HURDLE TECHNOLOGIES USING ESSENTIAL OILS AND HIGH HYDROSTATIC PRESSURE TO INACTIVATE *E. COLI* IN FRESH BEEF

By

Fatma Sahmurat

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Parameswarakumar Mallikarjunan, Chair

Joseph E. Marcy

Robert C. Williams

Robert Grisso

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Abstract

In this study, potential of high hydrostatic pressure (HHP) and essential oils (EOs) as natural antimicrobials was evaluated to produce *E. coli* safe and quality beef product. First, the individual and combined effects of antimicrobial activity (minimum inhibitory concentration) of basil, black cumin, cilantro, cumin, fenugreek, ginger, oregano, black pepper, rosemary, thyme, turmeric oil emulsions on *E. coli* ATCC 25922 with and without HHP treatment were evaluated. Cumin, oregano and thyme EOs showed highest antimicrobial activity against *E. coli* ATCC 25922. The synergy of selected EOs against *E. coli* ATCC 25922 was determined using the checkerboard method to obtain fractional inhibitory concentration index. Although their combinations did not show synergy, they expressed synergy when combined with HHP (400 MPa, 10 min, 20 °C) and the best combination was cumin and oregano EOs with HHP.

Effects of HHP & EO combinations on inactivation of *E. coli* ATCC 25922 in beef were investigated using response surface methodology (RSM). Statistical analysis showed the model was significant for predicting log reduction with high accuracy. The significant model terms were pressure and time. Compared to control, HHP/EO treated samples showed no-post growth when stored up to 120 days at 4°C. Presented results suggests that the combination of HHP and

antimicrobials has not only improved the process parameters (lowered pressure, time, and EO concentration) but also prevented recovery of *E. coli* ATCC 25922 during storage.

RSM was employed to analyze the synergistic effects of HHP and EOs on beef quality (color, texture and lipid oxidation). Color indices were significantly affected by pressure, time and their interactions. Above 400 MPa the discoloration was similar to cooked beef and EO addition did not help color improvement. However, EOs showed significant antioxidant activity on both treated and untreated samples during storage.

In conclusion, there is a great potential of HHP and EO combinations to enhance pathogen inactivation while keeping the quality of beef. Moreover, presence of EOs can prolong the shelf life of pressure treated beef. Therefore, the combination of HHP and EO is very promising for meat industry.

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General Audience Abstract

Meat is a natural source of protein, essential vitamins, which makes it a nutrient-rich source of a healthy diet as well as an ideal environment for food-borne pathogens and spoilage bacteria. It is therefore essential to preserve very perishable meat products in terms of microbial contamination. As an alternative to many preservation methods such as chilling, canning, curing, smoking, dehydrating and heat treatment, a non-thermal mild food preservation technology of high hydrostatic pressure processing (HHP) is proposed for inactivating the most common meat contaminant bacteria of *E. coli*. Essential oils (EOs) can provide a solution for pasteurization requirements and reducing quality losses associated with HHP treatment.

In this study the synergistic effect of selected EOs (basil, black cumin, cilantro, cumin, fenugreek, ginger, oregano, black pepper, rosemary, thyme, turmeric oil emulsions) and HHP technology on inactivation of *E. coli* ATCC 25922 on contaminated meat cuts were investigated. Experimental design and statistical analysis were conducted using response surface methodology (RSM). Combination of HHP/EO treated samples showed no-post growth of *E. coli* ATCC 25922 when meat samples were stored up to 120 days at 4°C. Presented results are suggesting that HHP in combination with EOs has increased the log reduction of *E.coli* and as well, decreased the quality losses (color, lipid oxidation textural analysis) compared to control samples where HHP is

applied alone. As a conclusion, this study shows that there is a great potential of HHP and EO combinations to enhance pathogen inactivation while keeping the quality of beef.

Dedication

To my beloved parents, Leyla and Adem Sahmurat,

And my precious sister Hulya,

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CHAPTER I

INTRODUCTION

Beef is an important source of protein and several essential vitamins. Red meat production has the largest segment of the U.S. agriculture and beef is the most preferred red meat with total consumption 25.8 billion pounds in 2012. Also, 9.4 % of beef production was exported in 2012 and $5.1billion value added in U.S. economy. Beef is mostly consumed as ground 7.5 billion tons at home and food serving facilities. Since beef is a nutrient rich food, raw beef and raw ground beef can easily spoil at ineligible conditions as microorganisms can rapidly grow in raw meat products. Especially ground beef can be easily cross-contaminated from process equipment and it rapidly loses quality. Because consumers prefer to eat juicier and tender beef, insufficient thermal treatment is common and it has been caused important health risks. For a safe beef meal, ground beef products should cook until the internal temperature of them reaches 160 °F and fresh beef should heat to 145°F with a 3 min rest time.

E. coli O157:H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes* are some of the foodborne organisms associated with meat-based products. All of those pathogenic bacteria are big treat to meat industry as they can spread via cross contamination by poor handling practices and poor sanitation. *E. coli O157:H7* has a low infectious dose as low as ten cell. It can easily make people sick, so scientist, food producers, consumers and regulation agencies have taken it seriously. If *E. coli* bacteria are found in beef, the beef either be destroyed or cooked before being sold to avoid any possibility of infection. Either action results in losses or additional costs to the industry.

Because fresh meat is vulnerable to severe heat treatments, non-thermal preservation technologies have drawn attention of the meat industry wishing to satisfy growing consumers demands for high quality, easy-to-handle, safe with natural flavor and color, as well as an extended shelf life without chemical preservatives, meat products. That's why the implementation of high hydrostatic pressure processing (HHP), which is a novel non-thermal preservation method, has a great potential in meat industry. HHP is an isostatic and adiabatic process which can be applied uniformly and instantly to the subject without no change the shape and integrity of the packaging intact. Since applying high hydrostatic pressure on food is adiabatic, this prevents the food from being heated which would modify its organoleptic properties.

High pressure treatment on meat and meat products has been a popular subject in the last decade for researchers. Although, it is well known that HHP induces the changes on muscle proteins of meat depending on the magnitude of pressure/temperature and the duration of the time, effects of these changes on the eating quality is not fully understood. HHP can cause partial denaturation of protein and increase the potential for protein breakdown with tenderization. HHP does little or no changes in protein profiles but it does strong modification on meat color and reduction on water holding capacity.

The effect of this technology on foodborne pathogenic bacteria has been widely studied and reported that most vegetative pathogens are susceptible to pressure, and pressure treatments achieve significant reductions. Apart from the numerous advantages that HHP technology presents, there are some drawbacks of high pressure on fresh meat products such as increased lipid oxidation and discoloration through protein denaturation. Mostly commercially available pressurized meat products are ready to eat foods except fresh minced beef now.

Even HHP is a good alternative to conventional heat treatment for meet safety; it still has disadvantageous effects on color characteristics and oxidative stability of meat and meat products. Both pressure and temperature regimes had significant effects on color, cook loss and lipid oxidation. Thus, recent researches have been focused on applying HHP in hurdle technologies to reduce the quality changes as providing the enough microbial inactivation.

A synergic effect on bacterial inactivation has been observed when HHP combined with some antimicrobials, such as bacteriocins, nisin and lysozyme. Furthermore, there has been some researches investigating the synergistic effect of HHP and essential oils or herb's extract as natural antimicrobials on different microorganisms in a variety of food systems.

Essential oils (EO) are volatile natural mixtures extracted from different plant parts, and are composed of terpenoid structures with broad activities. EOs are known to work as potential antimicrobial agents having the ability to control foodborne pathogenic bacteria Use of EOs in food and beverage industries has been exploited for decades. They have been shown to work as potent antibacterial agents because of the presence of bioactive volatile components. Besides, concerns on reducing chemical preservatives in food industry have been raised due to the adversary effects of chemical preservatives, resulting in the release of toxic materials inside the packed food products. Use of EOs can be an effective application as food preservatives naturally due to their potent antimicrobial nature. Undesirable organoleptic effects can be limited by careful selection of EO according to the type of food. Synergism and antagonism between components of EOs and food constituents require more study before these substances can be reliably used in commercial applications.

In recent years, the consumer demand for more natural foods has coerced producers to use natural food additives; such as, natural antimicrobials and antioxidants, instead of synthetic ones. Many researchers showed antimicrobial and antioxidant activity of essential oils' (EO) and suggested them to be used as natural antimicrobials and antioxidants in the food industry. Moreover, applying EOs in food products is adding them value as "functional food" because of their medicinal benefits.

It is apparent that minimal processing technologies such as HHP that will prevent contamination in meat based products needs to be developed to provide a strong alternative to severe heat treatment for sanitary purposes. A negative aspect such as induced lipid oxidation and discoloration needs to be reduced to improve the methodology. It has been suggested before high pressure should be combined with other preservation methods to improve the final product quality and investigated combination of HHP and antimicrobials. However, consumers are getting sensitive to purchase added synthetic preservatives and looking for more natural and safe products. That is why natural antimicrobials and antioxidants are getting popularity in food industry recent years.

Thus, in this study, it is proposed to extend the commercial shelf life of raw beef using HHP technology to inactivate *E. coli* ATCC 25922. Due to biosafety constraints, this particular strain of *E. coli* was selected as the surrogate of *E. coli* O157:H7. The study also investigated and optimized the synergistic effects of high hydrostatic pressure and natural antimicrobials during short and long term storage under refrigeration conditions (<4°C) with respect to microbial load, sensory, color and textural properties of product. It is suggested that applying high hydrostatic pressure and essential oils as natural antimicrobials on fresh beef meat products shall be an effective

preservation method to inactivate *E. coli* while adding functional value to raw beef products.

The specific objectives of this study are:

1. Evaluating the antimicrobial activity of wide variety of EOs and HHP, against *E. Coli* ATCC 25922.

2. Investigating synergetic effect of HHP and natural antimicrobials raw beef in means of inactivation of E coli

3. Determining effects of the combination of EO and HHP on color, lipid oxidation and textural properties of meat and microbial safety of products during the storage at 4°C.

4. Optimization of EO concentration and HHP operating parameters for meat products. Determining the synergistic effect of HHP and natural antimicrobials on E.coli log reductions, color, texture and sensory properties of meat.

The dissertation has three chapters focusing on each of the three objectives. The chapter 3 is focused on the effect of eleven essential oils and evaluated the minimum inhibitory concentration for each antimicrobial against the target bacterium. In addition, the chapter also include the fractional inhibitory concentration index to determine the synergistic effects of combining one or more antimicrobials against the target bacterium with or without HHP. The chapter 4 describes the effect of selected antimicrobials with HHP on the inactivation of *E. coli* ATCC 25922 in beef. The chapter evaluated the combination of HHP and EOs on beef quality and identified optimum process conditions and antimicrobial concentrations for effective reduction of the bacteria and maintenance or improvement of beef quality.

CHAPTER II

LITERATURE REVIEW

HIGH HYDROSTATIC PRESSURE

High hydrostatic pressure processing (HHP or Pascalization) is a non-thermal food preservation and processing technology that can inactivate food borne pathogens and spoilage micro-organisms without significantly altering organoleptic properties and nutritional value of foods. It is defined as a method of food preservation that involves subjecting food to a high hydrostatic pressure (300–700 MPa), with or without the addition of heat, to achieve microbial inactivation while achieving the consumer-desired qualities, i.e., retention of fresh- ness and nutritive value of food products. Pressures used are almost ten times greater than in the deepest oceans on Earth (Figure 1). (Rastogi 2013; Knorr 1999)

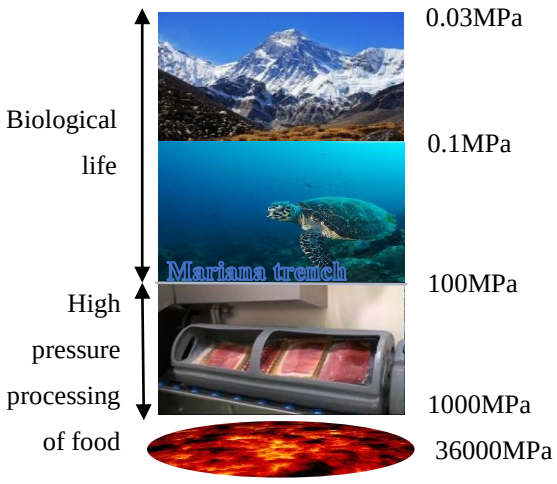

Figure 1 Schematic representation of the pressures used in food processing. (Adapted from Considine *et al.* 2008)

History

Although it is considered novel, the introduction of HHP in Biology was over a hundred year ago. Roger H. was the first to report that high pressure had an effect of reducing or destroying bacteria. He underlined two important phenomena: (a) the difference of behavior under pressure between microorganisms species (*Staphylococcus aureus* was not affected at 300 MPa whereas *Streptococcu*s was more sensitive with approximately one third of the initial population being killed), (b) the difference of sensitivity against pressure between the two bacterial forms of *Bacillus anthracis*, spores being more resistant than vegetative bacteria (Demazeau & Rivalain 2011).

The application of high pressure for food preservation was first tested as early as 1894 through the research efforts of Burt Hite at the West Virginia State University Agricultural Experiment Station in Morgantown, West Virginia. His research showed that pressures between 200 and 680 MPa could inactivate yeasts, molds, and spoilage bacteria in foods. However, he was not able to sterilize whole cow's milk to allow room temperature storage. This was Hite's original objective. Hite, and his associates, predicted that fruit juices, in large containers, could be sterilized by high pressure. Work showed that there would be little change in their fresh flavor during room temperature storage. (Balasubramaniam, Barbosa-Cánovas, and Lelieveld 2016; Yuste et al. 2001)

In Japan the first high-pressure processed foods were introduced to the food market in 1990. Then, M/s Fresherized Foods, Texas, US produce high-pressure preserved guacamole dip. There are a range of pressure-treated food products already on the market. They include fruit preparations, fruit juices, rice cakes and raw squid in Japan, fruit juices, especially apple juice in France and Portugal and guacamole and oysters in the USA. Concerning meat products, so far there are two Spanish meat companies using HHP equipment daily (Esteban Espun˜a, S.A. and Campofrio

Alimentacio´n, S.A.). In the USA, several meat companies have made this methodology available (e.g. Hormel Foods and Purdue Farms). (Hugas et al. 2002).

Governing Principles

The effects of high hydrostatic pressure on food chemistry and microbiology are governed by Le Chatelier's and isostatic principles. According to Le Chatelier's principle, a decrease in volume is enhanced by pressure, it is valid for reversible, and reaction equilibriums are shifted towards the most compact state, and the reaction rate constant is increased or decreased, depending on whether the "activation volume" of the reaction (i.e. volume of the activation complex less volume of reactants) is negative or positive. (Rastogi et al. 2007). The pressure effect on molecular systems can be predicted based on Eq. 1, where G is the Gibbs free energy, p is the pressure, T is the temperature, K is the equilibrium constant, R is the ideal gas constant, and V is the volume (Follonier et al. 2012).

$$\left(\frac{\partial \Delta G}{\partial p}\right)_T = \left(\frac{-\partial lnK}{\partial p}\right)_T RT = \Delta V \quad \textbf{(1)}$$

When pressure increases, a given equilibrium will be shifted to the side that occupies the smallest volume. In case of a non-equilibrium process, the pressure dependence of the reaction rate k is given by the activation volume $\Delta V^{\neq}$ (Eq. 2).

$$\left(\frac{-\partial lnK}{\partial p}\right)_T RT = \Delta V^{\neq} \quad \textbf{(2)}$$

Increasing the pressure can thus either accelerate or decelerate reactions depending on the sign of the activation volume $\Delta V^{\neq}$. This feature contrasts with the effect of temperature increase that is always accelerating (cf., Arrhenius law) (Follonier et al. 2012).

