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Food Preservation – A Biopreservative Approach

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ABSTRACT

Preservative agents are required to ensure that manufactured foods remain safe and unspoiled. Antimicrobial properties of essential oils (EOs) reveal that Gram-positive bacteria are more vulnerable than Gram-negative bacteria. A number of EO components have been identified as effective antibacterials, e.g. carvacrol, thymol, eugenol, cinnamaldehyde and cinnamic acid, having minimum inhibitory concentrations (MICs) at higher dilutions *in vitro*. EOs comprise a large number of components and it is likely that their mode of action involves several targets in the bacterial cell. The potency of naturally occurring antimicrobial agents or extracts from plants, ranges of microbial susceptibility and factors influencing antimicrobial action and their antioxidative properties, aimed at food preservation, are reviewed in this article. Methods employed for estimation of inhibitory activity, mode of action and synergistic and antagonistic effects are evaluated. The potential value of these agents as natural and biological preservatives is considered.

Keywords: essential oils, food safety, natural antimicrobials, natural flavor complexes, toxicity

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INTRODUCTION

Many food products are perishable by nature and require protection from spoilage during their preparation, storage and distribution to give them desired shelf-life. Because food products are now often sold in areas of the world far distant from their production sites, the need for extended safe shelf-life for these products has also expanded.

The development of food preservation processes has been driven by the need to extend the shelf-life of foods. Food preservation is a continuous fight against microorganisms spoiling the food or making it unsafe. Several food preservation systems such as heating, refrigeration and addition of antimicrobial compounds can be used to reduce the risk of outbreaks of food poisoning; however, these techniques frequently have associated adverse changes in organoleptic characteristics and loss of nutrients. Within the disposable arsenal of preservation techniques, the food industry investigates more and more the replacement of traditional food preservation techniques by new preservation techniques due to the increased consumer demand for tasty, nutritious, natural and easy-to-handle food products. Improvements in the cold distribution chain have made international trade of perishable foods possible, but refrigeration alone cannot assure the quality and safety of all perishable foods.

The most common classical preservative agents are the weak organic acids, for example acetic, lactic, benzoic and sorbic acid. These molecules inhibit the outgrowth of both bacterial and fungal cells and sorbic acid is also reported to inhibit the germination and outgrowth of bacterial spores.

In the production of food it is crucial that proper measures are taken to ensure the safety and stability of the product during its whole shelf-life. In particular, modern consumer trends and food legislation have made the successful attainment of this objective much more of a challenge to the food industry. Firstly, consumers require more high quality, preservative-free, safe but mildly processed foods with extended shelf-life. For example, this may mean that foods have to be preserved at higher pH values and have to be treated at mild-pasteurization rather than sterilization temperatures. As acidity and sterilization treatments are two

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crucial factors in the control of outgrowth of pathogenic spore-forming bacteria, such as *Clostridium botulinum*, addressing this consumer need calls for innovative approaches to ensure preservation of products. Secondly, legislation has restricted the use and permitted levels of some currently accepted preservatives in different foods. This has created problems for the industry because the susceptibility of some microorganisms to most currently used preservatives is falling.

An increasing number of consumers prefer minimally processed foods, prepared without chemical preservatives. Many of these ready-to-eat and novel food types represent new food systems with respect to health risks and spoilage association. Against this background, and relying on improved understanding and knowledge of the complexity of microbial interactions, recent approaches are increasingly directed towards possibilities offered by biological preservation.

Throughout the development of both Western and Eastern civilization, plants, plant parts, and derived oils and extracts have functioned as sources of food and medicine, symbolic articles in religious and social ceremonies, and remedies to modify behavior. Taste and aroma not only determine what we eat but often allow us to evaluate the quality of food and, in some cases, identify unwanted contaminants. The principle of self-limitation taken together with the long history of use of natural flavor complexes in food argues that these substances are safe under intended conditions of use.

Originally added to change or improve taste, spices and herbs can also enhance shelf-life because of their antimicrobial nature. Some of these same substances are also known to contribute to the self-defense of plants against infectious organisms (Kim *et al*. 2001).

In spite of modern improvements in food production techniques, food safety is an increasingly important public health issue (WHO 2002a). It has been estimated that as many as 30% of people in industrialized countries suffer from a food borne disease each year and in 2000 at least two million people died from diarrhoeal disease worldwide (WHO 2002a). There is therefore still a need for new methods of reducing or eliminating food borne pathogens, possibly in combination with existing methods. At the same time, Western society appears to be experiencing a trend of 'green' consumerism (Smid and Gorris 1999), desiring fewer synthetic food additives and products with a smaller impact on the environment. Furthermore, the World Health Organization has already called for a worldwide reduction in the consumption of salt in order to reduce the incidence of cardio-vascular disease (WHO 2002b). If the level of salt in processed foods is reduced, it is possible that other additives will be needed to maintain the safety of foods. There is therefore scope for new methods of making food safe which have a natural or 'green' image. One such possibility is the use of essential oils (EOs) as antibacterial additives.

Based on rich histories of use of selected plants and plant products that strongly impact the senses, it is not unexpected that society would bestow powers to heal, cure diseases, and spur desirable emotions, in the effort to improve the human condition. The perception that these products are "natural" and have a long history of use has, in part, mitigated the public's need to know whether these products work or are safe under conditions of intended use.

Until recently, EOs have been studied most from the viewpoint of their flavor and fragrance only for flavoring foods, drinks and other goods. Actually, however, EOs and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey 2001). It has long been recognized that some EOs have antimicrobial properties (Boyle 1955) and these have been reviewed in the past (Shelef 1983; Nychas 1995) as have the antimicrobial properties of spices (Shelef 1983) but the relatively recent enhancement of interest in 'green' consumerism has lead to a renewal of scientific interest in these substances (Tuley 1996). Besides antibacterial properties (Mourey and Canillac 2002; Rasooli and Razzaghi 2004; Rasooli and Owlia 2005), EOs or their components have been shown to exhibit antiviral (Bishop 1995), antimycotic (Mari *et al*. 2003), anti oxidative (Gachkar *et al*. 2006; Yadegarinia *et al*. 2006; Bektas *et al*. 2007a; Bektas *et al*. 2007b), antitoxigenic (Akgul *et al*. 1991; Juglal *et al*. 2002; Ultee and Smid 2001), antiparasitic (Pandey *et al*. 2000; Pessoa *et al*. 2002), and insecticidal (Karpouhtsis *et al*. 1998) properties. These characteristics are possibly related to the function of these compounds in plants (Mahmoud and Croteau 2002). The antibacterial properties of EOs and their components are exploited in such diverse commercial products as dental root canal sealers (Manabe *et al*. 1987), antiseptics (Cox *et al*. 2000) and feed supplements for lactating sows and weaned piglets (van Krimpen and Binnendijk 2001; Ilsley *et al*. 2002). It is therefore scientifically sound to evaluate the impact of EOs on food and food products safety.

Natural flavor complexes (NFCs) are mixtures of mainly low molecular weight chemical substances separated from plants by physical means such as distillation, extraction, and cold pressing. The most common NFCs are EOs. The EO is typically obtained by steam distillation of the plant or plant parts. With few exceptions, plants are dependent on their EO content for their unique aroma and gustatory profile. In other words, the volatile constituents of the plant isolated in the EO are primarily responsible for aroma and taste of the plant. For economic reasons, crude EOs are often produced via distillation at the source of the plant raw material and subsequently further processed at modern flavor facilities.

EOs, also called volatile or ethereal oils, are aromatic oily liquids obtained from plant flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots. They can be obtained by expression, fermentation, effleurage or extraction but the method of steam distillation is most commonly used for commercial production of EOs (van de Braak and Leijten 1999). The term 'essential oil' is thought to derive from the name coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim; he named the effective component of a drug *Quinta essentia* (Guenther 1948). An estimated 3000 EOs are known, of which about 300 are commercially important – destined chiefly for the flavors and fragrances market (van de Braak and Leijten 1999). Distillation as a method of producing EOs was first used in the East (Egypt, India and Iran) (Guenther 1948) more than 2000 years ago and was improved in the 9th century by the Arabs. By the 13th century EOs were being made by pharmacies and their pharmacological effects were described in pharmacopoeias (Bauer *et al*. 2001). The greatest use of EOs in the European Union (EU) is in food (as flavorings), perfumes (fragrances and aftershaves) and pharmaceuticals (for their functional properties) (van de Braak and Leijten 1999).

EOs and fractions are also formulated in shampoos, toothpaste, disinfectants, topical ointments and cosmetics. However, when used in foods, highly volatile plant EOs are sometimes lost during processing operations. Microencapsulation technology is one way these losses of EOs by volatilization can be prevented. This technique is being widely used in the pharmaceutical industry for controlled delivery of drugs. It is also currently used in the food industry for flavor stabilization. By encapsulating antimicrobial EOs, not only can they be protected from heat, but they also can be released in products at a controlled rate to deliver effective inhibitory concentrations over extended periods and thereby extend shelf-life.

This review presents the current understanding of the mode of action of these compounds and their possible applications in food protection.

INFLUENCE OF CHEMICAL COMPOSITION OF ESSENTIAL OILS ON THEIR ANTIMICROBIAL ACTIVITIES

Due to their natural origin, environmental and genetic factors will influence the chemical composition of the plant EOs. Factors such as species and subspecies, geographical location, harvest time, plant part used and method of isolation all affect chemical composition of the crude material separated from the plant.

Steam distillation is the most commonly used method for producing EOs on a commercial basis. Extraction by means of liquid carbon dioxide under low temperature and high pressure produces a more natural organoleptic profile but is much more expensive (Moyler 1998). The difference in organoleptic profile indicates a difference in the composition of oils obtained by solvent extraction as opposed to distillation and this may also influence antimicrobial properties. This would appear to be confirmed by the fact that herb EOs extracted by hexane have been shown to exhibit greater antimicrobial activity than the corresponding steam distilled EOs (Packiyasothy and Kyle 2002). EOs are volatile and therefore need to be stored in airtight containers in the dark in order to prevent compositional changes. The composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources (Juliano *et al*. 2000; Faleiro *et al.* 2002).

It was postulated that individual components of EOs exhibit different degrees of activity against gram-positives and gram-negatives (Dorman and Deans 2000) and it is known that the chemical composition of EOs from a particular plant species can vary according to the geographical origin and harvesting period (*vide supra*). It is therefore possible that variation in composition between batches of EOs is sufficient to cause variability in the degree of susceptibility of Gram-negative and Gram-positive bacteria.

The inherent activity of oil can be expected to relate to the chemical configuration of the components, the proportions in which they are present and to interactions between them (Dorman and Deans 2000; Marino *et al.* 2001; Delaquis *et al.* 2002). An additive effect is observed when the combined effect is equal to the sum of the individual effects.

Some studies have concluded that whole EOs have a greater antibacterial activity than the major components mixed (Gill *et al.* 2002; Mourey and Canillac 2002), which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence. The two structurally similar major components of oregano EO, carvacrol and thymol, were found to give an additive effect when tested against *S. aureus* and *P. aeruginosa* (Lambert *et al* 2001).

A mixture of cinnamaldehyde and eugenol at 250 and 500 µg/ml, respectively inhibited growth of *Staphylococcus* sp., *Micrococcus* sp. *Bacillus* sp. and *Enterobacter* sp. for more than 30 days completely, whereas the substrates applied individually did not inhibit growth (Moleyar and Narasimham 1992).

The oils with high levels of eugenol (allspice, clove bud and leaf, bay, and cinnamon leaf), cinnamamic aldehyde (cinnamon bark, cassia oil) and citral are usually strong antimicrobials (Davidson and Naidu 2000). Activity of sage and rosemary is due to borneol and other phenolics in the terpene fraction. The volatile terpenes carvacrol, *p*-cymene and thymol are probably responsible for the antimicrobial activity of oregano, thyme and savory. In sage, the terpene thejone and in rosemary a group of terpenes (borneol, camphor, 1,8 cineole, α-pinene, camphone, verbenonone and bornyl acetate) is responsible (Davidson and Naidu 2000).

Little information is available on interaction among constituents in EOs and the effects they have on antimicrobial activity. Phenolic components are responsible for antimicrobial action and other constituents are believed to have little activity. Dependability of EOs as antimicrobials could be improved if their content of active agents should be standardized by distillation (Delaquis *et al.* 2002). As a general observation, spice extracts are less antimicrobial than the whole spice but little quantitative data are available (Shelef 1983). Four studies are relevant. Lachowicz *et al.* (1998) found crude EO of basil more effective than components linalool and methyl chavicol either separately or together. Vardar-Unlu *et al.* (2003) found similar results following fractionation of extracts from thyme. In aqueous extracts from oregano or thyme there was little antimicrobial activity. Thus there appear to be interactive effects among constituents not extractable in the water-soluble phase and these components do not appear to be the phenolics normally considered to show the major antimicrobial activities. In contrast with the above studies, Delaquis *et al.* (2002) found that individual fractions of cilantro and dill EOs had greater antimicrobial activity than did the whole oil. In addition, they found cilantro fractions deficient in phenolics but enriched in long chain (C_6–C_{10}) alcohol and aldehydes that were particularly active against Gram-positive bacteria including *L. monocytogenes*. To broaden the antimicrobial spectrum, a fraction from cilantro oil with no activity against Gram-negative bacteria was combined with a eucalyptus fraction having broader activity. Additive or synergistic action was reported against all Gram-positive bacteria plus *Yersinia enterocolitica* and the mixture was antagonistic to *P. fragi*, *E. coli* O157:H7 and *Salmonella typhimurium*.

Bactericidal effects of cinnamaldehyde and thymol against *B. cereus* (Demo *et al* 2001; Kwon *et al*. 2003), as well as the development of synergistic effects between carvacrol or thymol and nisin have been also reported (Pol and Smid 1999; Periago and Moezelaar 2001; Periago *et al*. 2001). The effects of various concentrations of borneol, carvacrol, cinnamaldehyde, eugenol, menthol, thymol, and vanillin on the growth kinetics of activated *Bacillus cereus* INRA L2104 spores inoculated into tyndallized carrot broth were determined. Five microliters of cinnamaldehyde, 15 µl of carvacrol, or 30 mg of thymol per 100 ml of inoculated carrot broth completely inhibited bacterial growth for more than 60 days at 16°C. Lower concentrations of the three antimicrobials prolonged the lag phase and reduced both the exponential growth rate and the final population densities of cultures. The study of the sensory characteristics of the supplemented broths suggested that low concentration of cinnamaldehyde enhanced the taste of carrot broth, and that it did not have any adverse effect on the taste and smell of carrot broth at concentrations less than 6 µl 100 ml^{-1} (Valero and Giner 2006).

The major constituents of the oils of thyme and oregano species have been reported to be thymol, carvacrol and γ-terpinene. Thyme EO and its ingredients have been shown to exhibit a range of biological activities. Since EOs of thyme and oregano possess strong antibacterial and antimicrobial activity they can be used to delay or inhibit the growth of pathogenic microorganisms. These activities are mostly attributable to the presence of phenolic compounds such as thymol and carvacrol, and to hydrocarbons like γ-terpinene and *p*-cymene (Dorman and Deans 2000; Lambert *et al.* 2001; Aligiannis *et al.* 2001; Vardar-Unlu *et al.* 2003; Baydar *et al.* 2004). Thymol and carvacrol can be used alone or in combination during the treatment of oral infectious diseases because of their inhibitory activity on oral bacteria (Ditry *et al*. 1994; Kohlert *et al*. 2002). Thyme and oregano were found to inhibit aflatoxin production (Vaughn *et al*. 1993). Antispasmodic and antiplatelet aggregation activities were also reported with thyme constituents (Meister *et al*. 1999; Okazaki *et al*. 2002).

Monoterpenes are natural ten-carbon (C_{10}) compounds constructed from two isoprene molecules (C_5H_8, or hemiterpene), the five-carbon building-block of all terpenes. They are found in edible, medicinal and aromatic plants and are the main chemical constituents of their EOs. Plant volatile oils as well as their monoterpenoid constituents have been widely used as flavorings additives in foods and beverages, as fragrances in cosmetics, and as intermediates in the manufacture of perfume chemicals. They have also been em-

ployed as scent in household products (e.g., detergents, soaps, room air-fresheners and insect repellents) and as active ingredients in some old drugs (Leung and Foster 2003). Pinene, for instance, is one of the main constituents of a mixture of six monoterpenes used to dissolve gallstones (Ellis *et al*. 1984), and α-terpinene is one of the putative active ingredients of tea tree (*Melaleuca alternifolia*) oil, an antibacterial and antifungal remedy employed in both veterinary and human medicine (Dryden *et al*. 2004).

