curvature of the surfactant molecules (Bouchemal *et al*., 2004). Among the techniques of phase-inversion emulsification method, constant composition stands out. This technique allows the composition to be kept constant by varying the phase-inversion temperature (PIT) (Lee *et al*., 2014).

Ionotropic gelation is a simple method based on the complexation of positively charged polymers when in contact with specific poly-anions to form inter- and intra-molecular crosslinks. The encapsulation of many bioactive compounds has been a widely used technique. One example is chitosan, which has the property of coating the core of a bioactive compound and acting as a retardant in the rate of its release. Anionic compounds form a mesh structure by combining with polyvalent cations and chitosan, which induces gelation. Qi *et al*. (2004) used this method to evaluate the antibacterial activity *in vitro* of chitosan-copper nanoparticles against various microorganisms. Studied nanoparticles were prepared using ionic gelation of chitosan with tripolyphosphate anions. Copper ions were adsorbed onto chitosan by ion exchange resins chelating the surface to form copper nanoparticles. The antibacterial activity was evaluated against *E. coli*, *S*. Choleraesuis, *S*. Typhimurium, and *S. aureus* and was assessed by estimating the minimum inhibitory and the minimum bactericidal concentrations. Results demonstrated that studied chitosan-copper nanoparticles inhibited the growth of tested bacteria. Their study further revealed that exposing *S*. Choleraesuis to chitosan allows for rupture of cell membranes and causes cytoplasm leakage.

Polyelectrolyte complexation method is a widely used method to produce chitosan nanoparticles, which is based on the formation of polyelectrolyte complexes, and also requires a polyanionic chitosan polymer. There is no need of auxiliaries such as catalysts or initiator molecules and the reaction is usually performed in aqueous solution. The polyelectrolyte complexes are a particular class of electrostatically charged molecules with complementary ionizable groups. Sodium tripolyphosphate is the most widely used polyanion in the preparation of chitosan nanoparticles; however, the following polyanions have also been used: dextran sulfate, carboxymethylcellulose, heparin and DNA, while in the food area polyanionic compounds being used include alginate, xanthan gum, and carrageenan (Patil *et al*., 2010).

The properties and possibilities of obtaining networks of various shapes and sizes makes polyelectrolyte complexes highly usable in various fields, being the largest development in the biomedical field and management systems for new drugs, with also important applications as food antimicrobials.

CONCLUSIONS

The extraordinary scientific, technological, industrial, socio-economic, agro-food and health implications of nanotechnology are endless, only limited in the medium- to long-term by the imagination and creativity of scientists, technologists and engineers. In several countries, governments and companies are investing on hundreds of development projects in nanotechnology applied to food and agriculture.

Nanotechnology can be applied in most aspects of the food chain, both to improve safety and quality, and to produce novel food ingredients or supplements. Various methods have been developed for the formation of nanoparticles and specifically with chitosan, as they have been shown to display several advantages as carriers of bioactive substances. Production of nanoparticles with different methods has resulted in nanoparticles with different properties, including size and charge, which may affect their ability to focus and encapsulate. However, besides the method of preparation, they depend on other factors such as matrix material concentration, and reaction time, among others.

Indeed there is a stern increase in the amount of research aimed at the application of chitosan. There are expectations that in the near future, a formulation of nanoparticles based on chitosan as a carrier is to become marketable and accepted by the main regulatory agencies worldwide. However, more research is needed to understand the effect of food nanoparticles on humans. Little is known about how these particles are absorbed and excreted by the body and especially how they move through it.

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ACKNOWLEDGEMENTS

Author Soto-Chilaca gratefully acknowledges support for his PhD studies from Consejo Nacional de Ciencia y Tecnología (CONACyT) of Mexico and Universidad de las Américas Puebla, Mexico.

Conflicts of Interest

The authors declare no conflict of interest.