The second principle is the isostatic transmission. "Hydrostatic" refers to the equilibrium of fluids under the action of force or pressure. It indicates that the transmission of pressure is uniform and almost instantaneous independent of size, shape and composition of food and package. Mechanistically, pressure alters the distance between molecules having direct effect on distance – dependent interactions. For instance, van der Waals forces are one of those interactions strongly affected by pressure because their optimal working distance is altered by pressure, which disrupts the balance between attractive repulsive forces. Other interactions affected by pressure due to their working distance are hydrogen bonding, electrostatic, and hydrophobic interactions. Contrary, covalent bonds are unlikely to be affected by pressure because their working can be hardly reduced any further. Indeed, covalent bonds from primary structure of protein unaffected by pressure (up to 1500 MPa). The fact that high pressure does not alter covalent bonds has been the central hypothesis behind the preservation of activity of functional compounds (Balasubramaniam et al. 2016).

Effect on food components

High pressure affects only non-covalent bonds (hydrogen, ionic, and hydrophobic bonds), causes unfolding of protein chains, and has little effect on chemical constituents associated with desirable food qualities such as flavor, color, or nutritional content (Rastogi et al. 2007).

Although denaturation of proteins is induced either by heat, chemicals or high pressure the residual molecular structure can vary significantly. Temperature and/or chemical induced protein denaturation often unfold the complete protein irreversibly because of covalent bond breaking and/or aggregation of the molecule. In contrast, high pressure can leave parts of the molecule unchanged, indicating that the denaturation mechanisms are substantially different. In aqueous solution pressure affects mainly the tertiary and quaternary structure of proteins. Covalent bonds are rarely affected by high pressure and even α- helix or β-sheet structures appear to be almost incompressible (Knorr et al. 2006).

The application of pressure on proteins leads to different degrees of protein structure modification, i.e. primary, secondary, tertiary and quaternary structural levels. Pressure treatment induces unfolding of the protein structure and then folding after pressure release. (Bajovic et al. 2012).

Generally the primary structure is not modified by pressure, whereas secondary structure is only affected by very high pressure treatment , and tertiary and quaternary structures are modified from 100MPa (de Lamballerie et al. 2002). Pressure is able to affect the protein structure, at the secondary, tertiary and quaternary levels, leading in general to protein denaturation. Denaturation is a complex process involving intermediate forms such as the molten globule state leading to non-reversible denaturation, depending on the rate of compression and on the extent of secondary structure rearrangements (Hong et al. 2006). Thus, the native structure of a protein, i.e. the conformation that displays biological activity, is the result of a delicate balance between stabilizing and destabilizing interactions, within the polypeptide chains. The quaternary structure of protein, maintained by hydrophobic interaction, is the most sensitive to pressure. Moderate pressure below

150 MPa was found to favor the dissociation of oligomeric proteins. At 150-200 MPa, oligomeric dissociation occurs lower than those at which unfolding of monomers is observed. Pressure above 200 MPa induces unfolding of proteins and re-association of subunits from dissociated oligomers. Significant tertiary structure changes are observed beyond 200 MPa. However, reversible unfolding of proteins can occur at higher pressure (400 to 800 MP), showing that the volume and compressibility changes during denaturation are not completely dominated by hydrophobic effects (Hong et al. 2006).

Enzyme activity is an important parameter affecting quality, particularly of cut fruits and vegetables. Since enzymes are proteins, it is expected that application of pressure changes the structural conformation and may sometimes lead to loss of activity. Exposure to high pressure may inactivate or activate enzymes. Most work related to the effect of HHP on enzyme activity has been performed with respect to pectin esterase (PE) and polygalacturonase (PG) in intact, cut and pureed vegetables and juices. It was reported that the pressure- temperature ranges that inactivate tomato PG were between 300 and 600 MPa and 5 and 50ºC. However, PE activity in diced tomatoes markedly increased with application of 400 MPa pressure at 45 ºC (Balasubramaniam et al. 2016).

The primary effects of pressure on phospholipids can be observed on the temperatures of the transitions. Pressure favors the crystalline state, as a result of the Le Chatelier principle. As pressure increases at constant temperature, the lipid bilayer adapts by changing its conformation. A variety of pressure-induced phase transformations has been observed such as liquid-to-gel transition and gel-to-interdigitated gel transition. In contrast to Le Chatelier's principle, the volume of the lipid bilayer in liquid- to- gel transition increases with an increase in the fatty acyl chains (R-groups) length of the phospholipids. Pressures of 50-200 MPa cause the transition from the liquid-

crystalline to gel. At higher pressures (above 200 MPa), a second pressure-induced phase transition, called interdigitated phase, is observed, and the bilayer volume decreases by 5%, accompanied by a decrease in its thickness (Heinz & Buckow 2009; Knorr et al. 2006).

Meat application

E. coli O157:H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes* are some of the foodborne organisms associated with meat-based products. All of those pathogenic bacteria are big treat to meat industry as they can spread via cross contamination by poor handling practices and poor sanitation. When meat is ground, more of the meat is exposed to the harmful bacteria, and the risk of cross contamination increases. *E. coli* O157:H7 is one of the most important pathogens for beef. Between 1998 and 2010, The number of reported *E. coli* O157:H7 outbreak which is associated with consumption of raw beef and ground beef is 111 (CDC 2015). Almost five outbreaks occur every year. It has been reported that 41% of food-related *E. coli* O157:H7 outbreaks were associated with the consumption of contaminated ground beef (Dodd & D. 2009). *E. coli* O157:H7 was declared as an adulterant in raw ground beef by the USDA's Food Safety Inspection Service (FSIS) in 1994. If *E.coli* bacteria are found in beef, the beef either be destroyed or cooked before being sold to avoid any possibility of infection. Either action results in losses or additional costs to the industry. The U.S. Dept. of Agriculture-Food Safety and Inspection Services (USDA-FSIS) issued a letter-of-no-objection (LNO) for the use of HHP as an effective post-packaging intervention method in controlling *L. monocytogenes* in RTE meat and poultry products in 2003, HHP technology has been employed by many meat processors with great potential in terms of ensuring meat safety after packaging (Campus 2010).

Inactivation of *Escherichia coli* O15:H7 (EHEC) in ground beef at steady pressure (Podolak et al. 2006) and under different cyclic HHP operating conditions were investigated and up to 4.96 log CFU/g *E. coli* population reduction was reported when three 5-min cycles at 400Mpa was applied (Morales et al. 2008). High-pressure processing (HHP) reduced *E. coli* O157:H7 in ground beef by 3 log CFU/g and caused substantial sublethal injury resulting in further log reductions of bacteria during frozen storage (Black et al. 2010). Gill and Ramaswamy (2008) investigated the potential of HHP for killing *E. coli* O157 in ready to meat products and observed more than 4 log CFU/g initial reduction on *E. coli* numbers. Nevertheless, it is stated that *E. coli* O157 recovered with enrichment and immunomagnetic. During storage, the numbers of *E. coli* O157 increased on all the beef samples except Hungarian salami, which had a restrictive pH and water activity. No additional reduction was observed by increasing pressurization time. Omer et al (Omer et al. 2010) evaluated the effect of high pressure on the survival of verotoxigenic *E. coli* (VTEC) in two types of dry-fermented sausage and they reported that HHP treatment at 600 MPa for 10 min and at three cycles of 600 MPa for 200 s per cycle caused 2.9 and 3.3 log CFU/g *E. coli* reduction respectively. HHP treatment has also been shown to be effective on *Clostridium sporogenes* spores in ground beef at elevated temperatures. HP treatments at 700-900 MPa and temperatures at 80-100oC were examined; and D values ranging between 1.5 to 0.63 min at 100 °C and Z(T)(P) values of 520-563 MPa at 80-100 °C were reported. When results were compared with conventional thermal processing, HP treatment with elevated temperatures can destroy bacterial spores with a shorter time (Zhu et al. 2008). de Alba et al. (2013) has reported inactivation effect of HHP treatment on *Salmonella Enteritidis* inoculated beef carpaccio . He also looked for counts of *S. Enteritidis* during storage under temperature abuse conditions (8°C) for 30 days and found 5.94 log cfu/g *S.*

Enteritidis reductions in samples pressurized at 450 MPa for 10 min. Garriga et. al. (2002) proposed to add antimicrobials like bacteriocins to increase the death rate of foodborne bacteria [12]. Garriga, et al. (2002) stated that naturally contaminated meats contain levels of pathogenic and spoilage organisms at a very low levels and that treatment by 600 MPa of pressure is a valid process to inhibit or delay growth of spoilage microorganisms. Aymerich et al. (2008) reported high pressure treatment is generally in the range of 300–600 MPa for a short period of time, giving inactivation of >4 log units of the vegetative pathogenic and spoilage microorganisms.

Carlez et al. (1993) observed that pressurization above 200, 280, and 400 MPa for 20 min completely eliminated *Pseudomonas fluorescens* , *Citrobacter freundii* , and *L. innocua* , respectively in ground beef. *Pseudomonas sp*., *Lactobacillus sp*., and *coliforms* were completely inactivated after treatment at 400–450 MPa and recovered after storage at 3 °C in air. Jofré et al. (2009) evaluate the effect of the application of an HHP treatment of 600MPa to marinated beef loin challenged with food-borne pathogens (*L. monocytogenes, Salmonella enterica, S. aureus, Y. enterocolitica and Campylobacter jejuni*), spoilage microorganisms (slime producing LAB or the yeast *Debar- yomyces hansenii*) and the hygiene indicator *E. coli* and reported that the application of a 600MPa treatment effectively inactivated most of the microorganisms except for LAB, progressively decreased or maintained below the detection limit during the whole storage (120 days at 4 °C).

Apart from the numerous advantages that HHP technology presents, there are some drawbacks of high pressure on fresh meat products such as increased lipid oxidation and discoloration through protein denaturation (Campus 2010; Buckow et al. 2013; Aymerich et al.

2008; Bajovic et al. 2012; Cheftel & Culioli 1997; Simonin et al. 2012). Mostly commercially available pressurized meat products are ready to eat foods except fresh minced beef now (Bajovic et al. 2012).

Color change is one of the drawbacks in HHP treatments. As the pressure applied increases, the redness and total color difference becomes significant. Pressure higher than 300 MPa induces modifications of meat color parameters such a decrease of the total color difference while the redness of the 520 MPa samples decreases gradually, in relation to the increase of metmyoglobin (Jung et al. 2003). In order to overcome discoloration problem, various ingredients were added such as sodium chloride (NaCl), sodium tripolyphosphate (STPP) and beta-glucan (BG). However, additives showed little improvement on color characteristics but anti-oxidative property of beta-glucan in meat system was revealed (Omana et al. 2011). Induced lipid oxidation of beef is another problem in HHP treatment. Aiming to increase the meat quality after HHP treatment, many researchers have tried to add functional ingredients such as phosvitin (Jung et al. 2013) or vegetable oil (Y. Jung et al. 2012). Addition of phosvitin was reported to synergistically reduce microbial growth and delayed the lipid oxidation when applied with HHP. However, it had no on the color changes of raw ground beef attributable to HHP (Jung et al. 2013). On the contrary, addition of vegetable oil followed by the application of HHP has been proven to inhibit the bacterial growth and lipid oxidation (Y. Jung et al. 2012). HHP treatment of raw or cooked meat and meat products have attracted researcher as it reduces the total viable cell count of bacteria in meat and meat products (Hassan et al. 2002).

Even HHP is a good alternative to conventional heat treatment for meet safety; it still has disadvantageous effects on color characteristics and oxidative stability of meat and meat products.

Both pressure and temperature regimes had significant effects on color, cook loss and lipid oxidation. An increase in TBARS values was observed at the higher pressure levels (300, 400 MPa) (McArdle et al. 2010). But still, TBARS values were found to be lower in pressurized samples compared to cooked samples. The reported results of McArdle and his co-workers showed that HHP alters meat quality to a lesser extent than conventional cooking, thereby minimizing the processing impact (McArdle et al. 2011).

ESSENTIAL OILS

Essential oils (EO) are volatile natural mixtures extracted from different plant parts (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots), and are consisted of secondary metabolites such as phenolics, phenolic acids, quinones, flavonoids, tannins, coumarins, terpenoids and alkaloids.(Hyldgaard et al. 2012; Seow et al. 2014; Burt 2004). Secondary metabolites naturally provide defense mechanism to plant against microorganisms, insects and herbivores. They can either always present in the plant's cell or can be produced in response to invasion of microorganism or insects (Lai & Roy 2004). The estimated number of essential oils (EOs) are known is 3,000 and 300 are commercially used by the flavor and fragrance industries (Bassolé & Juliani 2012)

De la Croix in 1881 evaluated the antibacterial properties of secondary metabolites first time (Burt 2004).However, spices and herbs have been traditionally used for medicinal purpose for thousands of years. The first written document about medicinal use of plant is a Sumerian clay slab from around 3,000 BC years and comprised 12 drug recipes including over 250 plants. Also, Chinese, Egyptians, Africans and Native Americans have used herbs for healing (Petrovska 2012).

Mostly EOs is used as flavoring agents in food, essences in perfumes and additive in cosmetics. Also it is used in pharmaceutical products for their functional properties (Juneja et al. 2012; Adorjan & Buchbauer 2010; Isman 2000; Gurib-Fakim 2006). Use of plant essential oils in food and beverage industries has been exploited for decades. They have been shown to work as potential antibacterial agents because of the presence of bioactive volatile components (Bajpai et al. 2012). The use of EOs as biopreservatives is a matter of great interest for the food industry since consumers prefer natural additives instead of synthetic ones. That is why many studies have been performed on this subject in the last few years (Lang & Buchbauer 2012).Moreover, they have recently begun to receive much attention as possible sources of safe and natural alternative medicines once again because they have been known to possess various medicinal activities, including antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticarcinogenic (Shaaban et al. 2012).

In recent years, the consumer demand for more natural foods has coerced producers to use natural food additives; such as, natural antimicrobials and antioxidants, instead of synthetic ones. Many researchers showed antimicrobial and antioxidant activity of essential oils' (EO) and suggested them to be used as natural antimicrobials and antioxidants in the food industry. Furthermore, applying EOs in food products is adding them value as "functional food" because of their medicinal benefits.

The functional properties of EOs is extremely diverse depending on the source, quality, extraction procedure, etc. EOs generally obtained by hydrodistillation, steam distillation, hydrodiffusion or CO2 extraction. The microwave irradiation (or microwave assisted process) and extracting oils using a mechanical and thermochemical reaction are the methods developed more

recently for EO's extraction as well (21).

Undesirable organoleptic effects can be limited by careful selection of EO according to the type of food. Synergism and antagonism between components of EOs and food constituents require more study before these substances can be reliably used in commercial applications. If the active substances are to be added to foods in greater concentrations than is currently normal practice for flavorings, further safety studies may be necessary. A significant number of essential oils and phytochemicals are bioactive against foodborne pathogens in vitro, and to a smaller degree, in foods. To prevent off flavor or other undesirable sensory qualities, essential oils have to be carefully selected according to the specific food type. Synergism between different phytochemicals or other chemical compounds has to be investigated further before they can be applied commercially. A compound should be checked for its safety limits if its antimicrobial activity depends on a concentration greater than the usual for flavorings. Despite having disadvantages, essential oils prove beneficial with their broad antimicrobial activities and lack of apparent resistance development (Seow et al. 2014; Burt 2004).

The constituent of EOs and their antibacterial properties

EOs are usually characterized by a strong odor and these mixtures consist of more than 200 constituents which can be grouped basically into two fractions of volatile (90–95%) and nonvolatile fractions (5-10%). Volatile fraction of EOs consists of monoterpenes and sesquiterpene hydrocarbons and their oxygenated derivatives, along with aliphatic aldehydes, alcohols, and esters. The nonvolatile residue mainly contains hydrocarbons, fatty acids, sterols, carotenoids, waxes, cumarines, and flavonoids (Preedy 2016). The active compounds can be divided into four

groups according to their chemical structure: terpenes, terpenoids, phenylpropenes, and "others (Hyldgaard et al. 2012). Interactions between these components may lead to antagonistic, additive or synergistic effects. Some studies have demonstrated that whole EOs usually have higher antibacterial activity than the mixtures of their major components, suggesting that the minor components are critical to the synergistic activity, though antagonistic and additive effects have also been observed (Bassolé & Juliani 2012).