IN VITRO ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OILS

A large number of studies have examined the *in vitro* antimicrobial activity of spices, herbs and naturally occurring compounds from other sources. Plant EOs have been widely tested against both Gram-positive and -negative bacteria. For example, Farag *et al*. (1989) examined the antimicrobial activity of the oils of sage, thyme and rosemary leaves, caraway fruits, clove flower buds, and cumin fruits against three Gram-negative bacteria (*P. fluorescens*, *E. coli*, and *Serratia marcescens*) and four Gram-positive bacteria (*S. aureus*, *Micrococcus* spp., *Sarcina* spp., and *B. subtilis*). They found that the EOs from sage, cumin, rosemary and their principal components had no or very little effect against Gram-negative bacteria, but oil of caraway was moderately effective against this group. Oils from clove and thyme were highly active at a concentration of 0.75–1.5 mg /ml against *S. aureus* and *Micrococcus* spp., while only small inhibition zones were reported for Gram-negative bacteria. In general, Gram-negative bacteria were more resistant to EOs than Gram-positive bacteria, with the oils being effective even at low concentration (0.25–12 mg /ml) against the Gram-positive organisms. In similar work it was also found that mint EO was more effective against Gram-positive bacteria than against Gram-negative bacteria (Sivropoulou *et al*. 1995; Iscan *et al*. 2002). Delaquis *et al*. (2002) reported that Gram-positive bacteria were more sensitive to the EOs of dill, cilantro, coriander and eucalyptus than Gram-negative bacteria. It is well established that essential or volatile oils from plant sources have wide spectra of antimicrobial action (Alzoreky and Nakahara 2002; Packiyasothy and Kyle 2002). The composition, structure as well as functional groups of the oils play an important role in determining their antimicrobial activity. Usually compounds with phenolic groups are most effective (Dorman and Deans 2000). Among these, the oils of clove, oregano, rosemary, thyme, sage and vanillin have been found to be most consistently effective against microorganisms. Most studies investigating the action of whole EOs against food spoilage organisms and food borne pathogens agree that, generally, EOs are generally more inhibitory against Gram-positive than against Gram-negative bacteria (Marino *et al*. 2001). That gram-negative organisms are less susceptible to the action of antibacterials is perhaps to be expected, since they possess an outer membrane surrounding the cell wall (Ratledge and Wilkinson 1988), which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara 1992). While this is true of many EOs, there are some such as oregano, clove, cinnamon and citral; which are effective against both groups (Skandamis *et al*. 2002). However, not all studies on EOs have concluded that gram-positives are more susceptible (Wilkinson *et al*. 2003). There are also some non-phenolic constituents of oils such as allyl isothiocyanate, AIT; which are more effective (Ward *et al*. 1998) or quite effective against Gram-negative bacteria as in garlic oil (Yin and Cheng 2003). A study testing 50 commercially available EOs against 25 genera found no evidence for a difference in sensitivity between Gram-negative and Gram-positive organisms (Deans and Ritchie 1987). However, a later study using the same test method and the same bacterial isolates but apparently using freshly distilled EOs, revealed that Gram-positive bacteria were indeed more susceptible to two of the EOs tested and equally sensitive to four other EOs than were Gram-negative species (Dorman and Deans 2000). Of the Gram-negative bacteria, Pseudomonads, and in particular *P. aeruginosa*, appear to be least sensitive to the action of EOs (Ruberto *et al*. 2000; Senatore *et al*. 2000; Tsigarida *et al*. 2000; Dorman and Deans 2000; Pintore *et al*. 2002; Wilkinson *et al*. 2003). Pseudomonads consistently show high or often the highest resistance to these antimicrobials such as linalool/chavicol (Smith-Palmer *et al*. 1998), terpenoids/carvacrol/thymol (Griffin *et al*. 1999), oregano (Skandamis *et al*. 2002), *Capsicum* or bell pepper (Careaga *et al*. 2003) and annatto, (Galindo-Cuspinera *et al*. 2003). Nonetheless, since pseudomonads are so frequently responsible for spoilage of food stored at low temperatures they have often been used as targets, and at high concentrations some EO components have been reported to be effective (Careaga *et al*. 2003).

Infections caused by *Campylobacter* in humans are considered to be the result of ingestion of contaminated foods of animal origin, mainly poultry products and raw milk, or untreated water (Moore *et al*. 2002; Park 2002). Successful steps to reduce the occurrence of *Campylobacter* on poultry could have a major effect on reduction of foodborne illness. In a recent study, a proprietary mixture of herbs (Protecta II) at 2% (w/v) was used in poultry chill water and reduced the numbers of both *Campylobacter* and *E. coli* by 2 log cfu/ml in carcass rinses (Dickens *et al*. 2000). Friedman *et al*. (2002) evaluated 96 different naturally occurring plant oils and oil compounds against *C. jejuni* in iron-supplemented brucella agar. The oils of marigold taegetes, ginger root, jasmine, patchouli, and gardenia were most effective with bactericidal activity (BA) assessed as BA50's (concentration of oil at which a 50% reduction of total cfu was observed) ranging from 0.003% to 0.007%. Like plant EOs and oil-derived compounds, garlic-derived organosulphur compounds have also shown antimicrobial activity. When evaluated against *C. jejuni* in ground beef, diallyl sulphide and diallyl disulphide at 20 μM showed a significant reduction with final viable numbers of 1.63 log cfu /g and 1.26 log cfu/g, respectively, compared to 7.54 log cfu/g in untreated controls during 6 d storage at 15°C (Yin and Cheng 2003).

Recently there has been significant interest in the development of secondary preservation steps that could reduce *L. monocytogenes* viability and growth in refrigerated ready-to-eat foods (Rocourt *et al*. 2003). Four recent studies examined the effects of different natural antimicrobials on this organism in broth media. Of the agents tested isoeugenol was most effective, giving a 4.6 log reduction of *L. monocytogenes* numbers at 100 ppm in conjunction with use of freeze thaw cycles at −20°C (Cressy *et al*. 2003). Cilantro oil was more effective than hydroxycinnamic acids, with MICs of cilantro against *L. monocytogenes* of 0.02–0.07% (v/v) in Brain Heart Infusion (BHI) broth at 24°C (Gill *et al*. 2002) as compared with MICs of 0.2–0.27% (w/v) for 4 hydroxycinnamic acids (Wen *et al*. 2003). A GRAS carotenoid pigment used in butter and cheese, annatto, was least effective (MIC 1.25% v/v) against this organism (Galindo-Cuspinera *et al*. 2003). Differences in strain susceptibility were evident and cilantro oil was ineffective against *L. monocytogenes* when used on the surface of inoculated ham at a concentration of 6% (v/v) of the enrobing gel (Gill *et al*. 2002).

The antibacterial activity of EOs is influenced by the degree to which oxygen is available. This could be due to the fact that when little oxygen is present, fewer oxidative changes can take place in the EOs and/or that cells obtaining energy via anaerobic metabolism are more sensitive to the toxic action of EOs (Paster *et al*. 1990). The antibacterial activity of oregano and thyme EOs was greatly enhanced against *S. typhimurium* and *S. aureus* at low oxygen levels (Paster *et al*. 1990). The use of vacuum packing in combination with oregano EO may have a synergistic effect on the inhibition of *L. monocytogenes* and spoilage flora on beef fillets; 0.8% v/w oregano EO achieved a 2–3 log initial reduction in the microbial flora but was found to be even more effective in samples packed under vacuum in low-

permeability film when compared to aerobically stored samples and samples packaged under vacuum in highly permeable film (Tsigarida *et al*. 2000). Similarly, the lethal effect of clove and coriander EOs on *A*. *hydrophila* on pork loin steak stored at 2 and 10°C was more pronounced in vacuum packed pork than on samples stored in air (Stecchini *et al*. 1993). Oregano EO was found to delay microbial growth and to suppress final counts of spoilage microorganisms in minced beef under modified atmosphere packaging (MAP, 40% CO_2, 30% N_2 and 30% O_2) when, in contrast, no pronounced inhibition was evident in beef packed under air (Skandamis and Nychas 2001).

Cinnamon oil and clove oil are both natural preservative and flavouring substances that are not harmful when consumed in food products. Soliman and Badeaa (2002) found that ≤500 ppm of cinnamon oil can inhibit *A*. *flavus*, *A*. *parasiticus*, *A*. *ochraceus* and *Fusarium moniliforme* on potato dextrose agar. Matan *et al*. (2006) tested mixtures of cinnamon and clove oils for inhibitory activity against important spoilage microorganism of intermediate moisture foods. Four fungal species (*Aspergillus flavus*, *Penicillium roqueforti*, *Mucor plumbeus* and *Eurotium* sp.), four yeast species (*Debaryomyces hansenii*, *Pichia membranaefaciens*, *Zygosaccharomyces rouxii* and *Candida lipolytica*), and two bacteria species (*Staphylococcus aureus* and *Pediococcus halophilus*) inoculated separately on agar plates were sealed in a barrier pouch and exposed to EO volatiles under a modified atmosphere of low O_2 (< 0.05–10%) and high CO_2 (20% or 40%), with the balance being N_2. *A*. *flavus* and *Eurotium* sp. proved to be the most resistant microorganisms. Cinnamon and clove oils added between 1000 and 4000 µL at a ratio of 1:1 were tested for minimum inhibitory volume (MIV) against molds and yeasts. The gas phase above 1000 µL of the oil mixture inhibited growth of *C*. *lipolytica* and *P*. *membranaefaciens*; 2000 µL inhibited growth of *A*. *flavus*, *P*. *roqueforti*, *M*. *plumbeus*, *Eurotium* sp., *D*. *hansenii*, and *Z*. *rouxii*, while inhibition of *A*. *flavus* required the addition of 4000 µL. Higher ratios of cinnamon oil/clove oil were more effective for inhibiting the growth of *A*. *flavu*s. Citrus EOs can have very pronounced antimicrobial activity, even if their complexity and variability make difficult to correlate their action to a specific component, also in relation to possible antagonistic and synergistic effects. For this reason, Caccioni *et al*. (1998) proposed a holistic approach to explain the antimicrobial capabilities of such EOs, whose performances could be the result of a certain quantitative balance of various components. Citrus oxygenated monoterpenes are among the molecules with the highest antifungal activity (Caccioni and Guizzardi 1994) and citral was the most active compound against *Penicillium digitatum* and *P*. *italicum* (Caccioni *et al*. 1995).

Origanum vulgare L., Lamiaceae family, is being used in traditional medicine systems in many countries (Sagdic *et al*. 2002; Sahin *et al*. 2004). *Origanum vulgare* L. has been known as having many therapeutic properties (diaphoretic, carminative, antispasmodic, antiseptic, tonic) and its antimicrobial activity has currently received a renewed interest. It has been widely used in agricultural, pharmaceutical and cosmetic industries as a culinary herb, flavoring substances in food products, alcoholic beverages and perfumery for its spicy fragrance (Dorman and Deans 2000; Novak *et al*. 2000; Aligianis *et al*. 2001). Some researchers have found antimicrobial activity in *O*. *vulgare* L. (Skandamis *et al*. 2002; Baydar *et al*. 2004; Chun *et al*. 2004; Nostro *et al*. 2004). The oils extracted from plants of the genus *Origanum* have been shown to have antimicrobial activity *in vitro* and in food (Aligiannis *et al*. 2001). Souza *et al*. (2007) reported the effectiveness of *O*. *vulgare* L. EO to inhibit the growth/survival of various food spoiling yeasts. Anti-yeast activity was studied by determining the MIC by solid medium diffusion and microplate bioassay, as well as observing the effect of the EO MIC on the yeast cell viability. *O*. *vulgare* EO showed effectiveness to inhibit the growth of all assayed yeasts with MIC values for the most ones of 20 and 0.6 µL/mL when determined, respectively, by solid medium diffusion and microplate bioassay. Solid medium diffusion MIC presented statistically significant inhibitory effects ($P < 0.05$) on yeast cell viability, mainly when interacting with *Candida albicans* and *Candida krusei*. On the other hand, the microplate MIC just provided statistically significant inhibitory effects on the cell viability when interacting with *C*. *krusei*.

It is well established that bacterial biofilms exhibit more resistance to antimicrobial treatments than the individual cells grown in suspension (Knowles and Roller 2001; Chavant *et al*. 2004). Lebert *et al*. (2007) investigated bactericide solutions effective on spoilage and pathogenic bacteria while preserving technological bacteria. Two compounds of EO (thymol and eugenol), one EO of *Satureja thymbra* and two industrial biocides (PE 270–30, Brillo) were tested on technological strains (*Staphylococcus equorum*, *Staphylococcus succinus* and *Lactobacillus sakei*) grown in monoculture biofilm and on a mixed biofilm of pathogenic bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*) and spoilage bacteria (*Pseudomonas fragi*, *Escherichia coli*). Biofilm cultures were performed in glass fibre filters for 24 h at 20°C before application of biocides. Thymol and eugenol had no effect on the mixed biofilm. *S*. *thymbra* (2%) was highly effective on spoilage strains (5 log reduction), and *S*. *equorum* (4 log reduction) was moderately effective on pathogens (2.3 log reduction) and not effective on *S*. *succinus* and *L*. *sakei* (0.5 log reduction). PE-270-30 with 10% Na_2SO_4 decreased spoilage bacteria (5.1 log reduction), maintained the technological bacteria, but did not reduce the pathogens. The disinfectant Brillo (3%) killed all the strains. Their results showed the difficulty in obtaining a biocide that is effective in destroying spoilage and pathogenic bacteria while preserving technological bacteria. Lebert *et al*. (2007) concluded that EOs could be a good alternative for eradicating spoilage bacteria in the food environment where they are often found at high levels.

Antimicrobial packaging is a form of active packaging that could extend the shelf-life of product and provides microbial safety for consumers. It acts to reduce, inhibit, or retard the growth of pathogen microorganisms in packed foods and packaging material. In order to control undesirable microorganisms on food surfaces: (1) volatile and nonvolatile antimicrobial agents can be incorporated into polymers or (2) coating or adsorbing antimicrobial onto polymer surfaces can be applied (Appendini and Hotchkiss 2002). Several compounds have been proposed for antimicrobial activity in food packaging, including organic acids, enzymes such as lysozyme, and fungicides such as benomyl, imazalil and natural antimicrobial compounds such as spices (Tharanathan 2003). These compounds carry mostly antimicrobial and some antioxidant properties. Natural compounds, such as nisin and lysozyme, have been studied as potential food preservatives added to the edible films that are safe for human consumption (Padget *et al*. 2000; Hoffman *et al*. 2001; Dawson *et al*. 2002; Cagri *et al*. 2004; Min *et al*. 2005). Some spice EOs incorporated into packaging materials can control microbial contamination in beef muscle by reducing the growth of *Escherichia coli* O157:H7 and *Pseudomonas* spp. (Oussallah *et al*. 2004). The use of edible films to release antimicrobial constituents in food packaging is a form of active packaging. Antimicrobial properties of whey protein isolate (WPI) films containing 1.0–4.0% (wt/vol) ratios of oregano, rosemary and garlic EOs were tested against *Escherichia coli* O157:H7 (ATCC 35218), *Staphylococcus aureus* (ATCC 43300), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (NCTC 2167) and *Lactobacillus plantarum* (DSM 20174). Ten millilitres of molten hard agar was inoculated by 200 µl of bacterial cultures (colony count of 1×10^8 CFU/ml) grown overnight in appropriate medium. Circular discs of WPI films containing spice extracts, prepared by casting method, were placed on a bacterial lawn. Zones of inhibition were measured after an incubation period. The film containing oregano EO was the most effective against these bacteria at 2% level than those containing garlic and rosemary extracts

(P <0.05). The use of rosemary EO incorporated into WPI films did not exhibit any antimicrobial activity whereas inhibitory effect of WPI film containing garlic EO was observed only at 3% and 4% level (P <0.05). The results of this study suggested that the antimicrobial activity of some spice extracts were expressed in a WPI based edible film (Seydim and Sarikus 2006).

The experiments indicate that spices with strong antimicrobial activity include allspice, cinnamon, clove, mustard and vanillin. Among herbs, the following are most antimicrobial: basil, oregano, rosemary, sage and thyme. Spices and herbs which show limited antimicrobial activity include: anise, bay (laurel), black pepper, cardamom, cayene (red pepper), celery seed, chili powder, coriander, cumin, curry powder, dill, fenugreek, ginger, juniper oil, mace, marjoram, mint, nutmeg, paprika, sesame, spearmint, tarragon, and white pepper (Davidson and Naidu 2000).

IN VITRO ANTIMICROBIAL ASSAY METHODS

Tests of antimicrobial activity can be classified as diffusion, dilution or bioautographic methods. It appears that no standardized test has been developed for evaluating the antibacterial activity of possible preservatives against food-related microorganisms, although the need for such has been indicated (Davidson and Parish 1989). The NCCLS method for antibacterial susceptibility testing, which is principally aimed at the testing of antibiotics has been modified for testing EOs (NCCLS 2000). Researchers adapt experimental methods to better represent possible future applications in their particular field. However, since the outcome of a test can be affected by factors such as the method used to extract the EO from plant material, the volume of inoculum, growth phase, culture medium used, pH of the media and incubation time and temperature (Rios *et al*. 1988), comparison of published data is complicated (Friedman *et al*. 2002). The minimum inhibitory concentration (MIC) is cited by most researchers as a measure of the antibacterial performance of EOs. The definition of the MIC differs between publications and this is another obstacle to compareson between studies. In some cases, the minimum bactericidal concentration (MBC) or the bacteriostatic concentration is stated, both terms agreeing closely with the MIC. Screening of EOs for antibacterial activity is often done by the disk diffusion method, in which a paper disk soaked with EO is laid on top of an inoculated agar plate. This is generally used as a preliminary check for antibacterial activity prior to more detailed studies. Factors such as the volume of EO placed on the paper disks, the thickness of the agar layer and whether a solvent is used vary considerably between studies. This means that this method is useful for selection between EOs but comparison of published data is not feasible. The agar well test in which the EO is deposited into wells cut into the agar can be used as a screening method when large numbers of EOs and/or large numbers of bacterial isolates are to be screened (Dorman and Deans 2000). In order to make bacterial growth easier to visualise, triphenyl tetrazolium chloride may be added to the growth medium (Elgayyar *et al*. 2001; Mourey and Canillac 2002). The strength of the antibacterial activity can be determined by dilution of EO in agar or broth. The published studies using dilution in agar have used different solvents to incorporate the EO in the medium (Pintore *et al*. 2002), different volumes of inoculum (1-100 µl) (Juven *et al*. 1994; Prudent *et al*. 1995), which is sometimes dotted (Pintore *et al*. 2002) and sometimes streaked (Farag *et al*. 1989) onto the agar surface. Despite these variations, the MICs of EOs determined by agar dilution generally appear to be in approximately the same order of magnitude (Pintore *et al*. 2002). In broth dilution studies a number of different techniques exist for determining the endpoint, the most used methods are that of optical density (OD) measurement and the enumeration of colonies by viable count. The former method has the advantage of being automated; the latter is labour intensive. A very useful development has been the adoption of microwell titre plates containing broth to which 0.15% (w/v) agar is added to suspend partially soluble antimicrobials in the colloidal agar matrix (Mann and Markham 1998). This method continues to be used (Gill *et al*. 2002; Wen *et al*. 2003) and as originally described the broths contained resazurin dye as a redox indicator to give a visual signal reflecting bacteria growth. A prerequisite is that each test strain must be calibrated for its ability to reduce the resazurin dye before tests are conducted since rates may vary. The optimum test cell concentration is about one log lower than the level necessary to reduce resazurin to a pink/purple colour. Microwell titre plates are attractive for this type of study because only small reaction volumes (≤300 µl/test) are needed, replicate tests are easily prepared using multichannel pipettors, and multi-well plates lend themselves to adoption of protocols where two or more antimicrobials can be used simultaneously in gradients to examine reactants for interactive (synergistic or antagonistic) effects. The checker board assay typifies the approach used (Wen *et al*. 2003). A patented color indicator based on resazurin has been used to determine the MICs for methanolic extracts of plant materials (Salvat *et al*. 2001) and EOs (Burt and Reinders 2003) and the method can be automated by measuring the end point by fluorescence instead of visual means (Lancaster and Fields 1996). Triphenyl tetrazolium chloride has been used for visual end point determination in the study of tea tree oil in broth but, although it is an indicator of bacterial growth, the color change did not fully correlate with the MIC (Carson *et al*. 1995b).

The rapidity of a bactericidal effect or the duration of a bacteriostatic effect can be determined by time-kill analysis (survival curve plot) whereby the number of viable cells remaining in broth after the addition of EO is plotted against time. The most frequently used methods for this are measurement of OD and viable count after plating out onto agar. Decimal reduction value (D value has been employed to find out microbial elimination time by others (Gachkar *et al*. 2006; Yadegarinia *et al*. 2006).

One feature of test methods that varies considerably is whether or not an emulsifier or solvent is used to dissolve the EO or to stabilize it in water-based culture media. Several substances have been used for this purpose: ethanol (Marino *et al*. 2001; Packiyasothy and Kyle 2002; Walsh *et al*. 2003; Wen *et al*. 2003), methanol (Onawunmi 1989), Tween-20 (Hammer *et al*. 1999), Tween-80 (Mourey and Canillac 2002; Bassole *et al*. 2003; Wilkinson *et al*. 2003), sorbitan monolaurate (Mann and Markham 1998), acetone in combination with Tween-80 (Prudent *et al*. 1995), polyethylene glycol (Pintore *et al*. 2002), propylene glycol (Negi *et al*. 1999), *n*-hexane (Senatore *et al*. 2000), dimethyl sulfoxide (Firouzi *et al*. 1998; Iscan et al. 2002; Vardar-Unlu *et al*. 2003) and agar (Delaquis *et al*. 2002; Gill *et al*. 2002; Burt and Reinders 2003). While controls to monitor for possible solvent effects on bacterial viability should be included when solvents are used, there is always the possibility for interactive effects (e.g. quenching) that cannot be evaluated with a solvent-only control (Lambert *et al*. 2001). However, a number of researchers found it unnecessary to use an additive (Tassou *et al*. 2000; Dorman and Deans 2000; Elgayyar *et al*. 2001; Lambert *et al*. 2001; Canillac and Mourey 2001; Cimanga *et al*. 2002; Mejlholm and Dalgaard 2002). One study employed vigorous shaking in phosphate saline buffer to suspend EOs (Friedman *et al*. 2002).

The hydrophobicity of phenolics limits the value of agar disc/diffusion tests for estimating antimicrobial potency accurately. Since EOs are characterized as being volatile, methods that test the antimicrobial activity of such agents in their vapor phase have been outlined (Zaika 1988), and modified (Weissinger *et al*. 2001). In the latter study agar discs were inoculated with test bacteria and placed on a glass slide inserted into a glass jar along with horseradish EO that had been placed on a watch glass. The jar was sealed and placed at the desired temperature for exposure to EO vapor. Viability of exposed cells on the agar surface was

later assessed. The system worked well and allowed for quantification of the inhibitor in the vapour phase. Filter paper impregnated with AIT was used by Weissinger *et al*. (2001) to expose segregated alfalfa seed to vapor while both were enclosed in a glass jar. Iscan *et al*. (2002) used a combination of microdilution, agar diffusion and a bioautography bioassay to examine mint EO for activity against plant and human pathogens. For the bioassay, samples were subjected to thin layer chromatography (TLC) on parallel plates. One plate was chemically developed and the other was coated in agar, inoculated with the test organism and incubated at 37°C for 24 h. The latter plate was sprayed with 1% tetrazolium violet, incubated 1 h at 37°C and inhibition zones visualized against the colored background. In another modified agar method, Bagamboula *et al*. (2003) added dried, course ground herbal and spice plant material directly to agar which was then solidified, surface inoculated with a single organism and incubated. This method is also useful for screening agents. However, there is concern that EOs may only be partially released during grinding, leaving some trapped in cell compartments and unavailable for reaction with target organisms. Most researchers currently use agar or broth dilution series (Davidson and Naidu 2000; Rauha *et al*. 2000) or both for comparative purposes. Although tube macrodilution and diffusion from inhibitor-impregnated paper discs on agar surfaces are still used (Galindo-Cuspinera *et al*. 2003), there is heavy reliance on microwell plate systems containing inhibitors and target organisms in broth. Inhibitor effectiveness is measured by differences in rates of change of optical density from controls and bacterial numbers in non-turbid wells are monitored by plating on agar with enumeration after incubation. However, systems based on absorbance in broth lack sensitivity below 5 log bacteria /ml. This has largely been overcome by application of impedance measurement using inoculated broth. These systems can also be used to distinguish between bacteriostatic and bactericidal effects (Wan *et al*. 1998; Skandamis *et al*. 1999; Marino *et al*. 2001). Walsh *et al*. (2003) used conventional broth dilutions monitored for changes in absorbance at 540 nm as well as MICs determined by placing 1 µl spots of bacterial culture (6 log cfu/ml) on agar plates containing natural antimicrobial inhibitors (eugenol and thymol) using a multi-channel inoculator. After incubation, tests were compared to control plates without inhibitors.

There is clearly still a need for the adoption of standardized protocols for the evaluation of natural antimicrobials during *in vitro* tests to avoid the generation of contradictory results (Cowan 1999). However, it is unlikely that a single standard method will have general appeal. This is because studies often have different purposes and objectives, and frequently employ different experimental designs. These translate into different needs with respect to incubation temperature (4-37°C), pH (4-8) and length of exposure (hours or weeks). There are other factors, which influence experimental outcomes during potency testing of natural antimicrobials. These include: variability in composition or content of active agents that result from agronomic history, variety differences, and maturity of the plant material studied (Gill *et al*. 2002); physical and chemical characteristics of the antimicrobial itself (hydrophobicity, volatility, compatibility in the test system); presence of protein, starch or lipid that may complex with and neutralize antimicrobial activity or partition the agent away from its target (Davidson and Naidu 2000); and the inoculum size, genus of microorganism, species and even strain susceptibility as well as previous culture history (Gill *et al*. 2002; Bagamboula *et al*. 2003; Wen *et al*. 2003). It is probable that prior stress of cells by exposure to unfavorable conditions may change susceptibility of cells to natural antimicrobials, but this is an area still to be investigated (Gill *et al*. 2002). Incomplete control over the factors identified above will lead to generation of inconsistent results. Perhaps the greatest source of variation in study results arises from the use of unstandardized natural antimicrobials of different potency and composition.

AFLATOXIN THREAT AND HERBAL CONTROL

Many microorganisms have been found on intermediate moisture foods (IMF) including: *Aspergillus flavus* and *Penicillium roqueforti* on bread (Nielsen and Rios 2000); *Eurotium* sp. on bakery products (Suhr and Nielsen 2004); *Staphylococcus aureus* on honey (Mundo *et al*. 2004); *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* on syrups, fruit concentrates and jams (Andrews *et al*. 1997); red halophilic cocci on salt cured fish and solar salt (Pradsad and Seenayya 2000); *Pichia membranaefaciens* on mayonnaise and cheeses; *Candida albicans* on dried meat (Pitt and Hocking 1997); and *Mucor plumbeus* on cheeses (Taniwaki *et al*. 2001a, 2001b). Molds growing on IMF may produce toxins such as citrinin, aflatoxin, and requefortine c that can be hazardous to humans (Taniwaki *et al*. 2001a, 2001b). These toxins are produced during the growth of the microorganism on the food substrate (Filtenborg *et al*. 1996). *Aspergillus ochraceus* produces ochratoxin A (OTA) which is responsible for nephropathies in pigs and humans. Due to their ability to grow in almost all food products, yeasts and moulds can generate off-flavors, produce toxin, and cause discoloration and proteolysis through the action of various enzymes like lipases and proteases. The most important feature of moulds from a food safety perspective is their ability to produce mycotoxins. Fungi are the main agents of spoilage of bakery products. As with other foods, bakery products which contain natural preservatives are becoming more common. However, as with bacteria, fungi are more resistant to these natural antimicrobials when challenged in foods (Lopez-Malo *et al*. 2002). Aflatoxins, the most dangerous mycotoxins, are toxigenic secondary metabolites of fungi produced by certain strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius* which is phenotypically similar to *A. flavus* but with a distinctive bullet shaped sclerotia (Fente *et al*. 2001). Other species that produce aflatoxins in minute quantities include *A. peudotamarii*, *A. bonbysis* and *A. ochraceoroseus* (Fente *et al*. 2001). The hazardous nature of aflatoxins to human and animals necessitate the need for establishment of control measures and tolerance levels by national and international authorities. About 4.5 billion people in developing countries are chronically exposed to the uncontrolled amounts of aflatoxin (Williams *et al*. 2004). Current control measures are aimed at controlling fungal growth and mycotoxin formation in stored grains by physical methods (aeration, cooling and modified atmospheres), chemical treatments with ammonia, acids and bases or with food preservatives (Jackson and Bullerman 1999) and by biological methods (Blackwell *et al*. 1999). These methods require sophisticated equipment and expensive chemicals or reagents.

Plants derived EOs are also known to possess antifungal activity (Soliman and Badeaa 2002), and generally they are more active against fungi than Gram-positive bacteria (Shelef 1983). As an example of antimycotic activity, the oil of *Ocimum gratissimum* leaves was fungicidal at 78 ppm for *Microsporum gypseum* and *Trichophyton rubrum*, but a concentration of 312 ppm was required to inhibit growth of *Candida albicans* and *Cryptococcus neoformans* (Amvam Zollo *et al*. 1998). Many EOs and aroma compounds were also able to reduce or control mould mycotoxin production. Ochratoxin A accumulation by *Aspergillus ochraceus* was inhibited by eugenol (Basilico and Basilico 1999), which also prevented citrinin formation by *P. citrinum* in cheese (Vazquez *et al*. 2001). Chao *et al*. (2000) evaluated antifungal activity of 45 different plant oils against *C. albicans*, *Aspergillus niger* and *Rhizopus oligosporus*. Of the 45 oils, those of coriander, cinnamon bark, lemongrass, savory and rosewood were effective against all three microorganisms. Other oils showed selective activity against the three organisms. Some of them were effective against *C. albicans* only. Angelica and pine oils used in this study were not effective against *A. niger* and *R. oligosporus* which have a symbiotic relationship with the mycorhizae associated with

the plants from which the oils were isolated (*Angelica archangelica* L. and *Pinus sylvestris* L.), respectively. In addition to inhibition of vegetative growth, the other oils also inhibited production of mycotoxins by fungi. Thyme, anise and cinnamon oils were able to inhibit production of aflatoxins, ochratoxins A and fumonisin in broth at 2%. Anise, fennel and caraway oils also showed fungicidal effects against *A. flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium moniliforme*. Anise oil showed fungicidal activity at 500 ppm, while caraway and fennel oil at 2000 ppm were required to inhibit all four fungi (Soliman and Badeaa 2002). Some plant EOs have also been shown to inhibit mycelial growth and conidial germination. The oils of thyme, oregano, dictamus and marjoram totally inhibited mycelial growth at 250-400 µg/ml, while 250 µg/ml inhibited conidial germination of *Penicillium digitatum*. The oils of lavender, rosemary and sage gave 29.5%, 24.0% and 9.0% (% of untreated control) mycelial inhibition, respectively, at 1000 µg /ml. Citral, citronellal and eugenol prevented aflatoxin production in cultures of *A. flavus* in the first days of incubation but, after 2 weeks, toxin concentration was greater than in the control (Mahmoud 1994). This result, although not confirmed by other studies, has to induce some criticism in the application of such compounds in food. Their use in food preservation can not rescinds from the comprehension of the action mechanisms of the molecules as well as the adaptation of microorganisms towards the harsh environment and their growth kinetics. EOs of sweet basil (*Ocimum basilicum*), cassia (*Cinnamomum cassia*), coriander (*Coriandrum sativum*) and bay leaf (*Laurus nobilis*) at 1-5% (v/v) concentration in palm kernel broth inoculated with spore suspension (10^6/ml) of *Aspergillus parasiticus* CFR 223 were evaluated for their potential in the control of aflatoxigenic fungus *A. parasiticus* CFR 223 and aflatoxin production. Healthy sorghum grains (120/treatment) immersed in the oils and distributed in three petri dishes with wet cotton wool were also inoculated with spore suspension (10^6/ml) of *A. parasiticus* CFR 223 and assayed for grain protection. Sweet basil oil at optimal protective dosage of 5% (v/v) was fungistatic on *A. parasiticus* CFR 223 and aflatoxins produced *in vitro* and on fungal development on sorghum grains ($P \leq 0.05$) with a residual effect that lasted for 32 days. In contrast, oil of cassia and bay leaf stimulated the mycelia growth of the fungus in vitro but reduced the aflatoxin concentration ($B_1 + G_1$) of the fungus by 97.92% and 55.21% respectively, while coriander oil did not have any effect on both the mycelia growth and aflatoxin content of the fungus. The combination of cassia and sweet basil oil at half their optimal protective dosages (2.5% v/v) completely inhibited the growth of the fungus. The feasibility of implementing these results to control aflatoxins was examined by the addition of whole and ground dry basil leaves at 5% and 10% (w/w), respectively, to 10 g sorghum, groundnut, maize and melon seed after 35 days storage period. It was found that the addition of whole and ground basil leaves markedly reduced aflatoxin contamination; however, 10% (w/w) of whole leaves was more effective as the reduction in aflatoxin was between 89.05% and 91%. The findings showed that aflatoxins can be controlled by co-storing whole sweet basil leaves with aflatoxin infected foods (Atanda *et al*. 2007). EO extracted from the leaves of *Chenopodium ambrosioides* Linn. (Chenopodiaceae) was tested against the aflatoxigenic strain of test fungus *Aspergillus flavus* Link. The oil completely inhibited the mycelial growth at 100 µg/ml. The oil exhibited broad fungitoxic spectrum against *Aspergillus niger*, *Aspergillus fumigatus*, *Botryodiplodia theobromae*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Cladosporium cladosporioides*, *Helminthosporium oryzae* and *Pythium debaryanum* at 100 µg/ml. The oil showed significant efficacy in inhibiting the aflatoxin B1 production by the aflatoxigenic strain of *A. flavus*. During *in vivo* investigation it protected stored wheat from different storage fungi for one year (Rajesh *et al*. 2007). The EO from *O. gratissimum* has been reported to be a potential food preservative with a pH dependent superiority against potassium sorbate. The EOs extracts from *Cymbopogon citratus*, *Monodora myristica*, *Ocimum gratissimum*, *Thymus vulgaris* and *Zingiber officinale* were investigated for their inhibitory effect against three food spoilage and mycotoxin producing fungi, *Fusarium moniliforme*, *Aspergillus flavus* and *Aspergillus fumigatus*. Five strains of each fungus were tested. The EO from *O. gratissimum*, *T. vulgaris* and *C. citratus* were the most effective and prevented conidial germination and the growth of all three fungi on corn meal agar at 800, 1000 and 1200 ppm, respectively. Moderate activity was observed for the EO from *Z. officinale* between 800 and 2500 ppm, while the EO from *M. myristica* was less inhibitory. These effects against food spoilage and mycotoxin producing fungi indicated the possible ability of each EO as a food preservative. A comparative test on the preservative ability of the EO from *O. gratissimum* and potassium sorbate against *A. flavus* at pH 3.0 and 4.5 showed that the EO remained stable at both pH, whereas the efficacy of potassium sorbate was reduced at higher pH (Ngueface *et al*. 2004). Aflatoxin B1 (AFB1) is a highly toxic and carcinogenic metabolite produced by *Aspergillus* species on agricultural commodities (Leontopoulos *et al*. 2003). Allameh *et al*. (2001) reported more than 50% inhibition of aflatoxin production at 50% (v/v) neem extract concentration. It has been demonstrated that addition of neem leaf extract above 10% (v/v) effectively inhibited aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus*. Under such conditions the mycelial dry weight was not affected (Zeringue and Bhatnagar 1990). Namazi and co-workers (2002) demonstrated that 0.9%-1% ammonia inhibited fungal growth together with aflatoxin production. Aflatoxin production by *A.parasiticus* was reported to be significantly inhibited by *Thymus eriocalyx* and *Thymus x-porlock* EOs (Rasooli and Razzaghi 2004).