Terpenes

Terpenes are hydrocarbons formed through the combinations of several 5 carbon-base (C5) units called isoprene and the main terpenes are the monoterpenes (C10), most representative molecules constituting 90% of the EOs, and sesquiterpenes (C15) (Sánchez-González et al. 2011). Monoterpenes present in EOs may contain terpenes that are hydrocarbons (α-pinene), alcohols (menthol, geraniol, linalool, terpinen-4-ol, p-menthane-3,8-diol), aldehydes (cinnamaldehyde, cuminaldehyde), ketones (thujone), ethers [1,8-cineole (=eucalyptol)], and lactones (nepetalactone). Sesquiterpenes have a wide variety of structures, more than 100 skeletons, as the elongation of the chain to 15 carbons increases the number of possible cyclization (Regnault-Roger et al. 2012). Most terpenes do not show serious antimicrobial activity, such as p-Cymene, one of the most important components of thyme EO (Bagamboula et al. 2004).

Terpenoids

Terpenoids are terpenes containing oxygen or that have had their methyl groups moved or removed by specific enzymes (Nazzaro et al. 2013) Thymol, carvacrol, linalool, menthol, geraniol, linalyl acetate, citronellal and piperitone are the most common and well-known terpenoids. The

antimicrobial activity of most terpenoids is related to their functional groups, and the hydroxyl group of the phenolic terpenoids and the presence of delocalised electrons are important elements for their antimicrobial action (Nazzaro et al. 2013). The major components of common EOs and their antimicrobial activities are presented in Table 1.

Phenylpropenes

Phenylpropenes contain a six-carbon aromatic phenol group and a three-carbon propene tail from cinnamic acid, which is produced during the first step of phenylpropanoid biosynthesis. These compounds represent a relatively small portion of EOs. Eugenol, isoeugenol, vanillin, safrole and cinnamaldehyde are the most studied phenylpropenes (Nazzaro et al. 2013; Hyldgaard et al. 2012).

Table 1 Minimum Inhibitory concentrations (MICs) of essential oils against *E. coli*

Essential oil	**MIC value**	**Essential oil**	**MIC value**
			(Hammer et al. 1999)
Rosewood	0.12 (%v/v)	Black pepper	>2.0 (%v/v)
Celery seed	2.0 (%v/v)	Lavendar	0.25 (%v/v)
Frankincence	1.0 (%v/v)	Macadamia	>2.0 (%v/v)
Ylang ylang	2.0 (%v/v)	Tea tree	0.25 (%v/v)
Lime	2.0 (%v/v)	Cajuput	1.0 (%v/v)
Orange	>2.0 (%v/v)	Niaouli	0.25 (%v/v)
Petitgrain	0.25 (%v/v)	Peppermint	0.5 (%v/v)
Bergamot	1.0 (%v/v)	Spearmint	0.25 (%v/v)
Lemon	>2.0 (%v/v)	Basil	0.5 (%v/v)
Grapefruit	>2.0 (%v/v)	Evening primrose	>2.0 (%v/v)
Coriander	0.25 (%v/v)	Oregano	0.12 (%v/v)
Pumpkin	>2.0 (%v/v)	Geranium	0.25 (%v/v)

Lemongrass	0.06 (%v/v)	Aniseed	0.5 (%v/v)
Palmarosa	0.06 (%v/v)	Bay	0.12 (%v/v)
Citronella	0.5 (%v/v)	Pine	2.0 (%v/v)
Carrot seed	>2.0 (%v/v)	Apricot kernel	>2.0 (%v/v)
Eucalyptus	1.0 (%v/v)	Sweet almond	>2.0 (%v/v)
Fennel	0.5 (%v/v)	Rosemary	1.0 (%v/v)
Wintergreen	0.5 (%v/v)	Sage	0.5 (%v/v)
Juniper	>2.0 (%v/v)	Clary sage	>2.0 (%v/v)
French lavender	0.5 (%v/v)	Sandalwood	>2.0 (%v/v)
Thyme	0.12 (%v/v)	Clove	0.25 (%v/v)
			(Prabuseenivasan et al. 2006)
Cinnamon	1.6 mg/ml	Lime	6.4 mg/ml
Clove	1.6 mg/ml	Orange	12.8 mg/ml
Geranium	6.4 mg/ml	Rosemary	6.4 mg/ml
Lemon	6.4 mg/ml		
			(Kim et al. 1995)
Citral	500 μg/mL	Perrilaldehyde	500 μg/mL
Carvacrol	500 μg/mL	Eugenol	1000 μg/mL
Geraniol	500 μg/mL	Linalool	1000 μg/mL
Terpineol	1000 μg/mL	Citronellal	>1000 μg/mL
			Burt (2004)
Oregano	0.0625 (%v/v)	Thyme oil, white	2.5 (%v/v)
Thyme oil, red	1.250 (%v/v)		
			(Delaquis et al. 2002)
Cilantro	0.40 (%v/v)	Eucalyptus	0.27 (%v/v)
Dill	0.47 (%v/v)	Coriander	0.23 (%v/v)
			(Olasupo et al. 2003)
Carvacrol	1.5 mmol/L	Eugenol	2.5 mmol/L
Cinnamic acid	5.0 mmol/L	Thymol	1.2 mmol/L

HURDLE CONCEPT

Food products can be inhibited by various means: these include reduction in water activity (aw), low temperature, reduction of pH, addition of competitive microorganisms and addition of preservatives. Combinations of these various means can be used. The use of combinations is called "hurdle effect" (Shalini & Singh 2014). Using an intelligent mix of hurdles it is possible to improve not only the microbial stability and safety but also the sensory and nutritional quality as well as the economic aspects of a food. For the latter it is important that the water content in the product be compatible with its microbial stability, and if an increased water content (aw) is compensated for by other hurdles (e.g., pH, Eh) the food will usually become more economical to manufacture (Leistner & Gould 2002).

A synergic effect on bacterial inactivation has been observed when HHP combined with some antimicrobials, such as bacteriocins, nisin and lysozyme (Garriga et al. 2002; Hauben et al. 1996; Ananou et al. 2010; Jofré, Aymerich, et al. 2008; de Alba et al. 2013; Jofré, Garriga, et al. 2008; Abriouel et al. 2014; Marcos et al. 2008; Pérez Pulido et al. 2012; Vercammen et al. 2011). Also, there was a synergy between high pressure and salt or nitrite has been reported. Because high pressure can limit the addition of salt and nitrite while ensuring extended shelf life, it is promising for the manufacture of meat products (Duranton et al. 2012). Furthermore there has been some researches investigating the synergistic effect of HHP and essential oils or herb's extract as natural antimicrobials on different microorganisms in a variety of food systems. For example thyme oil and rosemary oil in combination with HHP (400 MPa, 5 min) significantly ($p<0.05$) reduced the

concentrations of aerobic mesophilic bacteria in cracked table olives (Pina-Pérez et al. 2009), the stability of cold-smoked sardine muscle was improved by coating the muscle with functional gelatin-based edible films enriched with an oregano or a rosemary extract and they were able to slow lipid oxidation, but they failed to slow microbial growth. Gelatin–chitosan films were most effective at reducing microbial growth (Gomez-Estaca et al. 2007). It has been reported that the combination of HP with the addition of soy sauce and/or olive oil is an effective technology that can improve chemical, health, sensory qualities and safety of chicken breast (Kruk et al. 2014). High pressure (600 MPa) was applied on olive oil and grape seed oil (10% of meat weight) added beef loin and it is indicated that the addition of vegetable oil followed by the application of HP enhances the safety of beef loin [37]. Another research has demonstrated that the combination of antimicrobial food ingredients (Ethanol extracted from garlic, leeks, onions, and ginger powder) and HP treatment might help improve the efficiency of sterilization in meat systems (S. Jung et al. 2012). The effect of HHP treatment against *S. aureus* was enhanced significantly by cinnamon and clove oils in rice pudding. Viable counts obtained for the combined treatments of HHP and cinnamon oil or clove oil were significantly lower ($P < 0.05$) by 1.3 and 1.8 log cycles, respectively, compared to the single HHP treatment (Pérez Pulido et al. 2012).

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CHAPTER III

SYNERGISTIC EFFECT OF HIGH PRESSURE PROCESSING AND ESSENTIAL OILS ON INACTIVATION OF *E. COLI* ATCC 25922

Fatma Sahmurat[a], P. Kumar Mallikarjunan[a*] Hande Kaya-Celiker[a]

Robert Williams[b]

[a]Biological Systems Engineering, [b]Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA (sahmurat@vt.edu, [*]kumar@vt.edu, hande@vt.edu, rcwilli3@vt.edu)

**: Correspondence should be sent to:*
Kumar Mallikarjunan, 205 Seitz Hall, Virginia Tech, Blacksburg, VA 24061
Phone: (540) 231-7937 Fax: (540) 231-3199 Email: kumar@vt.edu

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ABSTRACT

The individual and combined effects of antimicrobial activity of basil (*Ocimum basilicum*), black cumin (*Nigella sativa*), cilantro (*Coriandrum sativum L.*), cumin (*Cuminum cyminum*), fenugreek (*Trigonella foenumgraecum*), ginger (*Zingiber officinale*), oregano (*Origanum vulgare*), black pepper (*Piper nigrum*), rosemary (Rosmarinus officinalis), thyme (*Thymus vulgaris*), turmeric (*Curcuma longa*) emulsions (EOs) on *E. coli* ATCC 25922 with and without high hydrostatic pressure (HHP) treatment were evaluated. The minimum inhibitory concentration (MIC) of EOs against *E. coli* ATCC 25922 were determined by broth macro dilution method. Only basil, cilantro, cumin, oregano and thyme EOs were found effective against *E. coli* ATCC 25922 and their MIC values were 2500, 5000, 1250, 625 and 1250 ppm respectively without any HHP treatment. Three of selected EOs with the lowest MIC values (cumin, oregano and thyme essential oils) were further treated with HHP (at 400 MPa, 20 °C for 10 min) and MIC values were evaluated as 625 ppm, 156.25 ppm and 625 ppm, respectively.

The in-vitro synergy of three selected EOs when combined against *E. coli* ATCC 25922 was determined using the checkerboard method to obtain a fractional inhibitory concentration index (FICI). FIC index was used to interpret the test results as follows: synergism, ≤0.8; additive or indifference, >0.8–4; and antagonism, >4. All the possible combinations tested showed (TO, CO, TC, TOC) synergistic interactions for HHP treated samples (FICITO=0.64, FICICO=0.38, FICITC=0.63, FICITOC=0.64). However none of the untreated combinations revealed any synergy but additive. The FICI values for untreated TO, CO, TC, TOC combinations are 1.12, 1, 1.12, and 1 respectively. These results suggest that combination of HHP processing and essential oils

addition as natural antimicrobials have potential to be hurdle technology that enhances each other effectiveness against the pathogens.

INTRODUCTION

The most common pasteurization method to inactivate pathogenic microorganisms and deteriorative enzymes to prevent spoilage of food is thermal processing. However, high temperatures in thermal processes results in nutrient degradation, textural and organoleptic changes, which indeed are unwanted changes for fresh products. In last couple of decades, consumer demands were motivated towards for convenient, healthy, minimally processed and safe food products which guided food industry to find non-thermal, natural preservation methods capable of inactivating microorganisms and enzymes while keeping the freshness of food. High pressure processing (HHP) is a non-thermal mild food preservation technology for inactivating contaminants, such as yeasts, molds and most vegetative bacteria including most spoilage and pathogenic bacteria by short time application of high pressures (100 to 1000 MPa) usually at relatively low temperatures (San Martín et al. 2002; Guerrero-Beltrán et al. 2005).

In HHP treatment, food product is exposed to an instant and uniform transmission of the pressure independent of the volume of the product, thus pressure is said to be "isostatic" (Rastogi 2013; Rendueles et al. 2011; Hugas et al. 2002). Isostatic gas pressure is maintained in a high pressure containment vessel and penetrates into the food product without any further need for energy input, which provides an energy efficient process. Pressures of between 100 and 600MPa are generally applied at low or mild temperatures. HHP works according to Le Chatelier principle and at relatively low temperatures, covalent bonds are almost unaffected by the pressure. The hydrophobic and ionic interactions are affected primarily by pressure which alters the tertiary and quaternary structures of molecules (Balasubramaniam et al. 2016; Campus 2010; Tao et al. 2012).

Microbial inactivation is one of the main concerns in food preservation to improve the microbial safety of food products. Many reports have demonstrated the inactivation mechanism of HHP on microorganisms which targets the cell membrane. It is generally accepted that HHP destroys or dissociates the functionality of the cell wall and the cytoplasmic membrane, which leads to leakage of the intracellular constituents through the destabilized cell membrane (Mañas & Mackey 2004). However it is not enough for total destabilization of cell which leads to sub-lethal injury on microorganisms, even at lower pressures than those required for their death (Somolinos et al. 2008). Previous studies indicated that in case of gram negative bacteria, the destabilized membrane was rapidly restored after pressure release (Hauben et al. 1996). On the other hand, sub-lethally injured cells by pressure become more susceptible to antimicrobial substances and combined effect of applied pressure and antimicrobial agents on destabilization of cell membrane and its function can lead higher levels of inactivation (Somolinos et al. 2008; Oliveira et al. 2015). The permeability of cell membrane in injured cells is increased and this allows facilitated contact between antimicrobials and cell membrane. There are several studies showing the synergistic effect of HHP and bacteriocins in literature (Garriga et al. 2002; Kalchayanand et al. 2003), such as nisin (Yuste et al. 2002; Qi et al. 2010), nisin and lysozyme (Hauben et al. 1996; Masschalck 2000), enterocins A and B, sakacin K, pediocin AcH and nisin (Garriga et al. 2002), nisin and pediocin (Turgis et al. 2012) and enterocins and lactate–diacetate enterocins (Marcos et al. 2008).

High pressure processing does not necessarily change the taste, flavor or nutrient content of foods. There are many successful applications of HHP treatment on food products including fruit juices, hummus, dips, jams, guacamole, oysters, or ready-to-eat meat products (Lau & Turek, 2007). A high acid jam is the first commercial HHP treated product produced by Japanese company

Medi-Ya in the early 1990s (Koutchma & Koutchma 2014). As well, commercialized products of Orange juice (UltiFruit®, Pernod Ricard Company, France), avocado puree (Guacamole, Avomex Company, US), and sliced ham (Espuna Company, Spain) are on the market (Tewari et al., 1999). However, deteriorative problems associated with chemical constituents which lowers the sensorial and quality of food product, so as the consumer acceptance (Ortea et al. 2010).

Essential oils (EOs) are another alternative to observe a synergistic effect on spoilage bacteria when applied together with HHP. EOs can also provide a solution for quality losses associated with HHP treatment. EOs are extracted from plant material and considered as a secondary metabolites secreted as a defense mechanism against invading pathogens including insects, bacteria, fungi, and viruses (Hyldgaard et al. 2012; Lai & Roy 2004). The first study about the antimicrobial activity of EOs dates back to 1881, reported by De La Croix (Bassolé & Juliani 2012). Among 3000 known EOs, 300 of them are commercially available and used in pharmaceutical, argonomic, food, sanitary, cosmetic and parfume industries (Bakkali et al. 2008). As being natural plant originated antimicrobial agents, EOs have been very popular for food preservation in recent years. Consumer demand is increasing for safe food products and public health concerns about food-borne diseases are growing, increasing the need for more effective preservation strategies (Hyldgaard et. al., 2012). However their use in food preservation remains limited due to their strong aroma (Sánchez-González et al. 2011). Another main problem for using EOs is that they are often not potent enough, when applied alone, to provide an antimicrobial effect for food preservation. Synergistic strategies have been proposed to exploit the effectiveness, however, little is known about the mechanism for synergistic, additive, or antagonistic effects when blends of EOs are used (Hyldgaard et. al., 2012). There are studies in literature demonstrating the synergistic combinations of HHP and constituents

of crude EOs in preservation of food products (Espina et al. 2013) or synergistic effect of combined treatment on microbial reduction (Karatzas et al. 2001; Palhano et al. 2004).

In the present study we have investigated the antibacterial effects of certain essential oils, including basil (*Ocimum basilicum*), black cumin (*Nigella sativa*), cilantro (*Coriandrum sativum L.*), cumin (*Cuminum cyminum*), fenugreek (*Trigonella foenumgraecum*), ginger (*Zingiber officinale*), oregano (*Origanum vulgare*), black pepper (*Piper nigrum*), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), turmeric (*Curcuma longa*) emulsions (EOs) alone against *E.coli* ATCC 25922 and evaluated the synergistic effect of dual and triple combinations of cumin (*Cuminum cyminum*), oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) with and without HHP treatment.

MATERIAL AND METHODS

Essential oils. The essential oils used in this study were basil (Ocimum basilicum), black cumin (Nigella sativa), cilantro (Coriandrum sativum L.), cumin (Cuminum cyminum), fenugreek (*Trigonella foenumgraecum*), ginger (*Zingiber officinale*), oregano (*Origanum vulgare*), black pepper (*Piper nigrum*), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), turmeric (*Curcuma longa*). The oils were obtained from Eden Botanical (Petaluma, CA). According the certificates of analysis of EOs obtained from the company, rosemary, ginger, fenugreek EOs extracted by super critical CO_2 and the others by steam distillation (See Appendix III).

Test strains and cultures. The strain used in this work was *E. coli* ATCC 25922. The *E. coli* ATCC 25922 was obtained as Culti-loop™ from Thermo Scientific™ (Lenexa, KS). The loop was placed on tryptic soy agar (TSA, BD Difco, Detroit, Michigan) and incubated at 37±1 °C for 24 h.

The grown cultures were stored at 4°C and some were sub-cultured and maintained in tryptic soy broth (TSB, BD Difco, Detroit, Michigan).

Essential oil nano-emulsions. Stock nano-emulsions of EOs were prepared according to the method reported by (Ghosh et al. 2012; Sugumar & Singh 2016) as 50000 ppm oil-in-water using Tween-80 (Sigma, Saint Louis, MO, USA) as the emulsifying agent. Oil and Tween-80 were mixed at a ratio of 1:1 in an sterile aqueous phase, to give a final oil concentration of 50000 ppm. The coarse emulsion was initially mixed with a magnetic stirrer for two minutes under room temperature and sonicated using 20 kHz sonicator with maximum power output of 500 kW (Qsonica Sonicator Q500, Fisher Scientific, USA). Emulsions were sonicated at ambient temperature for 15 min with % 60 amplitude, where, each cycle consisted of 30 s pulses on and 1 s pulses off. In order to control temperature-rise in the emulsions, during the sonication emulsions hold in ice. Those stock emulsions stored in amber glass bottles at 4 ^{o}C at most 3 weeks and before each use they were sonicated for additional one minute.

Determination of minimum inhibitory concentration (MIC). The MIC for the oil emulsions was determined by modified broth macro dilution method according to the National Committee of Clinical Laboratories Standards (NCCLS) guidelines (American Society for Microbiology 2005). Two-fold serial dilution of all EOs (10000-78 ppm) were prepared in tubes containing Mueller Hinton Broth (MHB, Sigma Aldrich, Saint Louis, MO, USA) and inoculated with bacterial suspensions were adjusted to the 0.5 McFarland's standard (10^8 CFU/ml) as giving a final bacterial concentration of $1x10^7$ cfu/ml. Their final volume was set as 20 ml. For each concentration level negative controls (without microorganism) were prepared as well. All the tubes

were incubated at 37ºC for 24 h. MIC was determined as the lowest concentration showing no visible signs of growth. It is supported by the difference of absorbency of each samples at 600 nm by a spectrophotometer for t=0 and t=24h. All tubes centrifuged at 10000 rpm for 2 minutes and then re-suspended in 20 ml saline to eliminate any color and turbidity caused by EOs before absorbency reading. The negative controls of each sample were used for their own blank reading. All samples were tested in triplicate.

Determination of minimum bactericidal concentration (MBC). After 24 h incubation, 10 ml of samples, and their negative control samples were centrifuged at 10000 rpm for two minutes. The supernatant was discarded to remove the essential oil from the media and to eliminate its inhibition effect during the incubation. The pellet was re-suspended in 10 ml saline solution. Decimal dilutions of samples were prepared by transferring 100 µl of bacterial suspension in 900 µl of saline solutions. All dilutions mixed for 15 seconds by a vortex. 100 µl of each dilution was pipetted onto petri dishes containing plate count agar (PCA). Each dilution was spread on PCA plates using sterile cell spreader and incubated for 24 h at 37 °C. At the end of the incubation period, all of the petri plates containing between 30 and 300 colonies were selected and bacterial count calculated. The lowest concentration that demonstrates 99.9% reduction in CFU/ml of MHB was considered as the MBC value.

Determination of synergy between essential oil emulsions using checkerboard method. Two-dimensional and three dimensional checkerboard macro-dilution methods were used to determine synergy between cumin, thyme and oregano EOs' dual and triple combinations (Figure 1). The concentrations of the EOs used were started from their 2xMIC value and were serially

diluted in two-fold steps for two-dimensional test (7 level) and for three-dimensional test it started from their MIC values (4 level). The number of dilutions was decided based on the preliminary works. The final volume in each tube was 20 ml, comprised of 2 ml of *E. coli* ATCC 25922. Suspensions were adjusted to the 0.5 McFarland's standard (10^8 CFU/ml) as giving a final bacterial concentration of $1x10^7$ cfu/ml. Tubes were incubated at 37ºC for 24 hours. Then contaminated tubes and their negative controls checked visually to decide if there is a growth or not. The lowest total EOs concentration point, which did not show microbial growth, was picked to calculate FICI values.

The fractional inhibitory concentration (FIC) indices were calculated as $FIC_A + FIC_B$, $FIC_A + FIC_B + FIC_C$, where FIC_A , FIC_B and FIC_C are the respective MIC values of oil emulsions of A, B and C. Therefore FIC index was used to interpret the test results as follows: synergism, ≤0.8; indifference, >0.8–4; and antagonism, >4 (Stein et al. 2015). The fractional inhibitory concentration index (FICI) for each double (Eqn 3) or triple (Eqn 4) EO combination was calculated as follows:

$$\boldsymbol{FICI_{A/B}} = \frac{\boldsymbol{MIC_{A(combination)}}}{\boldsymbol{MIC_{A(alone)}}} + \frac{\boldsymbol{MIC_{B(combination)}}}{\boldsymbol{MIC_{B(alone)}}} \quad \textbf{(3)}$$

$$\boldsymbol{FICI_{A/B/C}} = \frac{\boldsymbol{MIC_{A(combination)}}}{\boldsymbol{MIC_{A(alone)}}} + \frac{\boldsymbol{MIC_{B(combination)}}}{\boldsymbol{MIC_{B(alone)}}} + \frac{\boldsymbol{MIC_{C(combination)}}}{\boldsymbol{MIC_{C(alone)}}} \quad \textbf{(4)}$$

High Pressure Processing. MIC, MBC and FIC indices were determined for samples treated with HHP. Nano-emulsions of selected EOs (Cumin, Thyme, Oregano) were blended with *E. coli* ATCC 25922 cultures in MHB for different EOs concentration ranging between 1250 and 19 ppm.

All media tubes were transferred into pre-sterilized (boiling in water for 15 min), 4mm, Nylon/Polyethylene heavy duty vacuum pouches (VACMASTER, Overland Park, KS, USA). Bags were sealed with a vacuum sealer (FoodSaver V3460) and sealed bags were immediately put in ice-chests and transferred to HHP unit.

High hydrostatic pressure treatments were performed at 400 MPa, 25° C for 10 min using a commercial scale high-pressure unit (Avure's Food Press QFP 35L-600, Columbus, OH). Water was used as the pressure-transmitting medium. Decompression occurred within 2-3 s. Pressure applied bags were unsealed under sterile conditions and broth medium were transferred to sterile test tubes, incubated at 37^{o}C for 24h and analyzed according to relevant procedures described earlier.

Statistical analysis. Independent samples t test was performed to compare absorbency values before and after incubation to prove microbial growth. IBM SPSS Statistic for Mac was used for this purpose. tests were performed within the confidence interval of 95% ($p<0.05$).

RESULTS AND DISCUSSIONS

MICs of EOs. The MIC and MBC values of EOs are demonstrated in Table 2. The EOs of black cumin (*Nigella sativa*), fenugreek (*Trigonella foenumgraecum*), ginger (*Zingiber officinale*), black pepper (*Piper nigrum*), rosemary (*Rosmarinus officinalis*), and turmeric (*Curcuma longa*) were resulted in microbial growth in TSB after 24h incubation at 37 °C, at their highest concentration of 10000 ppm. Microbial growth has also been seen in absorbency measurements that confirmed the findings. Thus, listed EOs were excluded from further study and MIC values

were accepted as being higher than 10000ppm. Similar results for black pepper and ginger were previously reported, as having a MIC value > 2.0 (% v/v) (>20000 ppm) (Hammer et al. 1999). Also Sahedeo and Vilas (2011) found black pepper ineffective against *Escherichia coli* (MTCC-119) and *Escherichia coli* (NCIM-2066). However, the MIC value of *Zingiber officinale* var. rubrum Theilade which is distributed mainly in Peninsular Malaysia, where it is known as halia bara, was found as 0.31 mg/mL (310 ppm) against *E. coli* ATCC 25922 (Sivasothy et al. 2011). *Nigella Sativa* EO was found ineffective on *E. coli* ATCC 25922 by Singh et al. (Singh et al. 2005) but Kokoska et al. reported that MIC values of *Nigella Sativa* EO, which were extracted by four different extraction methods, against *E. coli* ATCC 25922 in the range of <1024 and 256 μg/ml (1024 and 256 ppm). The MIC values of hydro-distilled and microwave extracted EO of rosemary (*Rosmarinus officinalis*) were determined as 7.5 and 1.88 mg/mL (7500 and 1880 ppm) respectively (Okoh et al. 2010). Because the composition of a particular essential oil may vary depending on the growing region, harvesting season and extraction method (Miguel 2010), previous research findings for MIC values of EOs have been inconsistent and contradictory. There is no MIC value study in the literature for fenugreek (*Trigonella foenumgraecum*), and turmeric (*Curcuma longa*) EOs against *E. coli*, up to our best knowledge. In overall, black cumin, fenugreek, ginger, black pepper, rosemary and turmeric EOs were considered as ineffective against *E. coli* ATCC 25922 in this research and they were excluded for future analyses.

The lowest concentrations of for cilantro (*Coriandrum sativum L.*) and basil (*Ocimum basilicum*) EOs which resulted in no visual growth were 2500 and 1250 ppm, respectively. However, absorbance measurement results showed statistically significant (p=0.05) difference between t=0 and t= 24 h incubated samples when negative control samples (only the EOs, no

microorganism) were used as blank. Therefore, MIC values for cilantro and basil EOs were decided as 5000ppm and 2500ppm. Shirazi et al. (2014) reported basil (*Ocimum basilicum*) EO's MIC value against *E. coli* PTCC 1330 [ATCC 8739] as 160 μg/ml (160 ppm) and Fei et al. (2011) reported this value as 1.25 μl/ml (1250 ppm) against *E. coli* ATCC 8739. There is no MIC value for cilantro (*Coriandrum sativum L.*) against *E. coli*, in the literature, up to our best knowledge.

The most effective EOs against *E. coli* ATCC 25922 were decided as cumin (*Cuminum cyminum*), oregano (*Origanum vulgare*), and thyme (*Thymus vulgaris*) EOs with 1250 ppm, 625 ppm, 1250 ppm MIC values, respectively. Similarly, the MIC value for cumin against *E. coli* ATCC 8739 and *E. coli* isolated from sausage were determined between 0.1-0.4 % (1000-4000 ppm) depending on the country of origin of cumin (Wanner 2010) . Thus far, the antimicrobial activity of oregano and thyme EOs has been studied widely because of their high antimicrobial activity. The MIC value of oregano EOs is in agreement with the one obtained by Burt and Reinders (2003). However Fournomiti et al. (2015) reported it as 219.9 mg/L (219.9 ppm) against *E. coli.* The MIC value of thyme (*Thymus vulgaris*) against *E. coli* was reported as 62.5 μg/mL (62.5 ppm) by Al-Bayati (2008) differs from the finding presented here.

The MIC values of cumin (*Cuminum cyminum*), oregano (*Origanum vulgare*), and thyme (*Thymus vulgaris*) EOs were evaluated in the combination of the HHP treatment at 400 MPa for 10 min and found that the MIC values of all EOs reduced by half, 625, 312.5 and 625 ppm, respectively after high pressure application against *E. coli* ATCC 25922.

MBCs of EOs. Since MBC can be equal or higher than MIC value, MBC was determined following MIC determination. The initial concentration of *E. coli* ATCC 25922 was $1x10^7$ CFU/ml

in all test tubes. Except for oregano, MBCs values of other EOs, which had determined MIC value, were equal to their MIC values. On the other hand the MBC value of Oregano was 2 times greater than its MIC value. Because the MBC:MIC ratio is less or equal to 4 for basil, cilantro, cumin, thyme and oregano EOs, they can be considered bactericidal against *E. coli ATCC* 25922. Their MBC for unpressurized samples, oregano, cumin and thyme were 1250, 1250 and 1250 ppm respectively. In the literature the MBC value of oregano was reported against *E.coli* ATCC 25922 and ATCC 25158 respectivel as 100 (De Martino et al. 2009) and 20000 ppm (Moreira et al. 2005). The MICs of cumin and thyme EOs against *E.coli* O157:H7 were found 3000 (Oroojalian et al. 2010) and 5000 (Selim 2011) ppm. With pressure treatment, oregano EO's MBC values decreased to 312.5 ppm and was equal to MIC.

Synergy between essential oil emulsions using checkerboard method. Three EOs, which were found as the most effective against *E. coli* ATCC 25922, oregano, cumin and thyme (O, C, and T) were tested in two and three dimensional checker board in combination, TO, CO, TC, TOC, with and without HHP treatment. Although none of untreated double EOs combinations revealed synergy, for pressurized combinations except TC displayed synergistic activity, giving FICI≤0.8. For untreated combinations, The FIC index values were found as $FICI_{TO}$=1.12, $FICI_{TC}$=1.12 and $FICI_{CO}$=0.75 (Table 3, Table 4 and Table 5). Only TO combinations evaluated before and it has been reported that they only had additive effect with the FICI of 1.17 (Gutierrez et al. 2008).

The triple combination of thyme, cumin and oregano EOs did not show any synergy as TO and TC combinations yet additive effect with the $FICI_{TOC}$=1 (Table 7). However, with HHP treatment a synergy was observed for the triple combinations, with FIC index value of $FICI_{TOC}$=

0.63 (Table 6). The lowest FICI was obtained for cumin and oregano combination with HHP treatment, $FICI_{CO}$=0.38 (Table 5) which can be concluded as the most effective synergistic combination. Oregano combinations with thyme and cumin for treated sample exhibit FICI of 0.62 and 0.38, respectively. It is fair to say that high pressure application resulted in increased cell wall permeability, so that cumin EO showed improved antimicrobial effect on *E. coli*. Oregano EOs is known to have an antimicrobial activity on membranes and walls of bacteria, either Gram negative or Gram positive. This is strongly revealed by electronic microscopy observations of damaged membrane integrity of bacteria and induced depletion of the intracellular ATP concentration (Rhayour et al. 2003; Oussalah et al. 2006). However, the antimicrobial activity mechanism of cumin EO is not studied extensively as oregano EO. Strong synergistic effect of oregano, cumin and HHP treatment indicates that, cumin became more effective after membrane got injured through high pressure application. Cytotoxic activity of cumin EOs might contribute to its antimicrobial effect (Tajkarimi & Ibrahim 2012).