ANTIOXIDATIVE PROPERTIES

Lipid peroxidation is a complex process occurring in aerobic cells and reflects the interaction between molecular oxygen and polyunsaturated fatty acids. Radicals are known to take part in lipid peroxidation, which causes food deterioration, aging organisms and cancer promotion (Ashok and Ali 1999). Antioxidants act as radical-scavengers, and inhibit lipid peroxidation and other free radical-mediated processes: therefore, they are able to protect the human body from several diseases attributed to the reactions of radicals (Takao *et al*. 1994). Use of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects (Cornwell *et al*. 1998), making attractive the search for antioxidant and scavenger natural compounds. The spoilage and poisoning of foods by oxidation and/or microorganisms is still a problem that is not yet overcome despite of the range of robust preservation techniques available. The screening of plant extracts and natural products for antioxidative activity has revealed the potential of higher plants as a source of new agents (Rios *et al*. 1988), to serve the processing of natural products. Free radicals provoked by various environmental chemicals as well as endogenous metabolism are involved in a number of diseases like tumors, inflammation, shock, atherosclerosis, diabetes, infertility, gastric mucosal injury, and ischemia due to the oxidative damage to DNA, lipids, and proteins and which can result in failure of cellular functions (Kasai *et al*. 2000). Consumption of antioxidants from plant materials that inhibit free radical formation or accelerate their elimination has been associated with a lowered incidence of these diseases as a consequence of alleviating the oxidative stress of free radicals (Leong and Shui 2002); accordingly, antioxidants have recently garnered increased research interest. Free radicals can result in food sourness, oil rottenness, and most industrial product aging. Many experiments have indicated that free radicals are necessary to support life, though they are also dangerous in biological tissues. Under normal physiological conditions, free radicals in the body will

undergo a process of production and continuous scavenging so as to sustain physiological equilibrium. Even when the free radicals generated in the body are in low concentrations, the body metabolism may be disordered and some diseases can be caused (Pietta 2000). Synthetic antioxidants have been used in the food industry since the 1940s, but trends in many health-related industries tend to shift preferences to natural sources. Therefore, investigation of natural antioxidants has been a major research interest for the past two decades as many research groups and institutions have been screening plant materials for possible antioxidant properties. Food composition data, necessary for epidemiological and nutritional studies, are merely representative of foodstuffs consumed in the raw state. Many food composition databases never take into consideration the fact that concentrations of nutrients and their activity may change through cooking practices such as blanching. This is of great importance, considering that only a small amount of vegetables is consumed in the raw state, whilst most need to be processed for safety and quality. Overwhelming scientific data, from epidemiological studies, indicate that diets rich in fruit, vegetables and grains are associated with a lower risk of several degenerative diseases, such as cancers (Steinmetz and Potter 1996) and cardiovascular diseases (Rimm *et al*. 1996). This association is often attributed to different antioxidant components, such as vitamin C, vitamin E, carotenoids, lycopenes, polyphenols and other phytochemicals. The most widely used synthetic antioxidants in food (butylated hydroxytoluene BHT, butylated hydroxyanisole BHA, propyl galate PG and tertiary butyl hydroquinone TBHQ) have been suspected to cause or promote negative health effects (Pokorny 1991). Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Since ancient times, spices in different types of food to improve flavors are well known for their antioxidant capacities (Madsen and Bertelsen 1995). In recent decades, the EOs and various extracts of plants have been of great interest as they have been the sources of natural products. In order to prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. But according to toxicologists and nutritionists, the side effects of some synthetic antioxidants used in food processing such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have already been documented. For example, these substances can exhibit carcinogenic effects in living organisms (Baardseth 1989). For this reason, governmental authorities and consumers are concerned about the safety of the food and also about the potential affects of synthetic additives on health (Reische *et al*. 1998). Hence, there is a growing interest in studies of natural additives as potential antioxidants. Many sources of antioxidants of plant origin have been studied in recent years. Among these the antioxidant properties of many aromatic plants and spices have shown to be effective in retarding the process of lipid peroxidation in oils and fatty foods and have gained the interest of many research groups. The use of EOs as functional ingredients in foods, drinks, toiletries and cosmetics is gaining momentum, both for the growing interest of consumers in ingredients from natural sources and also because of increasing concern about potentially harmful synthetic additives (Reische *et al*. 1998). Within the wide range of the above-mentioned products, a common need is availability of natural extracts with a pleasant taste or smell combined with a preservative action, aimed to avoid lipid deterioration, oxidation and spoilage by microorganisms. Those undesired phenomena are not an exclusive concern of the food industry, but a common risk wherever a lipid or perishable organic substrate is present. In fact, they induce the development of undesirable off-flavors, create toxicity and severely affect the shelf-life of many goods (Hirasa and Takemasa 1998). EO compounds, such as carvacrol and thymol, will both prevent the microbial and chemical deterioration when added to food (Ultee *et al*. 1999; Burt 2004; Mahmoud *et al*. 2004a, 2004b). Components of thyme, mainly thymol and carvacrol, were suggested to have antioxidant activity (Nakatani 2000; Lee and Shibamoto 2001; Miura 2002). The antimicrobial and antioxidant effect of these phenolic compounds can be enhanced by combining with other natural preservatives (Ramanathan and Das 1992; Yamazaki *et al*. 2004). The antioxidant activity found in the fractions of *Citrus sinensis*, is attributed to the presence of flavonoids and other phenolic compounds (Maria *et al*. 2006). The synthesis of several phenylpropanoid compounds (flavonoids, isoflavonoids, psoralens, coumarins, phenolics acids, chlorogenic acid, lignin and suberin) is induced in plants by biotic and abiotic stress, factors such as wounding, low temperature and attack of pathogens (Dixon and Paiva 1995). Phenolic compounds are known to constitute one of the most important groups of natural antioxidants, owing to their diversity and extensive distribution. They possess biological and chemical properties in common: reducing character, capacity of sequestering reactive oxygen species (ROS) and several electrophiles, for chelating metallic ions, tendency to self-oxidation and capacity for modulating the activity of some cell enzymes (Robards *et al*. 1999). Functions of diverse phenolic antioxidant in the diet have already been discussed (Astley 2003; Trewavas and Stewart 2003). The biological activity of phenylpropanoids and their function as antimicrobial agents are well recognized, as are their antiallergenic and anti-inflammatory properties, along with their antimutagenic action (Rice-Evans *et al*. 1996).

The number of antioxidant compounds synthesized by plants as secondary products, mainly phenolics, serving in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive, is currently estimated to be between 4000 and 6000 (Wollgast and Anklam 2000; Havsteen 2002). The phenolic content and composition of plants and the products produced from them depend on genetic and environmental factors, as well as post-harvest processing conditions (Cowan 1999). The antioxidant activities of phenolics are related to a number of different mechanisms, such as free radical-scavenging, hydrogen-donation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxyl. A direct relationship has been found between the phenolic content and antioxidant capacity of plants (Al-Mamary *et al*. 2002). There is intense interest in plant polyphenols as witnessed by the numerous papers devoted to various aspects of these compounds (Duthie and Crozier 2000; Harborne and Williams 2000; Tura and Robards 2002). Proestos *et al*. (2006) linked the phenolic fraction of plant extracts to their antioxidant capacity. The use of plants, herbs as antioxidants in processed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants (Madsen and Bertelsen 1995). They tend to be water soluble, because they frequently occur combined as glycosides and they are usually located in the cell vacuole (Harborne 1998). Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Pietta 2000). They also have metal chelation properties (Kahkonen *et al*. 1999). Their significance for the human diet and antimicrobial activity has been recently established (Rauha *et al*. 2000; Nychas *et al*. 2003). The antioxidant properties of these compounds are often claimed for the protective effects of plant-based beverages against cardiovascular disease, certain forms of cancer and photosensitivity reactions (Bravo 1998). It was also found that they inhibit human immunodeficiency viral replication (HIV), human simplex virus (HSV), glucosyl transferases of *Streptococcus mutans* (dental carries), ascorbate auto-oxidation (green tea), cytotoxic effects, tumor promotion and xanthine, monoamine oxidases (Mattila *et al*. 2000; Middleton *et al*. 2000; Havsteen 2002). These studies provide the basis for the present rapidly increasing interest for the use of natural antioxidants as functional food ingredients and/or as food supplements.

Extraction procedures to obtain active principles are mainly focused on the use of methanol or ethanol as solvents. Since active compounds in plants exhibiting biolo-

gical activity are in low concentrations, selective extraction methods should be used. Activity may be varied when different solvents are used for conventional extraction. Extraction with non-polar solvents, such as hexane, petrol ether, provided better antioxidant properties than did methanol or acetone (Chen *et al*. 1992; Chevolleau *et al*. 1992).

Presence of antioxidant component in plant materials is determined by many methods since the antioxidants act by different mechanisms. They play an important role for the scavenging of free radicals and chain-breaking and these types of compounds have been called primary antioxidants and act as deactivators of metals, inhibitors of lipid hydroperoxide breakdown, regenerators of primary antioxidants and quenchers of singlet oxygen (Koleva *et al*. 2002). Several methods have been recommended for the evaluation of antioxidant properties of plant materials and some methods in current use were compared (Koleva *et al*. 2002; Ou *et al*. 2002). 2,2′-Diphenyl-1-picrylhydrazyl radical (DPPH˙) assay is a well known method for the evaluation of free radical-scavenging activity. The method is sample polarity-independent very rapid, simple and reproducible (Koleva *et al*. 2002). On the other hand, fatty acid decomposition is one of the main causes of food spoilage and inhibition of fatty acid oxidation is an important issue in the food industry. Food preservers or antioxidants are mainly used as inhibitors of the oxidation of fatty acids. Therefore, the inhibition of linoleic acid oxidation can be measured in the presence of β-carotene that is used as a marker (Dapkevicius *et al*. 1998). Linoleic acid oxidation produces conjugated dienes and other volatile products that attack β-carotene and bleach its characteristic colour (pale yellow in aqueous emulsion). In general, both free radical-scavenging and inhibition of linoleic acid oxidation are desired in the food industry.

In a DPPH assay, *C. odorata*, *C. citratus*, *R. officinalis* and *C. longa* showed major effectiveness, with a radical inhibition ranging from 59.6 ± 0.42–64.3 ± 0.45%. In the β-carotene bleaching test, *C. odorata* (75.5 ± 0.53%), *R. officinalis* (81.1 ± 0.57%) and *C. longa* (72.4 ± 0.51%) gave the best inhibition results. Suggestions on relationships between chemical composition and biological activities are outlined (Sacchetti *et al*. 2005). *Mentha piperita* oil screened for antioxidant activities by DPPH free radical scavenging and β-carotene/linoleic acid systems showed greater antioxidant activity than *Myrtus communis* (Yadegarinia *et al*. 2006). Radical-scavenging and antioxidant properties of EOs from *Rosemarinus officinalis* and *Cuminum cyminum* were tested and compared to those of *Thymus x-porlock* EO. The radical scavenging performance of the rosemary oil was better than that of *Cuminum cyminum* (Gachkar *et al*. 2006). Owlia *et al*. (2007) reported antioxidant activity of EO from *Matricaria chamomilla* L.

The antioxidant activity of *Ruellia tuberosa* L. (Acanthaceae) was investigated by Chen *et al*. (2006). The methanolic extract (ME) and its four fractions of water (WtF), ethyl acetate (EaF), chloroform (CfF), and *n*-hexane (HxF) were prepared and then subjected to antioxidant evaluation. The results revealed that *R. tuberosa* possesses potent antioxidant activity. The antioxidant activities of the different fractions tested decreased in the order of EaF > CfF > ME > WtF > HxF according to the hydrogen peroxide-induced luminol chemiluminescence assay, and results were the same with the exception of the rank order of HxF and WtF according to the DPPH free radical-scavenging assay.

Eleven EOs, namely, *Cananga odorata* (Annonaceae), *Cupressus sempervirens* (Cupressaceae), *Curcuma longa* (Zingiberaceae), *Cymbopogon citratus* (Poaceae), *Eucalyptus globulus* (Myrtaceae), *Pinus radiata* (Pinaceae), *Piper crassinervium* (Piperaceae), *Psidium guayava* (Myrtaceae), *Rosmarinus officinalis* (Lamiaceae), *Thymus* × *citriodorus* (Lamiaceae) and *Zingiber officinale* (Zingiberaceae), were evaluated for their food functional ingredient related properties. These properties were compared to those of *Thymus vulgaris* EO, used as a reference ingredient. Antioxidant and radical-scavenging properties were tested by means of 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, β-carotene bleaching test and luminol-photochemiluminescence (PCL) assay. In the DPPH assay, *C. odorata*, *C. citratus*, *R. officinalis* and *C. longa* showed major effectiveness, with a radical inhibition ranging from 59.6 ± 0.42–64.3 ± 0.45%. In the β-carotene bleaching test, *C. odorata* (75.5 ± 0.53%), *R. officinalis* (81.1 ± 0.57%) and *C. longa* (72.4 ± 0.51%) gave the best inhibition results. Similar results were obtained for the same EOs in the PCL assay (Gianni *et al*. 2005).

Total antioxidant activity of water-soluble components in raw spinach was in the order of BI ≈ BM ≈ BPG > BP, whereas free radical-scavenging activity was in the order of BI > BPG > BM > BP (Amin *et al*. 2006).

Kartal and co workers (2007) examined the *in vitro* antioxidant properties of the EO and various extracts prepared from the herbal parts of *Ferula orientalis* A. (Apiaceae). The highest 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical-scavenging activity was found in the polar extract, e.g. methanol–water (1:1), obtained from non-deodorised materials with IC_{50} values at 99.1 μg/ml. In the β-carotene/linoleic acid assay, the deodorised acetone extract exhibited stronger activity than the polar one. The relative antioxidant activities of the extracts ranged from 10.1% to 76.1%, respectively. Extraction with methanol–water (1:1) mixture was concluded to be the most appropriate method in terms of higher extract yield, as well as effectiveness, observed in both assays. Although the EO showed antioxidative potential, it was not as strong as that of positive control (BHT).

Bektas *et al*. (2005) compared the antioxidant potentials of two *Thymus* species on the basis of the chemical compositions of EOs obtained by hydrodistillation. Using 2,2-diphenyl-1-picrylhydrazyl (DPPH), the free radical scavenging activity of the EO of *T. sipyleus* subsp. *sipyleus* var. *rosulans* was superior to var. *sipyleus* oil (IC_{50}=220 ± 0.5 and 2670 ± 0.5 μg/ml, respectively). In the case of β-carotene/linoleic acid assays, oxidation of linoleic acid was effectively inhibited by *T. sipyleus* subsp. *sipyleus* var. *rosulans* (92.0%), while the var. *sipyleus* oil had no activity. In the latter case, the linoleic acid inhibition rate of var. *rosulans* oil is close to the synthetic antioxidant BHT (96.0%).