Among the 11 essential oils investigated in the present study, only five of them showed antibacterial effect against study strain of *E. coli* ATCC 25922. Those EOs were basil, cilantro, cumin, thyme and oregano and based on their MIC values cumin, thyme and oregano EOs showed the strongest antibacterial activities towards *E. coli* ATCC 25922. Although the MBC value of oregano was 4 times greater than its MIC value, others' MBC and MIC value ratio was 1:1 and all five EOs can be considered bactericidal against *E. Coli* ATCC 25922 (Cutler et al. 1994).

HHP treatment enhances synergy between essential oils. The combinations, which did not display synergic effect without HHP, showed synergy once they pressurized. Only CO

combinations showed synergy among untreated combinations. However, the mode of action of HHP is not explained very well yet, most researchers think the key target is cell membrane.

For TO combinations both with and without HHP, no synergy observed. The essential oils of the oregano and thyme, whose major components are carvacrol and its isomer thymol (Burt et al. 2005) were reported as effective against strains of *E. coli* (Dorman & Deans 2000). It is concluded that they disintegrate the outer membrane of the gram-negative bacteria and cause releasing the lipopolysaccharide, so increase the permeability of the adenosine triphosphate in the cytoplasmic membrane. As a result they change the passive permeability of the cell (Rodriguez-Garcia et al. 2015). Therefore, HHP can trigger uptake of the antimicrobial compound into the cell and increase their antimicrobial activity. Because mainly thymol and carvacrol is also targeting the cell membrane the combination of TO did not reveal any synergy. On the other hand, with and without pressure treatment CO showed synergy. These results may show us once the cell injure, cumin penetrates into the cytoplasm and affects the vital metabolism. There was not any research found in the literature subjected to explain antimicrobial mechanism of cumin or its main component cuminaldehyde and it is needed to discover.

The evidence from this study suggests that HHP and EOs (alone or in combination) enhance each other's antimicrobial activation. Besides having better inhibition of pathogens, the limitations of their usage can be eliminated. Therefore, they have a great potential to use in hurdle concept to produce microbiologically safe and fresh likely food. Further studies regarding the mode of action of the combination of essential oils and high hydrostatic pressure would be worthwhile for better understanding.

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TABLES

Table 2 MIC and MBC values of EOs with or without high hydrostatic pressure treatment at 400 MPa for 10 min at 20 °C

	Untreated		HHP Treated	
	MIC	MBC	MIC	MBC
Cumin	1250	1250	625	625
Thyme	1250	1250	625	625
Oregano	625	1250	312.5	312.5
Basil	2500	2500	NA*	NA*
Cilantro	5000	5000	NA*	NA*
Black cumin	>10000	>10000	NA*	NA*
Black pepper	>10000	>10000	NA*	NA*
Fenugreek	>10000	>10000	NA*	NA*
Ginger	>10000	>10000	NA*	NA*
Rosemary	>10000	>10000	NA*	NA*
Turmeric	>10000	>10000	NA*	NA*

*The EOs which have high MIC value were eliminated for HHP treatment to reduce the sample size

Table 3 Checkerboard for oregano-thyme combination and FICI values. HHP treatment on left, untreated on right

		Thyme			
		625 ppm	312.5 ppm	156.25 ppm	78.125 ppm
Oregano	312.5 Ppm	FIC_T=1.00 FIC_O=1.00 FICI=2.00	FIC_T=0.50 FIC_O=1.00 FICI=1.50	FIC_T=0.25 FIC_O=1.00 FICI=1.25	FIC_T=0.12 FIC_O=1.00 FICI=1.12
	156.25 Ppm	FIC_T=1.00 FIC_O=0.50 FICI=1.50	FIC_T=0.50 FIC_O=0.50 FICI=1.00	FIC_T=0.25 FIC_O=0.50 FICI=0.75	FIC_T=0.12 FIC_O=0.50 FICI=0.62
	78.125 Ppm	FIC_T=1.00 FIC_O=0.25 FICI=1.25	FIC_T=0.50 FIC_O=0.25 FICI=0.75	FIC_T=0.25 FIC_O=0.25 FICI=0.50	FIC_T=0.12 FIC_O=0.25 FICI=0.38
	39.06 Ppm	FIC_T=1.00 FIC_O=0.12 FICI=1.12	**FIC_T=0.50** **FIC_O=0.12** **FICI=0.62**	FIC_T=0.25 FIC_O=0.12 FICI=0.38	FIC_T=0.12 FIC_O=0.12 FICI=0.25

		Thyme			
		1250 ppm	625 ppm	312.5 ppm	156.25 ppm
Oregano	625 ppm	FIC_C=1.00 FIC_O=1.00 FICI=2.00	FIC_C =0.50 FIC_O=1.00 FICI=1.50	FIC_C =0.25 FIC_O=1.00 FICI=1.25	**FIC_C =0.12** **FIC_O=1.00** **FICI=1.12**
	312.5 Ppm	FIC_C =1.00 FIC_O=0.50 FICI=1.50	FIC_C =0.50 FIC_O=0.50 FICI=1.00	FIC_C =0.25 FIC_O=0.50 FICI=0.75	FIC_C =0.12 FIC_O=0.50 FICI=0.62
	156.25 Ppm	FIC_C =1.00 FIC_O=0.25 FICI=1.25	FIC_C =0.50 FIC_O=0.25 FICI=0.75	FIC_C 0.25 FIC_O=0.25 FICI=0.50	FIC_C =0.12 FIC_O=0.25 FICI=0.38
	78.125 Ppm	**FIC_C =1.00** **FIC_O=0.12** **FICI=1.12**	FIC_C =0.50 FIC_O=0.12 FICI=0.62	FIC_C =0.25 FIC_O=0.12 FICI=0.38	FIC_C =0.12 FIC_O=0.12 FICI=0.25

- Grey areas are representing microbial growth observed samples

Table 4 Checkerboard for Thyme-cumin combination and FICI values. HHP treatment on left, untreated on right

		Thyme			
		625 ppm	312.5 ppm	156.25 ppm	78.125 ppm
Cumin	625 Ppm	FIC_C=1.00 FIC_O=1.00 FICI=2.00	FIC_C =0.50 FIC_O=1.00 FICI=1.50	FIC_C =0.25 FIC_O=1.00 FICI=1.25	FIC_C =0.12 FIC_O=1.00 FICI=1.12
	312.5 Ppm	FIC_C =1.00 FIC_O=0.50 FICI=1.50	**FIC_C =0.50** **FIC_O=0.50** **FICI=1.00**	FIC_C =0.25 FIC_O=0.50 FICI=0.75	FIC_C =0.12 FIC_O=0.50 FICI=0.62
	156.25 Ppm	FIC_C =1.00 FIC_O=0.25 FICI=1.25	FIC_C =0.50 FIC_O=0.25 FICI=0.75	FIC_C 0.25 FIC_O=0.25 FICI=0.50	FIC_C =0.12 FIC_O=0.25 FICI=0.38
	78.125 Ppm	FIC_C =1.00 FIC_O=0.12 FICI=1.12	FIC_C =0.50 FIC_O=0.12 FICI=0.62	FIC_C =0.25 FIC_O=0.12 FICI=0.38	FIC_C =0.12 FIC_O=0.12 FICI=0.25

		Thyme			
		625 ppm	312.5 ppm	156.25 ppm	78.125 ppm
Cumin	1250 Ppm	FIC_C=1.00 FIC_O=1.00 FICI=2.00	FIC_C =0.50 FIC_O=1.00 FICI=1.50	FIC_C =0.25 FIC_O=1.00 FICI=1.25	**FIC_C =0.12** **FIC_O=1.00** **FICI=1.12**
	625 Ppm	FIC_C =1.00 FIC_O=0.50 FICI=1.50	FIC_C =0.50 FIC_O=0.50 FICI=1.00	FIC_C =0.25 FIC_O=0.50 FICI=0.75	FIC_C =0.12 FIC_O=0.50 FICI=0.62
	312.5 Ppm	FIC_C =1.00 FIC_O=0.25 FICI=1.25	FIC_C =0.50 FIC_O=0.25 FICI=0.75	FIC_C 0.25 FIC_O=0.25 FICI=0.50	FIC_C =0.12 FIC_O=0.25 FICI=0.38
	156.25 Ppm	**FIC_C =1.00** **FIC_O=0.12** **FICI=1.12**	FIC_C =0.50 FIC_O=0.12 FICI=0.62	FIC_C =0.25 FIC_O=0.12 FICI=0.38	FIC_C =0.12 FIC_O=0.12 FICI=0.25

Table 5 Checkerboard for oregano-cumin combination and FICI values. HHP treatment on left, untreated on right

		Cumin			
		625 ppm	312.5 ppm	156.25 ppm	78.125 ppm
Oregano	312.5 Ppm	FIC_C=1.00 FIC_O=1.00 FICI=2.00	FIC_C =0.50 FIC_O=1.00 FICI=1.50	FIC_C =0.25 FIC_O=1.00 FICI=1.25	FIC_C =0.12 FIC_O=1.00 FICI=1.12
	156.25 Ppm	FIC_C =1.00 FIC_O=0.50 FICI=1.50	FIC_C =0.50 FIC_O=0.50 FICI=1.00	FIC_C =0.25 FIC_O=0.50 FICI=0.75	FIC_C =0.12 FIC_O=0.50 FICI=0.62
	78.125 Ppm	FIC_C =1.00 FIC_O=0.25 FICI=1.25	FIC_C =0.50 FIC_O=0.25 FICI=0.75	FIC_C 0.25 FIC_O=0.25 FICI=0.50	FIC_C =0.12 FIC_O=0.25 FICI=0.38
	39.06 Ppm	FIC_C =1.00 FIC_O=0.12 FICI=1.12	FIC_C =0.50 FIC_O=0.12 FICI=0.62	**FIC_C =0.25** **FIC_O=0.12** **FICI=0.38**	FIC_C =0.12 FIC_O=0.12 FICI=0.25

		Cumin			
		625 ppm	312.5 ppm	156.25 ppm	78.125 ppm
Oregano	625 ppm	FIC_C=1.00 FIC_O=1.00 FICI=2.00	FIC_C =0.50 FIC_O=1.00 FICI=1.50	FIC_C =0.25 FIC_O=1.00 FICI=1.25	FIC_C =0.12 FIC_O=1.00 FICI=1.12
	312.5 Ppm	FIC_C =1.00 FIC_O=0.50 FICI=1.50	FIC_C =0.50 FIC_O=0.50 FICI=1.00	FIC_C =0.25 FIC_O=0.50 FICI=0.75	FIC_C =0.12 FIC_O=0.50 FICI=0.62
	156.25 Ppm	FIC_C =1.00 FIC_O=0.25 FICI=1.25	**FIC_C =0.50** **FIC_O=0.25** **FICI=0.75**	FIC_C 0.25 FIC_O=0.25 FICI=0.50	FIC_C =0.12 FIC_O=0.25 FICI=0.38
	78.125 Ppm	FIC_C =1.00 FIC_O=0.12 FICI=1.12	FIC_C =0.50 FIC_O=0.12 FICI=0.62	FIC_C =0.25 FIC_O=0.12 FICI=0.38	FIC_C =0.12 FIC_O=0.12 FICI=0.25

Table 6 FICI table for Cumin-Oregano-Thyme Combination with HHP treatment

Cumin=625 ppm

Oregano	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm	Thyme: 78.125 ppm
312.5 Ppm	FIC_C=1 FIC_T=1 FIC_O=1 FICI=3	FIC_C=1 FIC_T=0.5 FIC_O=1 FICI=2.5	FIC_C=1 FIC_T=0.25 FIC_O=1 FICI=2.25	FIC_C=1 FIC_T=0.125 FIC_O=1 FICI=2.125
156.25 Ppm	FIC_C=1 FIC_T=1 FIC_O=0.5 FICI=2.5	FIC_C=1 FIC_T=0.5 FIC_O=0.5 FICI=2	FIC_C=1 FIC_T=0.25 FIC_O=0.5 FICI=1.75	FIC_C=1 FIC_T=0.125 FIC_O=0.5 FICI=1.625
78.125 Ppm	FIC_C=1 FIC_T=1 FIC_O=0.25 FICI=2.25	FIC_C=1 FIC_T=0.5 FIC_O=0.25 FICI=1.75	FIC_C=1 FIC_T=0.25 FIC_O=0.25 FICI=1.5	FIC_C=1 FIC_T=0.125 FIC_O=0.25 FICI=1.375
39.06 Ppm	FIC_C=1 FIC_T=1 FIC_O=0.125 FICI=2.125	FIC_C=1 FIC_T=0.5 FIC_O=0.125 FICI=1.625	FIC_C=1 FIC_T=0.25 FIC_O=0.125 FICI=1.375	FIC_C=1 FIC_T=0.125 FIC_O=0.125 FICI=1.25

Cumin=312.5 ppm

	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm	Thyme: 78.125 ppm	
312.5 Ppm	FIC_C=0.5 FIC_T=1 FIC_O=1 FICI=2.5	FIC_C=0.5 FIC_T=0.5 FIC_O=1 FICI=2	FIC_C=0.5 FIC_T=0.25 FIC_O=1 FICI=1.75	FIC_C=0.5 FIC_T=0.125 FIC_O=1 FICI=1.625	
156.25 Ppm	FIC_C=0.5 FIC_T=1 FIC_O=0.5 FICI=2	FIC_C=0.5 FIC_T=0.5 FIC_O=0.5 FICI=1.5	FIC_C=0.5 FIC_T=0.25 FIC_O=0.5 FICI=1.25	FIC_C=0.5 FIC_T=0.125 FIC_O=0.5 FICI=1.125	
78.125 Ppm	FIC_C=0.5 FIC_T=1 FIC_O=0.25 FICI=1.75	FIC_C=0.5 FIC_T=0.5 FIC_O=0.25 FICI=1.25	FIC_C=0.5 FIC_T=0.25 FIC_O=0.25 FICI=1	FIC_C=0.5 FIC_T=0.125 FIC_O=0.25 FICI=0.875	
39.06 Ppm	FIC_C=0.5 FIC_T=1 FIC_O=0.125 FICI=1.625	FIC_C=0.5 FIC_T=0.5 FIC_O=0.125 FICI=1.125	FIC_C=0.5 FIC_T=0.25 FIC_O=0.125 FICI=0.875	FIC_C=0.5 FIC_T=0.125 FIC_O=0.125 FICI=0.75	Oregano

Table 6 FICI table for Cumin-Oregano-Thyme Combination with HHP treatment (Continued)

Cumin=156.25 ppm

Oregano	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm	Thyme: 78.125 ppm
312.5 Ppm	FIC_C=0.25 FIC_T=1 FIC_O=1 FICI=2.25	FIC_C=0.25 FIC_T=0.5 FIC_O=1 FICI=1.75	FIC_C=0.25 FIC_T=0.25 FIC_O=1 FICI=1.5	FIC_C=0.25 FIC_T=0.125 FIC_O=1 FICI=1.375
156.25 Ppm	FIC_C=0.25 FIC_T=1 FIC_O=0.5 FICI=1.75	FIC_C=0.25 FIC_T=0.5 FIC_O=0.5 FICI=1.25	FIC_C=0.25 FIC_T=0.25 FIC_O=0.5 FICI=1	FIC_C=0.25 FIC_T=0.125 FIC_O=0.5 FICI=0.875
78.125 Ppm	FIC_C=0.25 FIC_T=1 FIC_O=0.25 FICI=1.5	FIC_C=0.25 FIC_T=0.5 FIC_O=0.25 FICI=1	FIC_C=0.25 FIC_T=0.25 FIC_O=0.25 FICI=0.75	**FIC_C=0.25** **FIC_T=0.125** **FIC_O=0.25** **FICI=0.625**
39.06 Ppm	FIC_C=0.25 FIC_T=1 FIC_O=0.125 FICI=1.375	FIC_C=0.25 FIC_T=0.5 FIC_O=0.125 FICI=0.875	**FIC_C=0.25** **FIC_T=0.25** **FIC_O=0.125** **FICI=0.625**	FIC_C=0.25 FIC_T=0.125 FIC_O=0.125 FICI=0.5