Orhan and co-workers (2007) examined *in vitro* anticholinesterase and antioxidant activities of 56 extracts prepared with petroleum ether, chloroform, ethyl acetate and methanol obtained from 14 *Salvia* species (*Salvia albimaculata* Hedge and Hub, *Salvia aucheri* Bentham var. *canescens* Boiss and Heldr, *Salvia candidissima* Vahl. ssp. *occidentalis*, *Salvia ceratophylla* L., *Salvia cryptantha* Montbret and Bentham, *Salvia cyanescens* Boiss and Bal., *Salvia frigida* Boiss, *Salvia forskahlei* L., *Salvia halophila*Hedge, *Salvia migrostegia* Boiss and Bal., *Salvia multicaulis* Vahl., *Salvia sclarea* L., *Salvia syriaca* L., *Salvia verticillata* L. ssp. *amasiaca*) growing in Turkey. The antioxidant activities were assessed by both chemical and enzymatic methods against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging and xanthine/xanthine oxidase (XO) system generated superoxide anion radical inhibition. Their data indicates that nonpolar extracts of *Salvia* species for anticholinesterase activity and the polar extracts for antioxidant activity are worth further phytochemical evaluation for identifying their active components.

It was reported that oxidative stress is associated with the pathogenesis of Alzheimer's disease (AD) and cellular characteristics of this disease are either causes or effects of oxidative stress (Vina *et al*. 2004). These evidences clearly show that oxidative stress, an early event in AD, may play a key pathogenic role in the disease (Zhu *et al*. 2004). Interestingly, intake of polyphenols through diets rich in fruits, vegetables and beverages such as red wine was stated to reduce incidence of certain age-related neurological disorders including macular degeneration and dementia (Commenges *et al*. 2000; Bastianetto and Quirion 2002).

Herbs and spices have been used for many centuries to improve the sensory characteristics and to extend the shelf-life of foods. As a result, considerable research has been carried out on the assessment of the antioxidant activity of

many herbs, spices and their extracts when added in a variety of foods and food model systems. Mate (*Ilex paraguariensis*) leaves contain many bioactive compounds, such as phenolic acids, which seem to be responsible for the antioxidant activity of green mate infusions, both *in vivo* and *in vitro* (Filip *et al*. 2000; Schinella *et al*. 2000; Bracesco *et al*. 2003; Markowicz-Bastos *et al*. 2006). The antioxidative effect of dietary *Oregano* EO and α-tocopheryl acetate supplementation on susceptibility of chicken breast and thigh muscle meat to lipid oxidation during frozen storage at −20°C for 9 months was examined. Dietary oregano EO supplementation at the level of 100 mg/kg feed was significantly ($P \leq 0.05$) more effective in reducing lipid oxidation compared with the level of 50 mg oregano EO k/g feed and control, but less effective ($P \leq 0.05$) compared with α-tocopheryl acetate supplementation (Botsoglou *et al*. 2003). Oregano, a characteristic spice of the Mediterranean cuisine obtained by drying leaves and flowers of *Origanum vulgare* subsp. *hirtum* plants, is well known for its antioxidative activity (Economou *et al*. 1991). Carvacrol and thymol, the two major phenols that constitute about 78-82% of the EO, are principally responsible for this activity (Adam *et al*. 1998; Yanishlieva 1999).

The antioxidant effect of two plant EOs (sage and rosemary EOs) and one synthetic antioxidant (BHT) on refrigerated stored liver pate (4°C/90 days) was evaluated. Pates with no added antioxidants were used as controls. Plant EOs inhibited oxidative deterioration of liver pates to a higher extent than BHT did (Estevez *et al*. 2007).

Oxidative reactions in foodstuffs are enhanced after cooking and refrigerated storage through the increase of their oxidative instability due to the degradation of natural antioxidants and the release of free fatty acids and iron from the haeme molecule (Kristensen and Purslow 2001; Estevez and Cava 2004). Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officianalis*) are popular Labiatae herbs with a verified potent antioxidant activity (Dorman *et al*. 2003). The antioxidant activity of sage and rosemary EOs is mainly related to two phenolic diterpenes: carnosic acid and carnosol which are considered two effective free-radical scavengers (Dorman *et al*. 2003; Ibanez *et al*. 2003). The antioxidant activity of these molecules has been compared to that from other recognized antioxidant substances, and Richheimer *et al*. (1999) indicated that the antioxidant potential of the carnosic acid was approximately seven times higher than that of BHT and BHA. Bektas and co workers (2007a) studied *in vitro* antioxidant activity of the EO of *Clinopodium vulgare* by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. In the first case, IC_{50} value of the *C. vulgare* EO was determined as 63.0 ± 2.71 μg/ml. IC_{50} value of thymol and γ-terpinene, the major compounds of the oil, was determined as 161 ± 1.3 μg/ml and 122 ± 2.5 μg/ml, respectively, whereas p-cymene did not show antioxidant activity. In β-carotene-linoleic acid system, *C. vulgare* EO exhibited 52.3 ± 1.19% inhibition against linoleum acid oxidation. Bektas *et al*. (2007b) screened the methanolic extracts of *Salvia verticillata* subsp. verticillata and *S. verticillata* subsp. Amasiaca for their possible antioxidant activity by two complementary test systems, namely DPPH free radical-scavenging and β-carotene/linoleic acid systems. In the first case, *S. verticillata* subsp. verticillata was superior to the subsp. amasiaca with an IC_{50} value of 14.5 ± 1.21 μg m/g. In the β-carotene/linoleic acid test system, inhibition capacity of *S. verticillata* subsp. *verticillata* was 74.4 ± 1.29%. Activity of rosmarinic acid was also screened for better establishing the relationship between rosmarinic acid level and antioxidant activity for the plant extracts. *S. verticillata* subsp. *verticillata* had the highest rosmarinic acid level with a value of 28.7 ± 0.89 μg m/g. There was a strong correlation between the rosmarinic acid level and antioxidant activity potential.

Honey has been reported to contain a variety of phenolics and represents a good source of antioxidants, which makes it a good food antioxidant additive and increases its usability potential in ethnomedicine (Al-Mamary *et al*. 2002; Aljadi and Kamaruddin 2004; Beretta *et al*. 2005; Kucuk *et al*. 2007). Several methods have been developed, in recent years, to evaluate the antioxidant capacity of biological samples (Rice-Evans *et al*. 1997; Schlesier *et al*. 2002). The total phenolic content of natural samples, such as plants and honey, reflects, to some extent, the total antioxidant capacity of the sample (Beretta *et al*. 2005). The most widely used antioxidant methods involve the generation of oxidant species, generally radicals, and their concentration is monitored as the present antioxidants scavenge them. Radical formation and the following scavenging are applied in 2,2-diphenyl-1-picrylhydrazyl (DPPH)- and superoxide radical-scavenging activity measurements (Gulcin *et al*. 2003). In radical-scavenging activity, the higher extract concentration required to scavenge the radicals means the lower antioxidant capacity. Ferric-reducing/antioxidant power (FRAP) is another widely used antioxidant activity measurement method, which has been used for the assessment of antioxidant and reducing power of many different samples, including honey (Aljadi and Kamaruddin 2004) and plant exudates (Gulcin *et al*. 2003).

ESSENTIAL OILS IN FOOD

Food composition and structure have a significant effect on the dynamic and final outcomes of an interaction. Naturally present ingredients can favor or inhibit the interactive cultures. Food composition can be manipulated to achieve the desired effect. In food products, the EOs have been used in bakery (Nielsen and Rios 2000), cheese (Vazquez *et al*. 2001), meat (Quintavalla and Vicini 2002) and fruit (Lanciotti *et al*. 2004), among others. The advantage of EOs is their bioactivity in the vapor phase, a characteristic that makes them useful as possible fumigants for stored commodity protection. Antimicrobial packaging is a form of active packaging that could extend the shelf-life of product and provides microbial safety for consumers. It acts to reduce, inhibit, or retard the growth of pathogen microorganisms in packed foods and packaging material. In order to control undesirable microorganisms on food surfaces: (1) volatile and non-volatile antimicrobial agents can be incorporated into polymers or (2) coating or adsorbing antimicrobial onto polymer surfaces can be applied (Appendini and Hotchkiss 2002). The coating can serve as a carrier for antimicrobial compounds and/or antioxidants compounds in order to maintain high concentrations of preservatives on the food surfaces (Siragusa *et al*. 1999; Oussallah *et al*. 2004). Although a small number of food preservatives containing EOs is commercially available, until the early 1990s very few studies of the activity of EOs in foods had been published (Board and Gould 1991). Since then a fair number of trials have been carried out with EOs in foods. There are reports of studies using diluted foods or food slurries (Pol *et al*. 2001; Smith-Palmer *et al*. 2001) and studies using dried herbs or spices or their extracts (Tassou *et al*. 1996; Hao *et al*. 1998a, 1998b). It has generally been found that a greater concentration of EO is needed to achieve the equivalent *in-vitro* effect in foods (Smid and Gorris 1999). The ratio has been recorded to be approximately twofold in semi-skimmed milk (Karatzas *et al*. 2001), 10-fold in pork liver sausage (Pandit and Shelef 1994), 50-fold in soup (Ultee and Smid 2001) and 25- to 100-fold in soft cheese (Mendoza-Yepes *et al*. 1997). An exception to this phenolmenon is *Aeromonas hydrophila*; no greater proportion of EO was needed to inhibit this species on cooked pork and on lettuce in comparison to tests *in vitro* (Stecchini *et al*. 1993; Wan *et al*. 1998). Several studies have recorded the effect of foodstuffs on microbial resistance to EOs but none appears to have quantified it or to have explained the mechanism, although suggestions have been made as to the possible causes. The greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster (Gill *et al*. 2002). Generally, the susceptibility of bacteria to the antimicrobial effect of EOs also appears to increase with a decrease in the pH of the

food, the storage temperature and the amount of oxygen within the packaging (Skandamis and Nychas 2000; Tsigarida *et al*. 2000). At low pH the hydrophobicity of an EO increases, enabling it to more easily dissolve in the lipids of the cell membrane of target bacteria (Juven *et al*. 1994). The physical structure of a food may limit the antibacterial activity of EO. A study of the relative performance of oregano oil against *S. typhimurium* in broth and in gelatine gel revealed that the gel matrix dramatically reduced the inhibitory effect of the oil. This was presumed to be due to the limitation of diffusion by the structure of the gel matrix (Skandamis *et al*. 2000). MICs for a particular EO on a particular bacterial isolate have been shown to be generally slightly lower in broth than in agar (Hammer *et al*. 1999). Research into the growth characteristics of *L. monocytogenes* and *Yersinia enterocolitica* in oil-in-water emulsions has shown that, depending on the mean droplet size of the emulsion, the bacteria can grow in films, in colonies or as planktonic cells (Brocklehurst *et al*. 1995). It is known that colonial growth restricts diffusion of oxygen (Wimpenny and Lewis 1977) and cells situated within a colony may be shielded to a certain extent by the outer cells from substrates in the emulsion. If the oil droplets in a food emulsion are of the appropriate size, it could be possible for bacteria growing within colonies to be protected from the action of EOs in this way.

Meat products

A high fat content appears to markedly reduce the action of EOs in meat products. It is generally supposed that the high levels of fat and/or protein in foodstuffs protect the bacteria from the action of the EO in some way. For example, if the EO dissolves in the lipid phase of the food there will be relatively less available to act on bacteria present in the aqueous phase (Mejlholm and Dalgaard 2002). Another suggestion is that the lower water content of food compared to laboratory media may hamper the progress of antibacterial agents to the target site in the bacterial cell (Smith-Palmer *et al*. 2001). Mint oil in the high fat products exhibited little antibacterial effect against *L. monocytogenes* and *S. enteritidis*, whereas in low fat food the same EO was much more effective (Tassou *et al*. 1995). Immobilising cilantro EO in a gelatine gel, however, improved the antibacterial activity against *L. monocytogenes* in ham (Gill *et al*. 2002). One study found that encapsulated rosemary oil was much more effective than standard rosemary EO against *L. monocytogenes* in pork liver sausage, although whether the effect was due to the encapsulation or the greater percentage level used was not further elucidated (Pandit and Shelef 1994). Certain oils stand out as better antibacterials than others for meat applications. Eugenol and coriander, clove, oregano and thyme oils were found to be effective at levels of 5-20 µl/g in inhibiting *L. monocytogenes*, *A. hydrophila* and autochthonous spoilage flora in meat products, sometimes causing a marked initial reduction in the number of recoverable cells (Tsigarida *et al*. 2000; Skandamis and Nychas 2001) whilst mustard, cilantro, mint and sage oils were less effective or ineffective (Gill *et al*. 2002; Lemay *et al*. 2002). In fish, just as in meat products, a high fat content appears to reduce the effectiveness of antibacterial EOs. For example, oregano oil at 0.5 µl/g is more effective against the spoilage organism *Photobacterium phosphoreum* on cod fillets than on salmon, which is a fatty fish (Mejlholm and Dalgaard 2002). Oregano oil is more effective in/on fish than mint oil, even in fatty fish dishes; this was confirmed in two experiments with fish roe salad using the two EOs at the same concentration (5-20 µl/g) (Koutsoumanis *et al*. 1999; Tassou *et al*. 1996). The spreading of EO on the surface of whole fish or using EO in a coating for shrimps appears effective in inhibiting the respective natural spoilage flora (Ouattara *et al*. 2001; Harpaz *et al*. 2003).

The activity of oregano EO against *Clostridium botulinum* spores has been studied in a vacuum packed and pasteurised minced (ground) pork product. Concentrations of up to 0.4 µl/g oregano EO were found not to significantly influence the number of spores or to delay growth. However, in the presence of low levels of sodium nitrite which delayed growth of bacteria and swelling of cans when applied alone, the same concentration of oregano EO enhanced the delay. The delay of growth was dependent on the number of inoculated spores; at 300 spores/g the reduction was greater than at 3000 spores/g (Ismaiel and Pierson 1990). Active packagings with the packaging materials delivering antimicrobials, can play an important role in satisfying current requirements because inhibitors are more effective when delivered in this manner. When AIT was used as an antimicrobial agent in active packaging of rye bread, it was found that 1 µl AIT completely inhibited the growth of *A. flavus*, *Penicillium commune*, *Penicillium corylophilum*, *Penicillium discolor*, *Penicillium polonicum*, *Penicillium roqueforti* and *Endomyces fibulige* (Nielsen and Rios 2000). Smith-Palmer *et al*. (2001) found hydrophobic plant EOs were more effective against *L. monocytogenes* in low fat (16%) that in high fat (30%) cheeses. Hasegawa *et al*. (1999) reported that AIT was more effective against *V. parahaemolyticus* in high fat (20.8%) than in low fat (0.4%) tuna tissue. The potential for intrinsic fat levels in food to moderate the antimicrobial activity of EOs is clear, and results from these two studies showed that interference can be expected at fat levels in food of >16%. Allyl isothiocyanate (AIT), a major antimicrobial component in mustard and horseradish oil, has been used in a number of foods against a variety of organisms. It has been found to be generally more effective against Gram-negative bacteria. In a study, Hasegawa *et al*. (1999) found AIT more effective in fatty (20.8%) than lean (0.4%) tuna meat suspension against 4 strains of *V. parahaemolyticus*. After 24 h of incubation, AIT at 152.6 µg /ml was able to inhibit only one strain in the lean suspension, but it reduced all strains below 10 cfu /ml in the fatty suspension. At 101.7 µg /ml, AIT inhibited 3 of the strains to the same level in the fatty suspension. The higher activity of AIT in fatty tuna meat flesh may be related to the high level of unsaturated fat. The main fatty acids of tuna flesh are *cis*-vaccenic, palmitic and docosahexaenoic acid, which may stabilize AIT in tuna tissue suspensions. AIT possesses strong antimicrobial activity against *E. coli* O157:H7 as well as *V. parahaemolyticus*. Nadarajah *et al*. (2002) killed 3.6 log cfu/g *E. coli* O157:H7 in ground beef with AIT (200–300 ppm) after 21 d at 4°C. The antimicrobial effectiveness of AIT against *E. coli* O157:H7 varied with storage temperature and inoculation level. There was very little inhibitory effect on the natural microflora. In subsequent work, Muthukumarasamy *et al*. (2003) examined the effectiveness of AIT at 1300 ppm in ground beef stored at 4°C under nitrogen with *Lactobacillus reuteri* against *E. coli* O157:H7. As an ingredient, AIT by itself eliminated 3 $\log_{10}$ cfu/g *E. coli* O157:H7 within 15 d and reduced 6 $\log_{10}$ cfu/g by 47 log cfu/g during 25 d storage. AIT did not interact synergistically with *Lb. reuteri* against *E. coli* O157:H7. When AIT was used in acidified chicken meat (0.1% w/w), it failed to exert a significant effect on the growth of *Brochothrix thermosphacta*, but it was able to delay growth of some LAB and aerobic mesophilic bacteria for at least 2 days (Lemay *et al*. 2002). In another similar study, when AIT was evaluated for its effectiveness in precooked roast beef against pathogenic bacteria (*E. coli* O157:H7, *L. monocytogenes*, *S. typhimurium*, and *S. aureus*) and spoilage bacteria (*Serratia grimesii* and *Lb. sakei*), it was found that pathogenic bacteria were inhibited by AIT at a concentration in the head space of 20 µl/l. *E. coli* O157:H7, *S. aureus* and *S. typhimurium* were most sensitive.