Cumin=78.125 ppm

Oregano	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm	Thyme: 78.125 ppm
312.5 Ppm	FIC_C=0.125 FIC_T=1 FIC_O=1 FICI=2.125	FIC_C=0.125 FIC_T=0.5 FIC_O=1 FICI=1.625	FIC_C=0.125 FIC_T=0.25 FIC_O=1 FICI=1.375	FIC_C=0.125 FIC_T=0.125 FIC_O=1 FICI=1.25
156.25 Ppm	FIC_C=0.125 FIC_T=1 FIC_O=0.5 FICI=1.625	FIC_C=0.125 FIC_T=0.5 FIC_O=0.5 FICI=1.125	FIC_C=0.125 FIC_T=0.25 FIC_O=0.5 FICI=0.875	**FIC_C=0.125** **FIC_T=0.125** **FIC_O=0.5** **FICI=0.75**
78.125 Ppm	FIC_C=0.125 FIC_T=1 FIC_O=0.25 FICI=1.375	FIC_C=0.125 FIC_T=0.5 FIC_O=0.25 FICI=0.875	FIC_C=0.125 FIC_T=0.25 FIC_O=0.25 FICI=0.625	FIC_C=0.125 FIC_T=0.125 FIC_O=0.25 FICI=0.5
39.06 Ppm	FIC_C=0.125 FIC_T=1 FIC_O=0.125 FICI=1.25	**FIC_C=0.125** **FIC_T=0.5** **FIC_O=0.125** **FICI=0.75**	FIC_C=0.125 FIC_T=0.25 FIC_O=0.125 FICI=0.5	FIC_C=0.125 FIC_T=0.125 FIC_O=0.125 FICI=0.375

Table 7 FICI table for Cumin-Oregano-Thyme Combination without HHP treatment

Cumin=1250 ppm

Oregano	Thyme: 1250 ppm	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm
625 ppm	FIC_C=1 FIC_T=1 FIC_O=1 FICI=3	FIC_C=1 FIC_T=0.5 FIC_O=1 FICI=2.5	FIC_C=1 FIC_T=0.25 FIC_O=1 FICI=2.25	FIC_C=1 FIC_T=0.125 FIC_O=1 FICI=2.125
312.5 Ppm	FIC_C=1 FIC_T=1 FIC_O=0.5 FICI=2.5	FIC_C=1 FIC_T=0.5 FIC_O=0.5 FICI=2	FIC_C=1 FIC_T=0.25 FIC_O=0.5 FICI=1.75	FIC_C=1 FIC_T=0.125 FIC_O=0.5 FICI=1.625
156.25 Ppm	FIC_C=1 FIC_T=1 FIC_O=0.25 FICI=2.25	FIC_C=1 FIC_T=0.5 FIC_O=0.25 FICI=1.75	FIC_C=1 FIC_T=0.25 FIC_O=0.25 FICI=1.5	FIC_C=1 FIC_T=0.125 FIC_O=0.25 FICI=1.375
78.125 Ppm	FIC_C=1 FIC_T=1 FIC_O=0.125 FICI=2.125	FIC_C=1 FIC_T=0.5 FIC_O=0.125 FICI=1.625	FIC_C=1 FIC_T=0.25 FIC_O=0.125 FICI=1.375	FIC_C=1 FIC_T=0.125 FIC_O=0.125 FICI=1.25

Cumin=625 ppm

Oregano	Thyme: 1250 ppm	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm
625 ppm	FIC_C=0.5 FIC_T=1 FIC_O=1 FICI=2.5	FIC_C=0.5 FIC_T=0.5 FIC_O=1 FICI=2	FIC_C=0.5 FIC_T=0.25 FIC_O=1 FICI=1.75	FIC_C=0.5 FIC_T=0.125 FIC_O=1 FICI=1.625
312.5 Ppm	FIC_C=0.5 FIC_T=1 FIC_O=0.5 FICI=2	FIC_C=0.5 FIC_T=0.5 FIC_O=0.5 FICI=1.5	FIC_C=0.5 FIC_T=0.25 FIC_O=0.5 FICI=1.25	FIC_C=0.5 FIC_T=0.125 FIC_O=0.5 FICI=1.125
156.25 Ppm	FIC_C=0.5 FIC_T=1 FIC_O=0.25 FICI=1.75	FIC_C=0.5 FIC_T=0.5 FIC_O=0.25 FICI=1.25	FIC_C=0.5 FIC_T=0.25 FIC_O=0.25 FICI=1	FIC_C=0.5 FIC_T=0.125 FIC_O=0.25 FICI=0.875
78.125 Ppm	FIC_C=0.5 FIC_T=1 FIC_O=0.125 FICI=1.625	FIC_C=0.5 FIC_T=0.5 FIC_O=0.125 FICI=1.125	FIC_C=0.5 FIC_T=0.25 FIC_O=0.125 FICI=0.875	FIC_C=0.5 FIC_T=0.125 FIC_O=0.125 FICI=0.75

Table 7 FICI table for Cumin-Oregano-Thyme Combination without HHP treatment (Continued)

Cumin=1250 ppm

Oregano	Thyme: 1250 ppm	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm
625 ppm	FIC_C=0.25 FIC_T=1 FIC_O=1 FICI=2.25	FIC_C=0.25 FIC_T=0.5 FIC_O=1 FICI=1.75	FIC_C=0.25 FIC_T=0.25 FIC_O=1 FICI=1.5	FIC_C=0.25 FIC_T=0.125 FIC_O=1 FICI=1.375
312.5 Ppm	FIC_C=0.25 FIC_T=1 FIC_O=0.5 FICI=1.75	FIC_C=0.25 FIC_T=0.5 FIC_O=0.5 FICI=1.25	FIC_C=0.25 FIC_T=0.25 FIC_O=0.5 FICI=1	FIC_C=0.25 FIC_T=0.125 FIC_O=0.5 FICI=0.875
156.25 Ppm	FIC_C=0.25 FIC_T=1 FIC_O=0.25 FICI=1.5	**FIC_C=0.25** **FIC_T=0.5** **FIC_O=0.25** **FICI=1**	FIC_C=0.25 FIC_T=0.25 FIC_O=0.25 FICI=0.75	FIC_C=0.25 FIC_T=0.125 FIC_O=0.25 FICI=0.625
78.125 Ppm	FIC_C=0.25 FIC_T=1 FIC_O=0.125 FICI=1.375	FIC_C=0.25 FIC_T=0.5 FIC_O=0.125 FICI=0.875	FIC_C=0.25 FIC_T=0.25 FIC_O=0.125 FICI=0.625	FIC_C=0.25 FIC_T=0.125 FIC_O=0.125 FICI=0.5

Cumin=625 ppm

Thyme: 1250 ppm	Thyme: 625 ppm	Thyme: 312.5 ppm	Thyme: 156.25 ppm	Oregano
625 ppm	FIC_C=0.125 FIC_T=1 FIC_O=1 FICI=2.125	FIC_C=0.125 FIC_T=0.5 FIC_O=1 FICI=1.625	FIC_C=0.125 FIC_T=0.25 FIC_O=1 FICI=1.375	FIC_C=0.125 FIC_T=0.125 FIC_O=1 FICI=1.25
312.5 Ppm	FIC_C=0.125 FIC_T=1 FIC_O=0.5 FICI=1.625	FIC_C=0.125 FIC_T=0.5 FIC_O=0.5 FICI=1.125	FIC_C=0.125 FIC_T=0.25 FIC_O=0.5 FICI=0.875	FIC_C=0.125 FIC_T=0.125 FIC_O=0.5 FICI=0.75
156.25 Ppm	FIC_C=0.125 FIC_T=1 FIC_O=0.25 FICI=1.375	FIC_C=0.125 FIC_T=0.5 FIC_O=0.25 FICI=0.875	FIC_C=0.125 FIC_T=0.25 FIC_O=0.25 FICI=0.625	FIC_C=0.125 FIC_T=0.125 FIC_O=0.25 FICI=0.5
78.125 Ppm	FIC_C=0.125 FIC_T=1 FIC_O=0.125 FICI=1.25	FIC_C=0.125 FIC_T=0.5 FIC_O=0.125 FICI=0.75	FIC_C=0.125 FIC_T=0.25 FIC_O=0.125 FICI=0.5	FIC_C=0.125 FIC_T=0.125 FIC_O=0.125 FICI=0.375

CHAPTER IV

SYNERGISTIC EFFECT OF HIGH PRESSURE PROCESSING AND ESSENTIAL OILS ON INACTIVATION OF *E. COLI* ATCC 25922 ON BEEF SAMPLE

Fatma Sahmurat[a], P. Kumar Mallikarjunan[a*] Robert Williams[b], Hande Kaya-Celiker[a]

[a] *Biological Systems Engineering, Virginia Polytechnic Institute and State University,*

[b] *Biological Systems Engineering, Virginia Polytechnic Institute and State University,*

*Blacksburg, VA, USA (sahmurat@vt.edu, *kumar@vt.edu, hande@vt.edu, rowilli3@vt.edu)*

**: Correspondence should be sent to:*
Kumar Mallikarjunan, 205 Seitz Hall, Virginia Tech, Blacksburg, VA 24061
Phone: (540) 231-7937 Fax: (540) 231-3199 Email: kumar@vt.edu

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ABSTRACT

Beef cuts, inoculated with *E. coli* ATCC 25922 having a final bacterial concentration of $1x10^7$ cfu/g sample were prepared and subjected to high hydrostatic pressure (HHP) and essential oils (EOs) of cumin seed (*Cuminum cyminum*) and oregano (*Origanum vulgare*) at room temperature. HHP parameters of pressure (200-400MPa) and time (1-15 min) and EOs concentrations (0-312.5 ppm for cumin and 0-156.25 for oregano EOs) were optimized for *E.coli* ATCC 25922 inactivation using response surface methodology (RSM). The quadratic equation for synergistic inactivation of HHP and EOs was calculated. By analyzing the Box–Behnken design response surface plots, the predicted log reductions were shown to be in good agreement with actual reductions (R^2= 0.9893). The antimicrobial activity of cumin EO was significantly affected by HHP treatment while oregano EO did not show improved activity after pressure treatment. The optimum process parameters were determined as HHP application at 495 MPA for 6.1 min and when cumin (158.6 ppm) and oregano (132.60ppm) were present for 5 log reduction. The number of surviving microorganisms in HHP/EO treated samples showed no-post growth when stored for 3 and 120 days at 4°C. Presented results suggests that the combined application of HHP and natural antimicrobials has not only improved the process parameters (lower pressure, time, EO concentration) but also prevented recovery of *E.coli* ATCC 25922 on raw meat samples.

INTRODUCTION

Meat as flesh of an animal that is intended for human consumption. Meat is composed of water, proteins, minerals, fats, vitamins and other bioactive components, and small amount of carbohydrates, which makes it an ideal environment for food-borne pathogens and spoilage bacteria (Chen et al. 2012). It is therefore essential to preserve meat with some preservation methods such as freezing, canning, curing, smoking, dehydrating and many others. For example, refrigeration of fresh meat (including storage) above or below the freezing point has been the traditional preservation method. Super-chilling technology, which stores meat just above the freezing point, has been used with success (Zhou et al. 2010). Traditionally, preservation methods can be grouped into three major categories (controlling by temperature, moisture or inhibitory processes), but in all, the major concern is the inhibiting microbial growth to maintain its safety and quality (Zhou et al. 2010). Other preservation methods required for minimizing the deteriorative changes in color, odor, texture and flavor of meat are also applied to market and distribute meet as “fresh” to the utmost.

Meat is perishable in nature, but this is further accelerated by some intrinsic factors, too. Generally, the pH of meat can range between 5.2 and 7.0 and most fresh meat has a water activity value higher than 0.85, which attracts the spoilage bacteria (Lawrie 1985). Hence, deterioration in quality is a common public health concern which led consumer demands and so as the meat processors to improve control steps to hurdle against microbial proliferation as well as the organoleptic quality (Lawrie 1985). The newest preservation technologies are including non-thermal inactivation technologies such as high hydrostatic pressure (HHP), a fresh approach to

meat packaging such as modified atmosphere packaging (MAP) and active packaging (AP), and addition of antimicrobial agents, and low dose irradiation (Jayasena & Jo 2013).

High hydrostatic pressure (HHP) processing involves an instant and uniform transmission of the pressure independent of the volume of the product (Hugas et al. 2002; Rastogi 2013; Rendueles et al. 2011). Pressures of between 100 and 600 MPa are generally applied at low or mild temperatures, and pressure affects the hydrophobic and ionic interactions within the cell components while does not affect the covalent bonds in molecules. Pressure applied alters the tertiary and quaternary structures of molecules (Balasubramaniam et al. 2016; Campus 2010; Tao et al. 2012). This technique has been shown to be effective for preserving a wide range of food products (San Martín et al. 2002); however, its effectiveness in fresh meat needs to be examined. HHP can modify the mechanical properties or color of meat as meat proteins undergo structural changes, especially, alters the state of the myoglobin (Cheftel & Culioli 1997; Carlez et al. 1995). Pressure applied impairs the functionality of the cell wall and the cytoplasmic membrane, which causes leakage of the intracellular constituents through the destabilized cell membrane (Mañas & Mackey 2004). Moussa et al (2007) showed condensation of nucleoids and aggregation of cytosolic proteins in *E. coli* K-12TG1 cells treated with 250-350 MPa at room and subzero temperatures, using staining cellular nucleoids and proteins and transmission electron microscopy (Moussa et al. 2007). Generally, loss of cytoplasmic membrane integrity is believed to be the reason for cell death in pressure applied microorganisms (Pagán & Mackey 2000; Somolinos et al. 2008) and several authors were confirmed that pressure induced injury to the cytoplasmic membrane results in intracellular material and osmotic responsiveness loss (Mañas & Mackey 2004; Pagán & Mackey 2000; Somolinos et al. 2008). However, pressure application is not enough for complete

destabilization of the cell. Many published reports have indicated the sub-lethal injury happens after HHP treatmen. Especially, in case of gram negative bacteria it was found that the destabilized membrane was rapidly restored after pressure release (Hauben et al. 1996). Since sub-lethally injured cells by pressure become more susceptible to antimicrobial substances, combination of processes (so-called hurdle technology (HT)) can be developed to attain certain objectives in terms of both microbial and organoleptic quality (Lawrie 1985).

The use of several hurdles in combination may act additively or synergistically to inhibit the spoilage bacteria or food-borne pathogens (Oliveira et al. 2015). With this in mind, Nisin was proposed to obtain synergistic lethal effect on both the Gram-negative stains *E.coli, P. fluorescens* and Gram-positive stains *L. innocua, L. viridescens* (Black et al. 2005). In fact, Nisin is not effective against gram negative bacteria (Qi et al. 2010). Here the synergistic effect occurs as pressure increases the permeability of outer cell membrane, the cytoplasmic membrane of vegetative cells adsorbs Nisin. Once in the cytoplasmic membrane, Nisin inactivates sulphydryl groups originating membrane disruption and leakage of cellular contents (Qi et al. 2010)

As like Nisin, plant-originated antimicrobial agents stand out for additional hurdle alternatives HHP improvements (Oliveira et al. 2015). Such a synergistic effect can be a confident defense against pressure resistant and/or spore forming strains. Moreover, cell recovery seen in sub-lethally injured cells can be minimized (Masana et al. 2015). Precisely at this point, the essential oils can be a good alternative for combined use of HHP plus natural antimicrobials.