Dairy products

A reaction between carvacrol, a phenolic component of various EOs, and proteins has been put forward as a limiting factor in the antibacterial activity against *Bacillus cereus* in milk (Pol *et al*. 2001). Protein content has also been put forward as a factor inhibiting the action of clove oil on *Salmonella enteritidis* in diluted low-fat cheese (Smith-Palmer *et al*. 2001). Carbohydrates in foods do not appear to protect bacteria from the action of EOs as much as fat and protein do (Shelef *et al*. 1984). A high water and/or salt level facilitates the action of EOs (Skandamis and Nychas 2000). Mint oil at 5-20 µl/g is effective against *S. enteritisdis* in low fat yoghurt and cucumber salad (Tassou *et al*. 1995). Mint oil inhibits the growth of yoghurt starter culture species at 0.05-5 µl/g but cinnamon, cardamom and clove oils are much more effective (Bayoumi 1992). Smith-Palmer *et al*. (1998) reported that the oils of clove, cinnamon and thyme were effective against *L. monocytogenes* and *S. enteritidis* in tryptone soya broth (TSB). The oils had MICs of 0.04%, 0.075% and 0.03%, respectively, against *L. monocytogenes*. Similarly, concentrations of 0.075%, 0.1%, and 0.04% were required to inhibit the growth of *S. enteritidis* in TSB. On the other hand, when Smith-Palmer *et al*. (2001) evaluated clove, cinnamon, thyme and bay oil for their activity against *L. monocytogenes* and *S. enteritidis* in both low (16%) and high fat (30%) cheese it was observed that the oils of clove and cinnamon were highly effective against *L. monocytogenes*. However, a 1% concentration of the oils was required to inhibit *L. monocytogenes* and reduce its number to <1.0 log cfu /ml within 3 days in low fat cheese. Clove oil at 1% was the only oil able to reduce viable numbers to <1.0 log cfu /ml in high-fat cheese. Generally they found that *L. monocytogenes* was more rapidly inhibited in low fat cheese than in high fat cheese. At 0.5%, all oils gave initial inhibition (ranging from <1.0 log cfu /ml for clove oil to 2.3 log for bay oil) of *S. enteritidis*, but this was followed by recovery of the bacteria during the subsequent storage period. However, at 1%, all oils were able to completely inhibit *S. enteritidis*, reducing numbers to below the detection limit in both low fat as well as in full fat cheese. The need for higher concentrations of oils in foods than in laboratory media to achieve inhibition may be related to the more complex nature of food. All the plant EOs tested were more effective in low than in high fat cheese. This may have resulted from the fat in the product providing a protective layer around the bacteria, or the lipid fraction may have absorbed the antimicrobial agent and thus decreased its concentration and effectiveness in the aqueous phase. Even though the oils did not eliminate large numbers of *L. monocytogenes*, Smith-Palmer *et al*. (2001) concluded that plant EOs could be used as natural antimicrobial agents in dairy products, since they prevented growth and reduced viability of *L. monocytogenes* and *S. enteritidis* in both low fat as well as full fat cheese.

Vegetables and fruits

The antimicrobial activity of EOs in vegetable dishes is benefited by a decrease in storage temperature and/or a decrease in the pH of the food (Skandamis and Nychas 2000). All EOs and their components that have been tested on vegetables appear effective against the natural spoilage flora and food borne pathogens at levels of 0.1-10 µl/g in washing water (Wan *et al*. 1998; Singh *et al*. 2002). Oregano oil at 7-21 µl/g was effective at inhibiting *Escherichia coli* O157:H7 and reducing final populations in eggplant salad compared to the untreated control. Although the salad recipe appears to have a high fat content, the percentage of fat was not stated (Skandamis and Nychas 2000). Vegetables generally have a low fat content, which may contribute to the successful results obtained with EOs. EOs of clove, cinnamon, bay and thyme were tested against *L. monocytogenes* and *S. enteritidis* in soft cheese diluted 1:10 in buffer. The former species was less easily inhibited in diluted full-fat cheese than in the low-fat version, indicating the protective action of fat. The level of fat in the cheese protected the bacterial cells to a different extent depending on which oil was used; clove oil was in fact more effective against *S. enteritidis* in full-fat than in low-fat cheese slurry (Smith-Palmer *et al*. 2001). Carvacrol and cinnamaldehyde were very effective at reducing the viable count of the natural flora on kiwifruit when used at 0.15 µl/ml in dipping solution, but less effective on honeydew melon. It is possible that this difference has to do with the difference in pH between the fruits; the pH of kiwifruit was 3.2–3.6 and of the melon 5.4–5.5 (Roller and Seedhar 2002). Carvacrol was also very effective at 0.15–0.75 µl/g in extending the lag phase and reducing the final population compared to a control whereas sage oil was ineffective at 0.2–0.5 µl/g against *B. cereus* in rice (Shelef *et al*. 1984; Ultee *et al*. 2000b). Cinnamaldehyde and thymol are effective against six *Salmonella* serotypes on alfalfa seeds when applied in hot air at 50°C as fumigation. Increasing the temperature to 70°C reduced the effectiveness of the treatment (Weissinger *et al*. 2001). This may be due to the volatility of the antibacterial compounds.

Sweet cherry shows severe problems for commercialization mainly due to incidence of decay and a fast loss of sensory quality, both for fruit and stem. Serrano *et al*. (2005) developed a package based on the addition of eugenol, thymol, menthol or eucalyptol pure EOs separately to trays sealed with polypropylene bags to generate a modified atmosphere (MAP= modified atmosphere packaging). In addition, cherries in MAP (without EOs) were selected and served as controls. All cherries were stored during 16 days at 1°C and 90% RH. Steady-state atmosphere was reached after 9 days of cold storage with 2–3% of CO_2 and 11–12% of O_2 with no significant differences between treated and control, with the exception of eucalyptol, in which significant increases in CO_2 and decreases of O_2 were obtained. When fruit quality parameters were determined, those treated with eugenol, thymol or menthol showed benefits in terms of reduced weight loss, delayed colour changes and maintenance of fruit firmness compared with control. Stem remained green in treated cherries while they became brown in control. However, cherries packaged with eucalyptol behaved even worst than control cherries, with generation of off-flavors, loss of quality and stem browning. Finally, the microbial analysis showed that all EOs reduced moulds and yeasts and total aerobic mesophilic colonies by 4- and 2-log cfu compared with control, respectively.

Yeasts are widely distributed in nature and are able to spoil many foods such as wines, cheese, vinegar, beverages, juices, fruits, salads, sugar and meat, causing changes in odor, color, taste and texture. *Candida*, *Pichia*, *Rhodotorula*, *Torulopsis*, *Saccharomyces*, *Zygossacharomyces*, *Hansenula* and *Trichosporon* are some important food spoiling yeasts (Wojtatowicz *et al*. 2002). Products are usually held at refrigerator temperature during their manufacture, storage and distribution. However, this provides opportunity for growth of psychrotolerant pathogens and spoilage bacteria. EO components have been used in vegetable-based food systems to explore their value as secondary preservatives (Shelef 1983). Wan *et al*. (1998) evaluated the antimicrobial activity of basil sweet linalool (BSL) and basil methyl chavicol (BMC) oil against a wide range of bacteria, yeasts and moulds in filter sterilized lettuce supernatant, with emphasis on inhibition of *A. hydrophila* and *P. fluorescens*. *A. hydrophila* is a psychrotroph and potential pathogen, which may occur in minimally processed foods at 4-6 log cfu/g and may produce cytotoxins. *P. fluorescens* is a psychrotrophic spoilage bacterium which can reduce the shelf-life of refrigerated fruits and vegetables. Both BMC and BSL were shown to have inhibitory effects against Gram-positive and Gram-negative bacteria, yeasts and moulds. While the growth of *A. hydrophila* was delayed by 0.063% (v/v) BMC, at 0.125% (v/v) this oil completely inhibited growth of the organism. BSL

was less effective as an antimicrobial than BMC and had an MIC of 1% (v/v) against *A. hydrophila*. The MIC of BMC against *P. fluorescens* was 2% (v/v), while that of BSL was >2%. Thus, *P. fluorescens* was again substantially more resistant to these agents.

Organoleptic aspects

If EOs were to be more widely applied as antibacterials in foods, the organoleptic impact would be important. Foods generally associated with herbs, spices or seasonings would be the least affected by this phenomenon and information on the flavor impact of oregano EO in meat and fish supports this. The flavor of beef fillets treated with 0.8% v/w oregano oil was found to be acceptable after storage at 5°C and cooking (Tsigarida *et al*. 2000). The flavor, odor and color of minced beef containing 1% v/w oregano oil improved during storage under modified atmosphere packaging and vacuum at 5°C and was almost undetectable after cooking (Skandamis and Nychas 2001). Oregano oil (0.05% v/w) on cod fillets produced a 'distinctive but pleasant' flavor, which decreased gradually during storage at 2°C (Mejlholm and Dalgaard 2002). Thyme and oregano oils spread on whole Asian sea bass at 0.05% (v/v) (sic) also imparted a herbal odor, which during storage up to 33 days at 0–2°C became more pronounced (Harpaz *et al*. 2003). The addition of thyme oil at up to 0.9% (v/w) in a coating for cooked shrimps had no ill effects on the flavor or appearance. However, 1.8% thyme oil in the coating significantly decreased the acceptability of the shrimps (Ouattara *et al*. 1997, 2001). Individual EO components, many of them being approved food flavorings, also impart a certain flavor to foods. On fish, carvacrol is said to produce a 'warmly pungent' aroma; citral is 'lemon-like' and geraniol 'rose-like' (Kim *et al*. 1995). Treatment of fresh kiwifruit and honeydew melon with 1 mM carvacrol or cinnamic acid has been found to delay spoilage without causing adverse organoleptic changes (Roller and Seedhar 2002).

Other factors

In addition to fat and protein, the pH of food systems is an important factor affecting the activity of oils. At low pH, the hydrophobicity of some EOs (for example, thyme oil and the phenolic oleuropein) increases and while they may tend to partition in the lipid phase of the food, they can also dissolve more easily in the lipid phase of the bacterial membrane and have enhanced antimicrobial action.

Leuconostoc species or yeasts and moulds are often the major cause of spoilage in packaged foods. Lachowicz *et al*. (1998) examined the inhibitory action of basil oil against the acid-tolerant food microflora. Commercial basil oil and oils prepared from four other varieties of basil (Anise, Bush, Cinnamon and Dark Opal) were used by the investigators. Anise basil oil (containing 44% linalool and 27% methyl chavicol) was tested against *Lactobacillus curvatus* and *S. cerevisiae* which can grow at low pH and spoil tomato-based foods. Growth of these two organisms at pH 4.2 was determined using an indirect impedance method. The addition of 0.1% anise oil increased the time for detection of growth (TDG) by about 51 h, while at the 1% level the growth of both organisms was completely inhibited over a 99 h test period. The antimicrobial activity of anise oil was then evaluated against these two organisms in tomato juice at 15°C. At 0.1% (v/v) anise oil, *Lb. curvatus* was reduced from 4 log cfu /mlto <1 cfu/ml at the end of first week and the inhibition was maintained during 4 weeks of further incubation. At the same concentration, anise oil was able to inhibit *S. cerevisiae* for only one week; by the second and third weeks the numbers of the yeast increased to 6 and 7 log cfu/ml, respectively. At 1% (v/v) oil, both organisms were completely inhibited and there was no growth of either organism during subsequent incubation. The EOs and leaves of sweet basil can be used for natural protection of grains and melon seeds against *Aspergillus* species if they are properly dried before storage. Bagamboula *et al*. (2003) postulated that the levels of EOs and their compounds necessary to inhibit microbial growth are usually higher in foods than in culture media. This is due to the interactions between phenolic compounds and the food matrix (Nychas and Tassou 2000). Partitioning of the hydrophobic antifungal EO components into the fat content of the food may prevent them from coming into contact with fungal cells growing in the hydrophilic regions in the food (Gill *et al*. 2002).

The critical analysis of literature data indicates that also citrus EOs could represent good candidates to improve the shelf-life and the safety of minimally processed fruits. In fact, it is well known that these oils can have a pronounced antimicrobial effect (Tassou *et al*. 1996; Dorman and Deans 2000). As flavoring principles, thyme volatiles such as thymol and carvacrol are present in low concentrations in human food. However, if the use of these compounds is extended to other applications that may require higher doses as well, the increased exposure of humans to these compounds is a matter of concern. The few data available in the literature mainly concern acute and short-term effects *in vivo* on different animal species, and suggest that such compounds may not pose a risk to human health (Stammati *et al*. 1999). Barakat *et al*. (2006) studied preservative effect of combined treatment with electrolyzed NaCl solutions and EO compounds on carp fillets during convectional air-drying. They concluded that such a treatment had stronger antimicrobial and antioxidant effects than all of the other treatments on carp fillets during drying, and could be a good alternative to artificial preservatives in food industry. In view of the published data on EOs in foods, the following approximate general ranking (in order of decreasing antibacterial activity) can be made: oregano/clove/coriander/cinnamon > thyme > mint > rosemary > mustard > cilantro/sage. An approximate general ranking of the EO components is as follows (in order of decreasing antibacterial activity): eugenol > carvacrol/cinnamic acid > basil methyl chavicol > cinnamaldehyde > citral/geraniol.

ESSENTIAL OILS, SYNERGISTIC, AND ANTAGONISTIC VIEW

EOs have been used in combination with other antibacterial agents and with a variety of treatments such as mild heat (Karatzas *et al*. 2000), hydrostatic pressure (Ogawa *et al*. 1998), sodium citrate and monolaurin (Blaszyk and Holley 1998). Ettayebi *et al*. (2000) reported the synergistic effects of EOs and nisin on *Bacillus cereus* and *Listeria monocytogenes*, and Pol and Smid (1999) found that the addition of lysozyme as a third preservative factor enhances the synergistic effect between carvone and nisin. Yamazaki *et al*. (2004) investigated plant-derived EO components in combination with nisin and diglycerol fatty acid esters for their antibacterial activity against *Listeria monocytogenes*. Fractions of cilantro, coriander, dill and eucalyptus EOs (each containing several components), when mixed in various combinations, resulted in additive, synergistic or antagonistic effects (Delaquis *et al*. 2002). The combined use of 2-3% NaCl and 0.5% clove powder (containing eugenol and eugenyl acetate) in mackerel muscle extract has been found to totally prevent growth and histamine production by *E. aerogenes*. The suggested mechanism for this is that eugenol increases the permeability of the cells after which NaCl inhibits growth by its action on intracellular enzymes (Wendakoon and Sakaguchi 1993). Antagonistic effects of salt were found with carvacrol and *p*-cymene against *B. cereus* in rice: carvacrol and *p*-cymene worked synergistically, but this effect was reduced when salt was added (1.25 g/l rice) (Ultee *et al*. 2000b). In the same study, soy sauce was shown to exhibit synergy with carvacrol. However, this synergy was also cancelled out by the presence of salt (Ultee *et al*. 2000b). Salt at 4% w/v in agar did not improve the antibacterial activity of cinnamaldehyde against Gram-positive and Gram-negative bacteria (Moleyar and Narasimham

1992). Combinations of oregano EO with sodium nitrite have been examined for their effect on growth and toxin production by *C. botulinum* (a combination of types A, B and E). Oregano oil acted synergistically with nitrite to inhibit growth in broth, whereas oregano oil applied alone at up to 400 ppm had no significant inhibitive effect on growth. The proposed mechanism of synergism depends on oregano EO reducing the number of spores which germinate and sodium nitrite inhibiting the outgrowth of spores. Both substances affect vegetative growth (Ismaiel and Pierson 1990).