EOs are extracted from plant material by means of expression, fermentation, extraction or steam distillation and considered as a secondary metabolites secreted as a defense mechanism

against invading pathogens including insects, bacteria, fungi, and viruses (Burt & Reinders 2003; Hyldgaard et al. 2012; Lai & Roy 2004). The biological properties and the flavor characteristics of EOs have provided their extensive use in food products for centuries. Regarding the meat and meat products, EOs from oregano, thyme, basil, marjoram, lemongrass, clove, rosemary, cinnamon, bay, sage, garlic, balm and ginger have been used as natural antimicrobials which lead successful inhibition of pathogens on meat products (Tsigarida et al. 2000; Skandamis & Nychas 2002; Fratianni et al. 2010; Karabagias et al. 2011; Dussault et al. 2014; Barbosa et al. 2009).

Terpenes and terpenoids, as well as aromatic and aliphatic constituents (Bakkali et al. 2008) of EOs are the active components that present antimicrobial effect. However, their use in food is limited because, for sufficient antimicrobial activity, high concentrations are needed, which indeed results in heavy odor and aroma. The hurdle theory of combining EOs or their constituents with HHP in the inactivation of target pathogens, under these circumstances, is gaining popularity. In literature, there is little research about HHP in combination with EOs for inactivation of spoilage bacteria and/or food-borne pathogens. HHP/citral combination against *E. coli* (Somolinos et al. 2008) and HHP/ carvacrol combination against *L. monocytogenes* (Karatzas et al. 2001) were reported. Palhano et al. (Palhano et al. 2004) studied the inactivation of the spores of *C. gloeosporioides* in saline solution by the use of high hydrostatic pressure, citral oil and lemongrass oil, alone and in combination and reported that using 0.75 mg/mL of citral or lemongrass oil reduce the needed pressure for effective inhibition from 350 MPa to 150 MPa. These promising results motivated the present study and in this study optimum HHP conditions (pressure and time of application) and EO concentrations (oregano, cumin) to inactivate *E. coli* ATCC 25922 in fresh meat product were aimed to found.

MATERIAL AND METHODS

Test strain and cultures. The test strain used in this study was *Escherichia coli* ATCC 25922 (Culti-loop™ from Thermo Scientific, US). The stock culture was prepared by sub-culturing the strain on tryptic soy agar (TSA) at 37±1 °C for 24 h and stored at 4°C at most for one month. Fresh cultures used in inoculation studies were sub-cultured from stock cultures on TBA plates, which further sub-cultured in tryptic soy broth (TSB). Test inoculum was prepared by transferring 1 ml of 24-hour old culture to 10 ml of 0.85% saline. The saline suspension was adjusted to an optical density of 0.1, which corresponds to 0.5 McFarland standard (1x 10^8 cfu/ml).

Essential oils. The essential oils (EOs) of cumin seed (*Cuminum cyminum*) and oregano (*Origanum vulgare*) were purchased from Eden Botanical (Petaluma, CA). In previous lab work, these two essential oils were shown to have the antimicrobial activity against *E. coli* ATCC 25922, and were proven to have synergistic effect on *E. coli* ATCC 25922 when applied with HHP (400MPa for 10 min).

Stock nano-emulsions of EOs were prepared as 50000 ppm oil-in-water emulsions using Tween-80 (Sigma) as the emulsifying agent using ultrasonication as described by Ghosh et. al. (Ghosh et al. 2012). Briefly, oil and Tween-80 were mixed at a ratio of 1:1 in an aqueous phase, to give a final oil concentration of 50000 ppm. The coarse emulsion was initially mixed with a magnetic stirrer for two minutes under room temperature and sonicated using 20 kHz sonicator with maximum power output of 500 kW (Qsonica Sonicator Q500, Fisher Scientific, USA). Emulsions were sonicated at ambient temperature for 15 min having %60 amplitude; and each cycle consisted of 30 s pulses on and 1 s pulses off. In order to control temperature rise in the

emulsions, during the sonication, emulsions were hold in ice bucket. Those stock emulsions stored in amber glass at 4^{o}C for at most 3 weeks and before each use they were sonicated for one minute. Stock solutions were diluted in water to give the desired concentrations.

Meat sample preparation. Two batches of whole sirloin samples (6 kg/batches) were obtained from Virginia Tech Meat Center (Blacksburg, VA, USA) 4 days after slaughtering. Vacuum packed samples stored at 4 °C for 3 days before experiments start.

Meat samples were cut into cubes of approximate 2 cm thickness using sterile knife. Randomly selected 5 pieces of samples were tested for presence of initial microbial load of untreated meat. It was confirmed that bacterial contaminants were absent.

Beef cuts were inoculated with *E. coli* ATCC 25922 so as to have a final bacterial concentration of $1x10^{7}$ cfu/g meat. Three batches of 4kg beef cuts were clustered in different containers and 400ml *E. coli* ATCC 25922 suspension (overnight cultures, which had been incubated in TSB, were diluted in saline solution to adjust the absorbency at 600nm to 0.5 McFarland's standard (10^{8} CFU/ml)) was added and mixed well for 5 min. All steps were performed aseptically.

Inoculated meat samples were kept in sterile zip-lock bags (double bags) and kept in the fridge for two hours. The initial microbial load of samples was determined using random sampling and the enumeration technique as described in microbial analysis and enumeration method section. The final concentration on each batch was found to be 7.8±0.09 log CFU/g meat. Beef cuts were divided into 40±5 g sub-samples and placed into pre-sterilized (boiling in water for 15 min), labeled

vacuum pouches. The amount of EOs that needs to be added in each vacuum bag was calculated carefully to have same amount of liquid in each bag.

High Pressure Processing. A high-pressure system (Avure's Food Press QFP 35L-600, Columbus, OH) having a 35 L capacity was used to treat the samples. All runs were performed at 4 °C and water ice mixture was used as transmitter medium. Based on the experimental design decided according to surface response methodology, nine runs were done one after another at the same day and the sample loaded on the pressure vessel for each run was adjusted to 30 bags. The highest adiabatic temperature rise was 13 °C in the vessel. After pressurization the samples were placed in ice filled container and immediately transferred and stored in refrigerator at 4 °C.

Microbial enumeration. Microbiological analysis was performed immediately after HHP treatment (t=0), three (t=3 days) and 120 days (t=120) after HHP treatment. 10 g meat samples from each bag were put in a stomacher bag, 90 ml peptone water was added and then the mixture was rubbed by hand for 2 min. 1ml of each sample suspension was taken and further diluted using peptone water and appropriate dilution were spread on plate count agar (PCA) plates. Plates were incubated at 37 °C for 24 h and colonies formed were counted.

Experimental design and HHP. The experimental design matrix was determined using Response surface methodology (RSM) using a statistical software (Design Expert version 10.0.1.0, Stat-Ease Inc, Minneapolis, MN) This empirical modelling approach is used to understand the quantitative relationship between multiple input variables of HP process parameters (pressure and time) and EOs concentrations and the output response of microbial log reduction to optimize the conditions to get 5 log reduction of *E. Coli* ATCC25922 on meat samples. Basically, RSM includes

mathematical and statistical procedures to design experimental matrix, model selection to fit results for best description and optimization on the fitted model (Chen & Chen 2008). The three-level, four-factorial Box–Behnken experimental design was applied to investigate and validate HP process parameters and EOs concentrations affecting the inactivation of *E. coli* ATCC 25922 in raw beef. The design consists of replicated 5 center points and the set of midpoints of each independent variable boundary values. Error assessment was determined from repeated data of center points. Four experimental factors were pressure (P, MPa), time (t, min), cumin concentration (CC, ppm) and oregano concentration (CO, ppm). The actual factor levels corresponding to coded factor levels are shown in Table 1.

Bacterial inactivation was assessed in terms of logarithmic reductions as the difference between counts after the treatments and the initial inoculum, i.e. Log (N/N_0). Mean values for the responses were fitted to a quadratic model to generate the regression equations. Analysis of variance (ANOVA) was performed to check the adequacy and accuracy of the fitted models and response surface plots were generated using the same software. The adequacy of the regression model was tested by normal probability plots of the residuals, the predicted versus actual plots, lack of fit test, and the coefficients of determination (R^2) values. Three-dimensional surface plots were obtained for the various responses by keeping two independent variables at zero level while the other two were varied.

The model constructed as a response function of the variables on the log reduction is a second order polynomial as follows:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_4 + \alpha_5 X_1^2 + \alpha_6 X_2^2 + \alpha_7 X_3^2 + \alpha_8 X_4^2 + \alpha_9 X_1 X_2 + \alpha_{10} X_1 X_3 + \alpha_{11} X_1 X_4 + \alpha_{12} X_2 X_3 + \alpha_{13} X_2 X_4 + \alpha_{14} X_3 X_4 \quad (5)$$

Where Y is the measured response associated with each factor level combination; α_0 to α_{14} are the regression coefficients; X_1, X_2, X_3 and X_4 are the factors.

RESULTS AND DISCUSSION

According to the Box Behnken design, 29 different experimental runs were determined and details were given in Table 2. The reduction of viability, which defined as log (N/N_0), where N_0 is the initial number of cells (inoculum level) and N is the final number of survivors after HHP treatment, ranged between 1.6 to 5.8 log reductions. *E. coli* ATCC 25922 was found to be completely inactivated (decreased below the detection limit) on the samples pressurized at 600 MPa regardless of the holding time in HP chamber. Different findings were reported in the literature as 2.9 log reduction for *E. coli* O103:H25 in sausage after 600 MPa for 10 min high-pressure treatment (Omer et al. 2010) and 3 log *E. coli* O157:H7 NCTC 12079 reduction in poultry meat, which was pressurized at 600 MPa at 20 ° C for 15 min (Lakshmanan et al. 2005). This might be due to the different effect of high pressure on different strains in different food matrices; still almost 8 log reduction at 600 MPa is remarkable. Among the trials at 400 MPa, the highest log reduction occurred for 8 min treatment on the sample which was including the highest cumin and oregano EOs concentrations (312.5 ppm and 156.25 ppm, respectively) and the lowest reduction observed for 1 min pressure treatment in the 78.125 ppm oregano applied sample.

The ANOVA results for log reduction values obtained from response surface second-order polynomial model [Eqn 6] are shown in Table 10. The predicted model is:

$$Y = 0.463 - 2.8\text{x}10^{-3} * \text{P} + 0.17107t + 3.2\text{x}10^{-3}C_C + 5.16\text{x}10^{-3}C_O + 1.70\text{x}10^{-5}P^2 - 7.7\text{x}10^{-3}t^2 - 5.93\text{x}10^{-6}C_C^2 - 1.74\text{x}10^{-5}C_O^2 - 5.36\text{x}10^{-5}\text{P}t - 9.6\text{x}10^{-7}\text{P}C_C - 5.76\text{x}10^{-6}\text{P}C_O + 9.83\text{x}10^{-5}tC_C + 1.37\text{x}10^{-4}tC_O - 2.05\text{x}10^{-6}C_CC_O \quad (6)$$

The presence of cumin EO exerted a significant influence on the HHP-inactivation of *E. coli* ATCC 25922. In previous studies of our group it was found that cumin EO is less effective than oregano EO against *E. coli* ATCC 25922, as having higher MIC value (MIC for cumin was found as 1250ppm and for oregano it was 625 ppm), and its effectiveness is increasing when high pressure is applied (Chapter 3). In this study, oregano EO showed no significant synergistic effect against *E. coli* ATCC 25922 when added together with cumin EO or treated with HHP on meet product. However, oregano and cumin EOs were found to have synergistic effect against *E. coli* ATCC 25922 when high pressure is applied in model medium of Muller Hinton Broth (MHB).

Being more effective than EOs, HHP parameters of pressure and time of application had significant ($P<0.01$) effect on the log reduction. Increasing the pressure resulted in a significant increase in the log reduction of *E. coli* ATCC 25922 in meat samples. This observation is consistent with earlier findings in literature, which demonstrated that increasing pressure, and time results in an increase in the log reduction in meat samples (Campus 2010; Neetoo & Chen 2010). Interactions of variables did not have a significant effect on inactivation of *E. coli* ATCC 25922.

The three-dimensional surface plots for the second-order polynomial model are shown in Figure 1. The coefficient of determination (R^2) of 0.9893 (Table 10) depicted that the RSM model explained a higher proportion of the experimental variability. The suitability of the model was further verified with the normal probability plot of log reduction of *E. coli* ATCC 25922 (Figure 2a), and the predicted versus actual log reduction plot (Figure 2b). Both plots lie approximately along the line of best fit, signifying that there were no problems with the normality and severity of outliers in the experimental data for the inactivation level of *E. coli* ATCC 25922. The lack-of-fit test was not significant, which further validates the model (Table 10). This model was also significant ($P<0.0001$) and can therefore be used to predict log reduction of *E. coli* ATCC 25922 on beef sample for HHP and oregano/cumin EOs combination treatment.

For short (3 days) term storage at 4 °C, the change on the logarithmic number of microorganism was between -0.77 and 0.62 when compare to the values right after the HHP treatment. For long (120 days) term storage at 4 °C, 1.8 log increase at most observed. In the literature, it was reported that after HHP treatment *E. coli* ATCC 25922 was completely recovered after 120 h of incubation at 25°C (Koseki & Yamamoto 2006). Also similar results were reported by Ohshima et al. (Ohshima et al. 2013). Jofré et al. (2009) inoculated *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, *Yersinia enterocolitica* and *Campylobacter jejuni*, and the spoilage lactic acid bacteria (LAB), *Escherichia coli* and the yeast *Debaryomyces hansenii* on slices of cooked ham, dry cured ham and marinated beef loin (containing 0.44 % water, sodium chloride 0.10 %, sodium tri-polyphosphate 0.02 %, sodium ascorbate 0.006 % and sodium nitrite 0.002 %) and applied 600MPa treatment. It is reported that high pressure application effectively inactivated most of the microorganisms, the counts of which, except for LAB that increased in

cooked ham and in beef loin, progressively decreased or maintained below the detection limit during the whole storage (120 days at 4°C) (Jofré et al. 2009).

Different optimum conditions listed on the Table 11 for targeting 5 log reduction while minimizing the pressure. Reducing the intensity of pressure is important to maintain the product quality. Also, lower pressure and shorter process time are more feasible and energy efficient. However, the desirability of the conditions were getting weaker as the pressure level being decreased. The most desirable optimum conditions were calculated as 520 MPa of pressure, 12.1 min of process time, 311.7 ppm of cumin's concentration, and 91.7 ppm of oregano's concentration. Furthermore, for better understanding of the effect of EOs on inactivation of *E. coli*, log reductions can be achieved without EOs are added on the second row of Table 11. Presence of EOs increased the log reduction approximately 0.86 unit.