The simultaneous application of nisin (0.15 µg/ml) and carvacrol or thymol (0.3 mmol/l or 45 µg/ml) caused a larger decline in viable counts for strains of *B. cereus* than was observed when the antimicrobials were individually applied. The maximum reduction of viability was achieved in cells that had experienced prior exposure to mild heat treatment at 45°C (5 min for exponentially growing cells and 40 min for stationary phase cells) (Periago *et al*. 2001). Carvacrol was found not to increase the sensitivity of vegetative *B. cereus* cells to pulsed-electric-field (PEF) treatment nor did it sensitize spores to nisin or PEF (Pol and Smid 1999). At pH 7 the synergistic action of nisin and carvacrol was significantly greater at 30°C than at 8°C, which would appear to indicate temperature-induced changes in the permeability of the cytoplasmic membrane (Periago and Moezelaar 2001). The mechanism of synergy is not known. Previously, it was hypothesised that carvacrol may increase the number, size or duration of existence of the pores created by nisin in the cell membrane (Pol and Smid 1999). Later it became clear that this was not so – the mechanism may lie in the enhanced dissipation of the membrane potential and a reduction in the pH gradient and intracellular ATP (Pol *et al*. 2002). The combined effect of carvone (5 mmol/l) and mild heat treatment (45°C, 30 min) on exponentially growing cells of *L. monocytogenes* grown at 8°C has been studied. Separately, the two treatments demonstrated no loss in viability but a decrease of 1.3 log units in viable cell numbers was recorded when they were combined. Cells grown at 35 or 45°C were not susceptible to the same combined treatment. The authors hypothesized that the phospholipid composition of the cytoplasmic membrane of cells grown at 8°C has a higher degree of unsaturation in order to maintain fluidity and function at low temperatures. This high degree of unsaturation causes the membranes of these cells to be more fluid at 45°C than the membranes of cells grown at that temperature. This increased fluidity would enable carvone to dissolve more easily into the lipid bilayer of cells grown at 8°C than into the bilayer of cells grown at 45°C. Membranes of cells grown at 45°C are less fluid because there is a 'normal' ratio of saturated to unsaturated fatty acids in their phospholipids and carvone is therefore less effective against them (Karatzas *et al*. 2000). Thymol and carvacrol have been shown to have a synergistic effect with high hydrostatic pressure (HHP). The viable numbers of mid-exponential phase *L. monocytogenes* cells were reduced more by combined treatment with 300 MPa HHP and 3 mmol /l thymol or carvacrol than by the separate treatments. Since HHP is believed to cause damage to the cell membrane, it is suggested that this common target is the root of the observed synergism (Karatzas *et al*. 2001). The presence of fat, carbohydrate, protein, salt and pH reaction influence the effectiveness of these agents in foods. Their antimicrobial potency is also reduced in foods with lower water activity. Some examples from *in vitro* work also show these effects are varied. While bovine serum albumin (BSA) neutralized the antimicrobial activity of thymol, oil of clove and tea tree oil were not substantially affected by organic matter or BSA, respectively (Davidson and Naidu 2000). In a food system, Gill *et al*. (2002) found cilantro oil at 6% with glycerol monolaurate or lecithin in a gelatin gel coating on ham became ineffective against *L. monocytogenes* even though cilantro showed considerable antilisterial action in broth. There are two examples where spice/herbal materials have been successfully used as either a dip on poultry carcasses (Dickens *et al*. 2000) or as a surface coating on salt water fish (Asian sea bass; Harpaz *et al*. 2003) to extend shelf-life. Carvacrol and thymol were found to have the strongest antilisterial properties, followed by eugenol, cinnamaldehyde and isoeugenol. The antilisterial activity of the other EOs (limonene, pinene, ally lisothiocyanate and linalool) was found to be low, even at the highest concentration used (0.1%). Among the diglycerol esters of fatty acids with different carbon chain lengths, diglycerol monolaurate was the most active against *L. monocytogenes*. A combined antilisterial effect was observed between nisin and the EOs (carvacrol, thymol and eugenol); moreover, the addition of diglycerol monolaurate as a third preservative factor led to further combined antilisterial activities between the EO constituents (carvacrol, thymol and eugenol) and nisin even at lower, sub-lethal concentrations. These results indicate that nisin and diglycerol monolaurate can be used to enhance the antilisterial activity of EOs, allowing for a reduction in the dosage used in food preservation and thereby resulting in the reduction of undesirable flavors. It can therefore reasonably be expected to produce a similar additive or synergistic effect on other appropriate preservatives in combination with EOs and nisin.

DAMAGE TO MICROBIAL CELLS

The chemical structure of the individual EO components affects their precise mode of action and antibacterial activity (Dorman and Deans 2000). EOs are mixtures of molecules often characterized by a poor solubility in water and by a high hydrophobicity. In addition, the different components of an EO can have antagonistic, synergistic or additive effects on microbial cells. The intrinsic variability in the composition of EOs can influence their overall antimicrobial activity leading to apparently contradictory results. Plant oils and extracts, primarily from clove, oregano, rosemary, thyme, tea tree (*Melaceuca alternafolia* from Australia) and sage have shown significant inhibitory activity, while less potent activity was shown by other plant materials. Expression of antimicrobial activity is often very clear, but the mechanism of antimicrobial action is incompletely understood. One of the more dramatic effects of inhibitory action appears in two separate reports where the outer of the two cell membranes of *E. coli* and *S. typhimurium* disintegrated following exposure to carvacrol and thymol (Helander *et al*. 1998). Similar observations were made by Lucchini *et al*. (1990) with these agents using a different strain of *E. coli* and *P. aeruginosa*. Yeast and Gram-positive bacteria showed no such changes in cell wall morphology. This was probably due to the solubility of lipopolysaccharides (LPS) in the outer membrane in phenolic-based solvents. Generally, the EOs possessing the strongest antibacterial properties against food borne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl)phenol) and thymol (Dorman and Deans 2000; Juliano *et al*. 2000; Lambert *et al*. 2001). It seems reasonable that their mechanism of action would therefore be similar to other phenolics. Juven *et al*. (1994) hypothesized that the inhibition of *S. thypimurium* and *S. aureus* by thyme oil was due to the hydrophobic and hydrogen bonding of its phenolic constituents to the membrane proteins, after its partition in the lipid bilayer. Moreover, the destruction of electron transport systems and the perturbation of membrane permeability were suggested by Tassou *et al*. (2000) as the fundamental mechanisms of action of mint EO against *S. aureus* and *S. enteritidis*. Carvacrol and thymol are hydrophobic compounds that dissolve in the hydrophobic domain of cytoplasmic membrane. They cause increased the permeability to ATP that results in lethal damage to the bacterial cell (Ultee *et al*. 1999; Burt 2004). The importance of the presence of the hydroxyl group in phenolic compounds such as carvacrol and thymol has been confirmed (Dorman and Deans 2000; Ultee *et al*. 2002). The relative position of the hydroxyl group on the phenolic ring does not appear strongly to influence the degree of antibacterial activity; the action of thymol against *B. cereus*,

Staphylococcus aureus and *Pseudomonas aeruginosa* appears to be comparable to that of carvacrol, for example (Lambert *et al*. 2001; Ultee *et al*. 2002). Thyme, oregano and dictamus oils were fungitoxic and this may have been due to formation of hydrogen bonds between the hydroxyl group of oil phenolics and active sites of target enzymes (Daferera *et al*. 2000). However, in one study carvacrol and thymol were found to act differently against Gram-positive and Gram-negative species (Dorman and Deans 2000). The significance of the phenolic ring itself (destabilised electrons) is demonstrated by the lack of activity of menthol compared to carvacrol (Ultee *et al*. 2002). In one study the addition of an acetate moiety to the molecule appeared to increase the antibacterial activity; geranyl acetate was more active against a range of Gram-positive and negative species than geraniol (Dorman and Deans 2000). As far as non-phenolic components of EOs are concerned, the type of alkyl group has been found to influence activity (alkenyl > alkyl). For example, limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) is more active than *p*-cymene (Dorman and Deans 2000).

Propidium iodide (Cox *et al*. 2000), the nucleic acid dye ethidium bromide (Lambert *et al*. 2001) as well as the self-quenching probe rhodamine *B* (Ultee *et al*. 2002) were used to monitor membrane integrity and uptake of EO components. An acetate-succinyl ester of carboxy fluorescein was used by Lambert *et al*. (2001) to follow changes in cytoplasmic pH following carvacrol challenge. Fluorescent probes have been used to study membrane changes. Work with fluorescent probes showed that EOs increased membrane permeability and that oil components actually dissolved in the membranes causing swelling and reduced membrane function. Although important, the extent of dilution in the membrane of the antimicrobial was unrelated to their overall antimicrobial activity in the case of carvacrol and its precursor *p*-cymene (Ultee *et al*. 2002). In this study, Ultee *et al*. (2002) concluded that to be effective against vegetative cells of *B. cereus* antimicrobials should have both a hydroxyl group on the phenolic ring as well as a system of delocalized electrons (i.e. presence of α–β double bonds) to elicit strong antimicrobial activity. Ultee *et al*. (2002) developed a model for carvacrol inhibition of bacterial cells where it acted as a protonphore uncoupler, facilitating K^+ efflux and destruction of the internal pH gradient. With the loss of the gradient, ATP levels were depleted and this led to cell death. Synergism between carvacrol and its biological precursor *p*-cymene has been noted when acting on *B. cereus* vegetative cells. It appears that *p*-cymene, a very weak antibacterial, swells bacterial cell membranes to a greater extent than carvacrol does. By this mechanism *p*-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are used together (Ultee *et al*. 2000a). Ultee *et al*. (2002) suggested that carvacrol exerts its activities by interacting with the cytoplasmic membrane via its own hydroxyl group, thus changing the permeability of membrane for protons and potassium ions.

Possible use of three different EO components as natural food preservatives was studied by examining their influence in the kinetics of growth from activated spores of four *Bacillus cereus* strains in tyndallized carrot broth over the temperature range 5–16°C. Selected low concentrations of carvacrol, cinnamaldehyde, or thymol showed a clear antibacterial activity against *B. cereus* in the vegetable substrate. The addition of 2 µl cinnamaldehyde or 20 mg thymol to 100 ml of broth in combination with refrigeration temperatures (≤8°C) was able to inhibit the outgrowth from activated spores of the psychrotrophic strain INRA TZ415 for at least 60 days, but only cinnamaldehyde did it even at the mild abuse temperature of 12°C. Five microliters of carvacrol per 100 ml of inoculated carrot broth, however, were unable to inhibit bacterial growth at 8°C (Valero and Frances 2006). Inhibitory effects of carvacrol, an antimicrobial compound present in the EO fraction of oregano and thyme, on growth and diarrheal enterotoxin production by *B. cereus* inoculated in brain heart infusion medium (BHI), cooked rice and mushroom soup have been published (Ultee *et al*. 2000b; Pol *et al*. 2001; Ultee and Smid 2001). Observations that higher concentrations were needed to achieve the same effect in food as in laboratory medium showed that carvacrol was less effective in a food matrix, most likely as a result of interaction with food components (Pol *et al*. 2001; Ultee and Smid 2001). These studies pointed out the potential use of carvacrol for preservation of foods, increasing the safety of the products. It is apparent that the generally greater resistance of Gram-negative bacteria to EOs (Davidson and Naidu 2000; Lambert *et al*. 2001; Walsh *et al*. 2003) is likely to be due in part to the greater complexity of the double membrane-containing cell envelope of these organisms in contrast with the single membrane-glycoprotein/techoic acid, or membrane-glycoprotein/β-glucan-based structures of Gram-positive bacteria and yeast, respectively. Sterols present in membranes of yeasts and fungi but absent from prokaryotic cells do not confer resistance against these antimicrobials (Vardar-Unlu *et al*. 2003). Resistance also seems to be related to the rate and extent of antimicrobial dissolution or ability to partition in the lipid phase of the membrane as discussed by Lambert *et al*. (2001), although this is not the complete explanation. In attempting to explain differences in the sensitivity of Gram-positive and -negative cells, differences in cell surface hydrophobicity have been suggested as contributing factors (Chao *et al*. 2000). Enhancement of EO antimicrobial action against both types of bacteria by ethylene diamine tetra acetic acid (EDTA) suggests a role for metal cations in resistance (Naidu 2000; Walsh *et al*. 2003). The use of several chelating agents, such as EDTA and other substances, have been proposed to destabilize the lipopolysaccharide layer of outer membrane of Gram-negative bacteria (Helander *et al*. 1997). Unfortunately, those agents that cause outer membrane permeabilization are often too toxic to be used as food ingredients. Damage to the bacterial cell wall and loss of cell contents can be studied by scanning electron microscopy (SEM) (Lambert *et al*. 2001; Skandamis *et al*. 2001; Burt and Reinders 2003). Careful preparation of the samples for SEM is necessary to ensure that the observed difference between control and treated cells are due to the effect of the EO and not to the preparation method.

Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Skandamis *et al*. 2001; Carson *et al*. 2002). The locations or mechanisms in the bacterial cell thought to be sites of action for EO components have been reported as: degradation of the cell wall (Helander *et al*. 1998); damage to cytoplasmic membrane (Ultee *et al*. 2000a, 2002); damage to membrane proteins (Juven *et al*. 1994; Ultee *et al*. 1999); coagulation of cytoplasm (Gustafson *et al*. 1998) and depletion of the proton motive force (Ultee *et al*. 1999; Ultee and Smid 2001). *Listeria monocytogenes* treated with EOs from two thyme species exhibited thickened or disrupted cell wall with increased roughness and lack of cytoplasm (Rasooli *et al*. 2006a). Transmission electron microscopy (TEM) of *A. niger* exposed to MIC levels of the oils showed irreversible damage to cell wall, cell membrane and cellular organelles (Rasooli *et al*. 2006b). TEM of *A. parasiticus* exposed to MIC level (250 ppm) of thyme oils showed irreversible damage to cell wall, cell membrane and cellular organelles (Rasooli and Owlia 2005). An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Sikkema *et al*. 1994). Karatzas *et al*. (2000) observed that a combined treatment with S-carvone (a terpene associated mainly to the *Labiatae* EOs) and mild heat treatment led to a decline in the viable number of *L. monocytogenes*. They explained the differences in the effectiveness of the treatment with the amount of

S-carvone which can dissolve in the cell membrane. In particular, the membrane solubility of S-carvone was dependent on the growth temperature before the treatment, which, in turn, influenced the membrane composition and fluidity. Leakage of ions and other cell contents can then occur (Cox *et al*. 2000; Lambert *et al*. 2001; Skandamis *et al*. 2001; Ultee *et al*. 2002; Carson *et al*. 2002;). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (Denyer and Hugo 1991).

Components of EO also appear to act on cell proteins embedded in the cytoplasmic membrane (Knobloch *et al*. 1989). Enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on these. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid–protein interaction; alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Juven *et al*. 1994; Sikkema *et al*. 1995). Some EOs have been found to stimulate the growth of pseudomycelia (a series of cells adhering end-to-end as a result of incomplete separation of newly formed cells) in certain yeasts. This could be an indication that EOs act on the enzymes involved in the energy regulation or synthesis of structural components (Conner and Beuchat 1984). Cinnamon oil and its components have been shown to inhibit amino acid decarboxylases in *Enterobacter aerogenes*. The mechanism of action was thought to be the binding of proteins (Wendakoon and Sakaguchi 1995). Indications that EO components may act on proteins were also obtained from studies using milk containing different protein levels (Pol *et al*. 2001).

There is overwhelming consensus that aromatic and phenolic compounds exert their antimicrobial effects at the cytoplasmic membrane by altering its structure and function (Sikkema *et al*. 1995). Efflux of K^+ is usually an early sign of damage (Walsh *et al*. 2003) and is often followed by efflux of cytoplasmic constituents (Cox *et al*. 2000; Davidson and Naidu 2000; Lambert *et al*. 2001; Ultee *et al*. 2002) including ATP (Brul and Coote 1999). The loss of the differential permeability character of the cytoplasmic membrane is frequently identified as the cause of cell death. Some workers have explored this further, reasoning that loss of membrane function is only part of the explanation for antimicrobial activity (Walsh *et al*. 2003). Other events which could lead to membrane dysfunction and subsequent disruption include dissipation of the two components of the proton motive force in cells (the pH gradient and the electrical potential) either by changes in ion transport or depolarization through structural changes in the membrane; interference with the energy (ATP) generation system in the cell; or enzyme inhibition preventing substrate utilization for energy production (Lambert *et al*. 2001; Ultee *et al*. 2002). In addition, Cox *et al*. (2000) showed that tea tree oil which contains terpinen-4-ol (a cyclic monoterpene believed primarily responsible for the antimicrobial activity) inhibited oxidative respiration in *E. coli*, *S. aureus* and a yeast at the MIC; and also induced membrane swelling and increased membrane permeability. Certainly, the ability of phenolics to interfere with cellular metabolism through a number of mechanims (substrate complexing, membrane disruption, enzyme inactivation and metal chelation) is well known (Cowan 1999). It is also evident that their ability to preferentially partition from water to membrane structures and penetrate the membrane are important factors which have a bearing on the sensitivity or resistance of exposed cells (Cox *et al*. 2000; Lambert *et al*. 2001). The results of another experiment (Gill and Holley 2003) with eugenol and cinnamaldehyde against *L. monocytogenes* and *Lb. sakei* are not consistent with a protonphore uncoupler mechanism. Eugenol, like carvacrol, is a substituted phenolic compound and cinnamaldehyde is a substituted aromatic compound. Treatment of un-energized cells of *L. monocytogenes* with bactericidal concentrations of eugenol (5 mM) or cinnamaldehyde (40 mM) prevented cellular ATP pools from increasing following addition of glucose. The treatment of energized *L. monocytogenes* cells with eugenol had no significant effect on cellular ATP pools, but the cellular ATP of cinnamaldehyde treated cells was rapidly depleted. The ATP pools of *L. monocytogenes* cells treated with 10 μM of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) responded identically to cells treated with cinnamaldehdye, not like eugenol treated cells. *Lb. sakei* responded to bactericidal eugenol treatment (10 mM) like *L. monocytogenes*, but was unresponsive to ≤0.4 M cinnamaldehyde or 100 μM CCCP. If eugenol functioned as a protonophore it would affect *L. monocytogenes* like CCCP. Protonophores rapidly deplete the cellular ATP of *L. monocytogenes* as the cell makes a futile attempt to reestablish the membrane proton motive force by exporting hydrogen ions with the F_1F_0 ATPase (Shabala *et al*. 2002). Though cinnamaldehyde does behave like CCCP, it cannot function as a protonophore as it does not possess a hydroxyl or acid group to act as a proton carrier. Since measurements of extracellular ATP were inconclusive, the results of Gill and Holley (2003) would be consistent with either membrane disruption by cinnamaldehyde or inhibition of either glucose uptake or utilization by both compounds. These differences in response to eugenol and cinnamaldehyde by *L. monocytogenes* and *Lb. sakei* may be due in part to differences in the solubility or permeability of the agents in the cell membrane. It is conceivable that small differences in antimicrobial concentration internally may determine the biochemical event that dominates to inhibit cell growth or cause death. It is clear that the bactericidal response of *Lb. sakei* and *L. monocytogenes* to eugenol and cinnamaldehyde involves energy generation by the cells. In other relevant work on the mode of action of natural antimicrobials Delaquis *et al*. (2002) found, in a study of fractionated hydroxycinnamic acids used against *L. monocytogenes*, that the presence of hydroxyl groups on long chain alcohols was correlated with inhibitory activity. This supports observations reported by Helander *et al*. (1998). In other work with yeast Fitzgerald *et al*. (2003) found that the antimicrobial functionality of vanillin was due to its aldehyde group. In their study of the mode of action of cinnamaldehyde against *Enterobacter aerogenes*, Wendakoon and Sakaguchi (1993) concluded that the antimicrobial inactivated decarboxylase enzymes in the cell. The non-phenolic isothiocyanates are also potent antimicrobials and have activity against a wide range of microorganisms. Their antimicrobial activity is believed to be due to the inactivation of extracellular enzymes through cleavage of disulfide bonds (Delaquis and Mazza 1995). Since the bioactivity of many aroma compounds is dependent, in the first instance, on their partition in plasma membrane, a key role in their toxicity has been attributed to the vapor pressure that can be considered an indirect measure of their hydrophobicity. Thus, the factors able to increase the vapor pressure of these substances can enhance their antimicrobial activity increasing their solubility in cell membranes (Gardini *et al*. 2001). Lanciotti *et al*. (1999) proved that the antimicrobial activity of hexanal, 2-(*E*)-hexenal and hexyl acetate is dependant on their vapor pressure and, consequently, positively affected by temperature rise. The literature data indicate that, although the sensitivity to the aroma compounds used varies with the target species considered and system composition, some aroma compounds such as hexanal and 2-(*E*)-hexenal are able to reduce significantly the growth potential of gram negative species in fruit based products (Lanciotti *et al*. 2003). These microorganisms, and particularly *Enterobacteriaceae*, have a high growth potential and specificity in minimally processed fruits attributable to their physiology and to the availability of specific carbon sources such as inositol and pinitol (O'Conner-Shaw *et al*. 1995). The sensitivity of *S. enteritidis* and *E. coli* to 2-(*E*)-hexenal and hexanal makes these molecules particularly interesting as antimicrobial agents. In fact, the inherent or acquired resistance of this

microbial group to many antimicrobial agents complicates their control in the environment and in food materials. The resistance of gram-negative bacteria is mainly due to the outer membrane, which acts as an efficient permeability barrier against macromolecules and hydrophobic substances (Helander *et al*. 1997) as well as to the high content in cyclopropanic fatty acids of the inner membrane (Chang and Cronan 1999). The citric acid used in the sliced apple pre-treatment probably enhanced the outer membrane destabilization, due to its chelating activity. Nevertheless, small hydrophobic compounds such as six carbon aldehydes, can enter, throughout porin proteins, into the deeper parts of Gram-negative bacteria without any alteration to the permeability of the outer membrane (Helander *et al*. 1997). This gives an extra value for the aroma compounds considered as antimicrobial agents in foods, because of its pronounced action against gram-negative bacteria. Moreover, the effectiveness at low levels, also under abuse temperature conditions, the natural occurrence in several fruits and edible vegetables, the possibility of using in unregulated doses as flavoring agents, make hexanal, 2-(*E*)-hexenal and hexyl acetate good candidates as antimicrobial agents to improve the safety of minimally processed fruits. The ability of a potentially active molecule to interact with the hydrophobic cell membrane can be regarded as the result of its intrinsic hydrophobicity, which increases with the hydrocarbon chain length, and its actual hydrophobicity, which provides an inverse measure of the water molecules surrounding its polar groups (Guerzoni *et al*. 1997). A temperature rise increases the tendency of a molecule to pass into the vapor phase and, consequently, its antimicrobial effects (Caccioni *et al*. 1997). Thus, the effect of temperature on the increase of vapor pressure and toxicity, can compensate for an eventual interruption of the chilling chain.

GENOTOXICITY OF ESSENTIAL OILS AND SAFETY CONCERNS

Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. For these reasons, consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable preservatives has been intensified (Skandamis *et al*. 2001). The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Schuenzel and Harrison 2002). EOs used as flavoring substances occupy an intermediate position in that they are composed of naturally occurring substances many of which are intentionally added to food as individual chemical substances. Because they are considered neither a direct food additive nor a food itself, no current standard can be easily applied to the safety evaluation of EOs. Also, there is a growing interest in organically produced foods that the general public associated with healthier food. Another problem is the use of animal waste as organic fertilizer, whether in organic or non-organic agriculture, that gives rise to concerns about the possible contamination of agricultural produce with pathogens (especially *E. coli* O157:H7) and the possible contamination of ground and surface water. The evaluation of the safety of EOs that have a documented history of use in foods starts with the presumption that they are safe based on their long history of use over a wide range of human exposures without known adverse effects. The close relationship of flavor complexes to food itself has made it difficult to evaluate the safety and regulate the use of EOs. For EOs, there is a requirement to specify the chemical constituents and their range of concentrations for the oil to be evaluated for GRAS. However, the chemical description represents the chemical composition of material considered for GRAS. It is not a required specification, since different batches of the commercial oil will not contain all listed constituents in the reference concentration ranges. Instead different batches will be required to exhibit upper concentration limits for congeneric group that comprises the EO. Phenolic components present in EOs have been known to possess antimicrobial activity and some are classified as Generally Recognized as Safe (GRAS) substances and therefore could be used to prevent post-harvest growth of native and contaminant bacteria. Phenolic components of oils sensitize the phospholipid bilayer of the cell membrane, causing an increase of permeability and leakage of vital intracellular constitutes or impairment of bacterial enzyme systems (Singh *et al*. 2002).

Some EOs and their components have been known to cause allergic contact dermatitis in people who use them frequently. Preventive measures may be needed to ensure the well-being of workers if these substances were to be used on a larger scale (Carson and Riley 2001; Bleasel *et al*. 2002). Some oils used in the fields of medicine, paramedicine and aromatherapy have been shown to exhibit spasmolytic or spasmogenic properties, although these are difficult to associate with a particular component (Madeira *et al*. 2002). A necessary part of the safety evaluation of an EO involves specifying the biological origin, physical and chemical properties, and other identifying characteristics of the substance being evaluated. An EO produced under good manufacturing practices (GMP) should be of a purity (quality) and chemical composition sufficiently high to represent a reasonable certainty of safety under conditions of intended use. Because the evaluation is based primarily on the actual chemical composition of the EO, specifications must necessarily include chemical assay for the EO in commerce. In addition to information on the origin of the EO and physical properties, specifications on composition link the chemical identity of the EO to its safety evaluation. Therefore data on the percent range or upper limit of concentration of congeneric groups in the EO, target constituents monitored in an ongoing quality control program, and the amount of trace unidentified constituents that stipulate the composition of the EO become key specifications linking the product distributed in the marketplace to the chemically-based safety evaluation.

The leafy parts of thyme and its EO have been used in foods for its flavor, aroma and preservation for many years. Very few studies have been performed on the mutagenicity and/or antimutagenicity of the ingredients of thyme. Although the genotoxic potential of thymol and carvacrol at non-toxic doses has been suggested to be weak in the DNA-repair test and the SOS-chromotest (Stammati *et al*. 1999), contradictory results have been reported with the Ames mutagenicity assay (Azizan and Blevins 1995; Stammati *et al*. 1999). Sevtap and co-workers (2005) studied the genotoxic potential of major compounds of thyme oil i.e. thymol, carvacrol, and γ-terpinene and of the methanolic extracts of thyme in human lymphocytes by single-cell gel electrophoresis. Also, the effects of these substances on the induction of DNA damage by 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ) and mitomycin C (MMC) were evaluated. No increase in DNA strand breakage was observed at thymol and γ-terpinene concentrations below 0.1 mM, but at the higher concentration of 0.2 mM significant increases in DNA damage were seen. Thymol and γ-terpinene significantly reduced the DNA strand breakage induced by IQ and MMC at the lower concentrations studied. Carvacrol, which is an isomer of thymol, seemed to protect lymphocytes from the genotoxic effects of IQ and MMC at non-toxic concentrations below 0.05 mM, but at the higher concentration of 0.1 mM carvacrol itself induced DNA damage. Also the constituents of the *n*-hexane and ethyl acetate fractions prepared from the concentrated aqueous methanolic extracts of *Thymus spicata* protected lymphocytes against IQ- and MMC-induced DNA damage in a concentration-dependent manner. Gomes-Carneiro *et al*. (2005) investigated the genotoxicity of β-myrcene, α-terpinene and (+) and (−)-α-pinene, monoterpenes found in a variety of plant volatile oils. β-myrcene, α-terpinene and α-pinene as well as plant

oils containing these hydrocarbon monoterpenes have been used as flavoring additives in foods and beverages, as fragrances in cosmetics, and as scent in household products. Mutagenicity was evaluated by the *Salmonella*/microsome assay (TA100, TA98, TA97a and TA1535 tester strains), without and with addition of an extrinsic metabolic activation system (rat liver S9 fraction induced by Aroclor 1254). Two dose-complementary assays were performed so that a broad range of doses, including a number of regularly-spaced doses in the non-toxic dose interval, were tested. No increase in the number of his^+ revertant colonies over the negative control values was observed in any of the four *S. typhimurium* tester strains. Their results indicated that β-myrcene, α-terpinene, and (+) and (−)-α-pinene are not mutagenic in the Ames test. Ipek *et al*. (2005) evaluated the genotoxic and antigenotoxic effects of the EO of *Origanum onites* L. and carvacrol that are used in medicine, flavoring of food and crop protection by Ames *Salmonella*/microsomal test. The mutagenic activity was initially screened using *Salmonella typhimurium* strains TA98 and TA100, with or without S9 metabolic activation. No mutagenicity was found in the oil to the both strains either with or without S9 mixture whereas significant mutagenic activity was induced by carvacrol generally in the absent of metabolic activity. The oil and its major constituent carvacrol were finally tested for their antimutagenic activity with 30 min standard preincubation time. It was shown that both of them strongly inhibited mutagenicity induced by 4-nitro-*o*-phenylenediamine and 2-aminofluorene in both strains with or without S9, respectively. These results indicate significant antimutagenicity of the EO and carvacrol *in vitro*, suggesting its pharmacological importance for the prevention of cancer.

Among the thousands of naturally occurring constituents so far identified in plants and exhibiting a long history of safe use, there are none that pose, or reasonably might be expected to pose a significant risk to human health at current low levels of intake when used as flavoring substances. When consumed in higher quantities, normally for other functions, some plants do indeed exhibit toxicity. Historically, humans have used plants as poisons (e.g., hemlock) and many of the intended medicinal uses of plants have produced undesirable toxicity. Even at high intake levels, the majority of the constituents show no carcinogenic potential (Smith *et al*. 2005a). In spite of the fact that a considerable number of EO components are GRAS and/or approved food flavorings, some research data indicate irritation and toxicity. For example, eugenol, menthol and thymol, when applied in root canal treatments, have been known to cause irritation of mouth tissues. The results of a cytotoxicity study on these compounds suggest that gum irritation may be related to membrane lysis and surface activity and that tissue penetration may be related at least partly to membrane affinity and lipid solubility (Manabe *et al*. 1987). Cinnamaldehyde, carvacrol, carvone and thymol appear to have no significant or marginal effects *in vivo* whilst *in vitro* they exhibit mild to moderate toxic effects at the cellular level. Genotoxicity data appear not to raise concern in view of the present levels of use (Stammati *et al*. 1999). Oregano and cranberry extract mix provide a GRAS-type phytochemical source with the potential to develop a natural and effective antimicrobial strategy against *Vibrio parahaemolyticus*. Different ratios of extract mixtures were optimized from oregano and cranberry, and evaluated for antimicrobial activity in laboratory medium and seafood products (Lin *et al*. 2005). The results indicated that the antimicrobial activity was enhanced in response to extract mixtures than individual extracts of each species. The efficacy was further improved by combination with lactic acid, which is also considered GRAS. These results show the potential of plant extracts to be antimicrobial and, when combined with lactic acid, they can be used as a more effective multiple-barrier food preservation system. Such a synergistic ingredient approach can have wide implications for improvement of food safety.

Many of the individual constituents of the EOs are themselves used as flavoring substances and pose no toxicological threat (Smith *et al*. 2005b). Besides, chronic studies have also been performed on over 30 major chemical constituents including methyl chavicol and cinnamldehyde and results have not revealed any safety concern (*Smith et al*. 2005b).

Hence, it is recommended that more safety studies be carried out before EOs are more widely used or at greater concentrations in foods that at present.

FUTURE RESEARCH

Foods preserved with natural additives have become popular due to greater consumer awareness and concern regarding synthetic chemical additives. This has led researchers and food processors to look for natural food additives with a broad spectrum of antimicrobial activity (Marino *et al*. 2001).

We have little doubt that the aroma compounds can represent a useful tool to increase the shelf-life and the safety of processed foods and fruits. Nevertheless, in relation to the composition of foods and fruits, further investigations are necessary to identify the conditions that maximize their activity without detrimental effects on the organoleptic properties of the product.

The antimicrobial molecules in complex mixture of EOs' compounds and their eventual interactions should be addressed. This will lead to increase in control of microbial growth, to minimize the impact of these substances on the flavor of food products and to avoid fluctuations in EOs activity due to meteorological, seasonal and geographical factors, as well as different compositions due to the plant type.

The stability of EOs during food processing will also need to be studied.

Standardization of test methods for testing antibacterials for use in food. This is a field where a selection of standard methods would accelerate the study of promising antibacterial components and their synergistic or antagonistic action with each other and with food ingredients.

Synergistic effects could be exploited so as to maximize the antibacterial activity of EOs and to minimize the concentrations required to achieve a particular antibacterial effect. Antagonism between EO and food ingredients is undesirable and research is needed so it can be avoided in practical applications.

Interactions between EOs and their components and other food ingredients and food additives need to be investigated. Clove and oregano oils can acquire a dark pigmentation when in contact with iron (Bauer *et al*. 2001); this may impose limitations on the public acceptability of the food products or preservatives.

Further elucidation of the mechanisms of antimicrobial actions of EOs would provide insights that may prove useful for technological applications.

Setting up specific active packaging able to release slowly over time in the headspace the selected molecules. The activity would be enhanced by a calibrated increase of EO vapor pressure in order to enhance its capacity to interact with the microbial cell membrane (Guerzoni *et al*. 1994; Gardini *et al*. 1997; Lanciotti *et al*. 1999).

The interaction between antimicrobials and packaging materials, rather than the food itself need research.

Development of microbial resistance to the presence of EOs in foods is important. Disadvantageous effects of addition of EOs on the safety of the food, such as influencing the stress tolerance of pathogens need special attention.

Possible consequences such as toxicity or other unwanted side effects on the human health of the use of EOs would need to be explored.

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