The role of HHP in food safety and inhibiting the growth of pathogens is well established. Indeed, sterilization of food products using high pressure at moderate temperature has gained acceptance, and many products are in market for commercial use. However, there are still many aspects under research, such as recovery of certain microorganisms after pressure application during storage and adverse effects of HHP on organoleptic quality of food product. Therefore, when applying HHP, the process parameters of pressure, temperature, and time must be considered carefully. The major problem for widespread use of HHP is that the complete control on certain microorganisms is not possible and for full decontamination, elevated temperature, longer exposure time needs to be carried out. Regarding the meat and meat products, process conditions must be carefully determined for enhanced microbial control and to keep organoleptic quality. Higher

pressure, temperature and exposure time can result in undesirable quality losses, especially in raw meat. However, moderate pressure application at room temperature can have a tenderizing effect on meat. Thus, the use of hurdle technologies to improve HHP parameters is not only a promising technology to lower the HHP parameters but also to increase shelf life of meat. Many EOs have been studied for their antimicrobial effect on meat and meat product. However, their strong flavor and odor limits their use in food industry. Thus in this study, we proposed synergistic effect of combining EOs with HHP technology can solve listed problems and optimum operation parameters and EO concentrations were designed using surface response methodology. Our results suggest that there is an additive effect of pressure application on activity of cumin EO. Also, our results showed that *E.coli* recovery after pressure application can be impeded when cumin and oregano EOs were added

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FIGURES

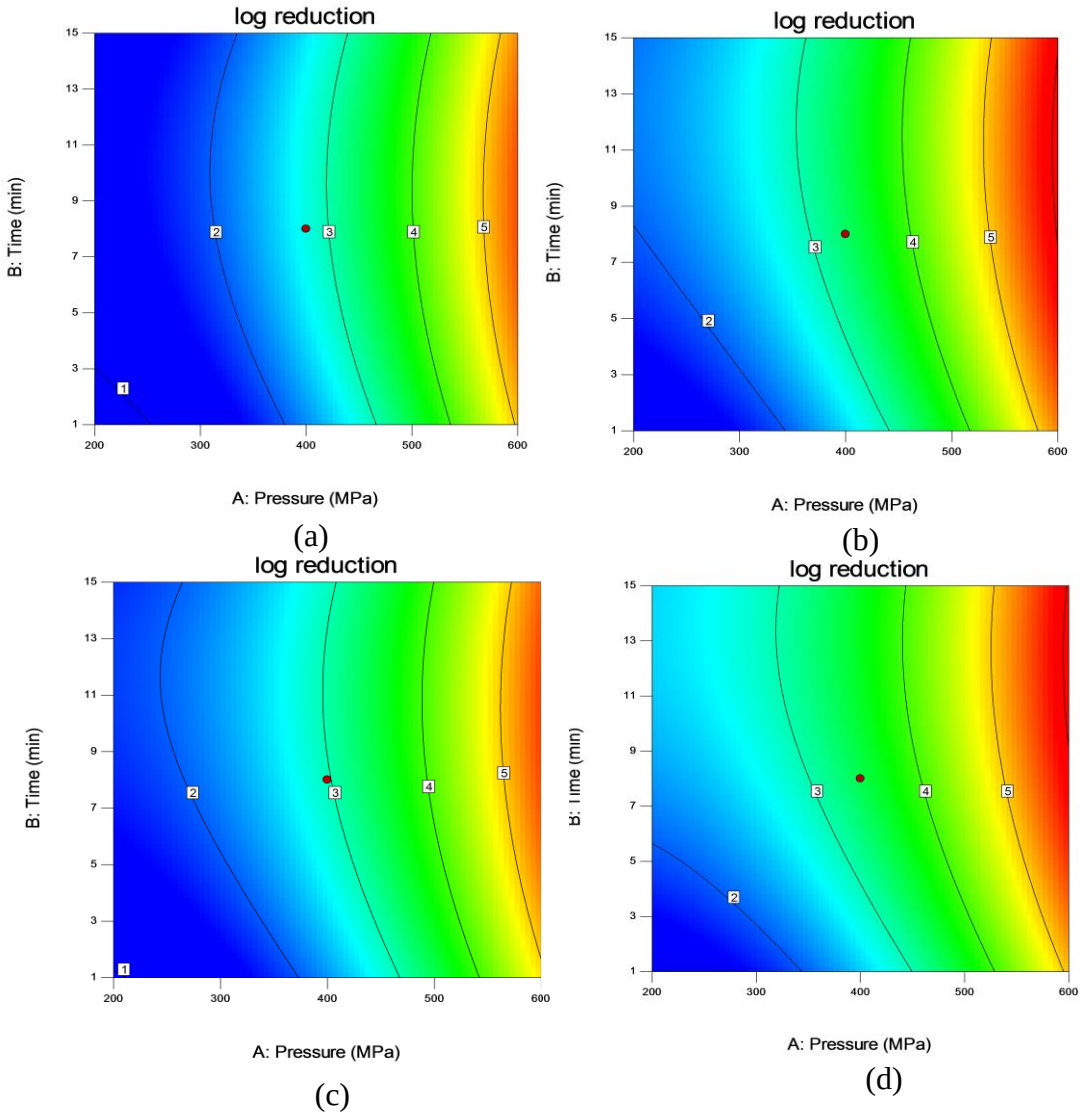

Figure 2 Response surface plots describing the effect of HHP variables (pressure, holding time) on log reduction of *E. coli* ATCC 25922. (a) No EO added; (b) only cumin EO added at highest level; (c) only oregano added at highest level; (d) both cumin and oregano EOs added at highest level

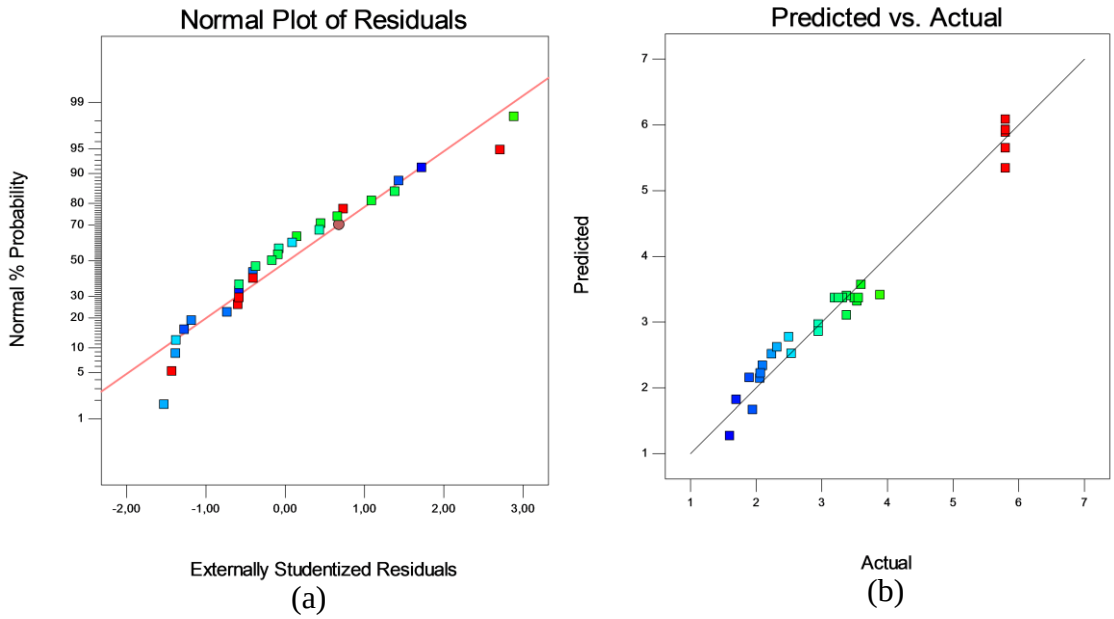

Figure 3 (a) Normal Plot of residual and (b) Predicted vs Actual values

TABLES

Table 8 Levels of factors chosen for the experimental design

			Actual levels of coded factor		
Factors	Symbols	Units	-1	0	+1
Pressure	P	MPa	200	400	600
Time	T	minute	1	8	15
Concentration of cumin EO	C_C	ppm	0	156.25	312.5
Concentration of oregano EO	C_O	ppm	0	78.125	156.25

Table 9 Box-Bohenken design matrix for the four variables and experimental and predicted results

A:Pressure MPa	B:Time min	C:Cumin Ppm	D:Oregano ppm	log reduction actual	log reduction predicted
200	1	156.25	78.125	1.6	1.2
200	8	0	78.125	2	1.8
200	8	156.25	156.25	2.1	2.3
200	8	312.5	78.125	2.1	2.4
200	8	156.25	0	1.7	1.9
200	15	156.25	78.125	1.9	2.4
400	1	312.5	78.125	2.3	2.8
400	1	0	78.125	2.1	2.4
400	1	156.25	0	2.5	2.6
400	1	156.25	156.25	2.2	2.6
400	8	156.25	78.125	3.3	3.6
400	8	312.5	0	3.5	3.6
400	8	156.25	78.125	3.5	3.6
400	8	312.5	156.25	3.9	3.7
400	8	0	156.25	3	3.1
400	8	0	0	2.5	2.9
400	8	156.25	78.125	3.3	3.6
400	8	156.25	78.125	3.2	3.6
400	8	156.25	78.125	3.6	3.6
400	15	156.25	156.25	3.4	3.8
400	15	156.25	0	3.4	3.5
400	15	0	78.125	3	3.2
400	15	312.5	78.125	3.6	4
600	1	156.25	78.125	>5.8	7.5
600	8	312.5	78.125	>5.8	8.6
600	8	0	78.125	>5.8	7.9
600	8	156.25	0	>5.8	8.3
600	8	156.25	156.25	>5.8	8.3
600	15	156.25	78.125	>5.8	8.5

Table 10 Regression coefficients and ANOVA of the models fitted for log reduction after high pressure treatment (t=0), short (t=3 days) and long term storage (t=120 days)

	t=0	t=3day	t=120 day
Constant	4.63E-01	1.73E-01	-2.62E+00
Pressure	-2.80E-03[a]	-3.06E-03	8.90E-03
Time	1.71E-01[c]	1.84E-01	8.94E-02
Cumin	3.20E-03[d]	4.32E-03	1.34E-03
Oregano	5.16E-03	5.29E-03	2.64E-03
Pressure * Time	-5.36E-05	1.25E-05	-
Pressure * Cumin	-9.60E-07	-3.28E-06	-
Pressure * Oregano	-5.76E-06	-6.56E-06	-
Time * Cumin	9.83E-05	-1.05E-04	-
Time * Oregano	1.37E-04	2.56E-04	-
Cumin * Oregano	-2.05E-06	-1.11E-05	-
Pressure^2	1.70E-05[a]	1.73E-05	-
Time^2	-7.70E-03[c]	-7.46E-03	-
Cumin^2	-5.93E-06	-4.30E-07	-
Oregano^2	-1.74E-05	-6.64E-06	-
R^2	0.9745	0.9244	0.7262
F value	38.19	12.22	11.93
P value (Prob>F)	<0.0001	<0.0001	<0.0001
Statistical significance	Significant[a]	Significant[a]	Significant[a]
Lack of fit	Not significant	Not significant	Not significant

[a] <0.0001; [b] <0.001; [c] <0.01; [d] <0.1

Table 11 Optimum conditions for targeting 5 log reduction while minimizing pressure.

	Combination I	Combination II	Combination III	Combination IV	Combination V
Log reduction	**5.00**	**4.75**	**4.25**	**3.83**	**3.47**
If no EOs Log reduction	*4.22*	*3.95*	*3.40*	*2.93*	*2.52*
Pressure (MPa)	520.0	501.3	459.2	419.7	380.0
Time (min)	12.1	12.2	12.4	12.6	12.8
Cumin (ppm)	311.7	312.5	312.5	312.5	312.5
Oregano (ppm)	91.7	95.1	102.9	110.3	117.8
Desirability	**0.765**	**0.635**	**0.578**	**0.555**	**0.549**

CHAPTER V

OPTIMIZATION OF HIGH HYDROSTATIC PRESSURE AND CUMIN-OREGANO ESSENTIAL OILS COMBINATION'S CONDITIONS ON QUALITY PARAMETERS OF BEEF STEAK USING RESPONSE SURFACE METHODOLOGY

Fatma Sahmurat[a], P. Kumar Mallikarjunan[a*] Hande Kaya-Celiker[a]

Joseph E. Marcy[b]

[a] *Biological Systems Engineering, Virginia Polytechnic Institute and State University,*

[b] *Biological Systems Engineering, Virginia Polytechnic Institute and State University,*

*Blacksburg, VA, USA (sahmurat@vt.edu, *kumar@vt.edu, hande@vt.edu, jmarcy@vt.edu)*

*: *Correspondence should be sent to:*
Kumar Mallikarjunan, 205 Seitz Hall, Virginia Tech, Blacksburg, VA 24061
Phone: (540) 231-7937 Fax: (540) 231-3199 Email: kumar@vt.edu

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ABSTRACT

The purpose of this study was optimizing the synergistic effects of high hydrostatic pressure and natural antimicrobials on meat quality. Therefore, the focus of these studies were to further understand the impact of pressure, processing time, oregano and cumin EOs on fresh surface color (instrumental and visual), oxidative stability (TBARS), and tenderness of beef. For this purpose Response Surface Methodology (RSM) was employed. Color indices were significantly affected by pressure, time and their interactions. Above 400 MPa the discoloration was similar to cooked beef and EO addition did not help color improvement. However, EOs showed significant antioxidant activity on both treated and untreated samples during long term storage while pressure and holding time found statistically not effective on lipid oxidation in terms of TBARS value. Oxidation was not increased immediately after the pressure treatment, but pressure induced lipid oxidation during long term storage of the meat. Oregano essential oil's concentration and its interaction with cumin are found statistically important ($P<0.01$). WBSF is the most common used indicator of tenderness of meat. The effect of pressure and EO's were found ineffective while only time significantly effective ($P<0.01$). EOs increased tenderness. However, the maximum value of WBSF is 3.5 kgf which is still reasonable for beef meat. The optimum process conditions were decided as pressure=200 MPa, time= 14.9 min, cumin concentrations=312.3 ppm, and oregano concentration= 156.2 with 0.552 desirability. RSM was found effective to analyze combination treatments involving hydrostatic pressure, and EOs concentration to control undesirable effects of pressure on beef.

INTRODUCTION

Beef is an important source of protein and several essential vitamins. Red meat production has the largest segment of the U.S. agriculture and beef is the most preferred red meat with total consumption 25.8 billion pounds in 2012. Also, 9.4 % of beef production was exported in 2012 and $5.1billion value added in U.S. economy (USDA's Economic Research Service 2014).

E. coli O157:H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes* are some of the foodborne organisms associated with meat-based products . All of those pathogenic bacteria are big treat to meat industry as they can spread via cross contamination by poor handling practices and poor sanitation. Because consumers prefer to eat more juicy and tender beef, insufficient thermal treatment is common and it has been caused important health risks. For a safe beef meal, ground beef products should cook until the internal temperature of them reaches 160 °F and fresh beef should heat to 145 F with a 3 min rest time. At this conditions, *E. coli* O157:H7 will destroy and the meal will be safe (Food and Drug Administration 2012; Food and Drug Administration 2014).

Because fresh meat is vulnerable to severe heat treatments, non-thermal preservation technologies have drawn attention of the meat industry wishing to satisfy growing consumers demands for high quality, easy-to-handle, safe with natural flavor and color, as well as an extended shelf life without chemical preservatives, meat products. That's why the implementation of high hydrostatic pressure processing (HHP) has a great potential in meat industry (Campus 2010; Chen et al. 2012). HHP is an isostatic and adiabatic process which can be applied uniformly and instantly to the subject without no change the shape and integrity of the packaging intact. The rising of

temperature in the subject is reported as approximately 3 °C per 100 MPa, depending on the composition of the food. Since applying high hydrostatic pressure on food is adiabatic, this prevents the food from being heated which would modify its organoleptic properties (Rendueles et al. 2011).

High-pressure processing has many advantages as a non-thermal technology used to improve food safety. For example, it can be applied at ambient or even lower temperatures, because of the isostatic transmission of pressure, the processed material experiences the pressure instantaneously with no gradient, resulting in uniform treatment irrespective of the size and geometry of the material and High-pressure modifies only noncovalent bonds and does not affect small molecules and leads to less degradation in the overall quality of processed foods in comparison with heat treated foods and it is a waste-free environmentally friendly technology (Rendueles et al. 2011; Norton & Sun 2008). The U.S. Dept. of Agriculture-Food Safety and Inspection Services (USDA-FSIS) issued a letter-of-no-objection (LNO) for the use of HHP as an effective post-packaging intervention method in controlling L. monocytogenes in RTE meat and poultry products in 2003, HHP technology has been employed by many meat processors with great potential in terms of ensuring meat safety after packaging (Campus 2010; Koutchma 2014). HHP is gaining popularity as an alternative to thermal processing as proving milder effects on sensory and nutritional quality of foods (Campus 2010; Chen et al. 2012; Buckow et al. 2013). The eligibility of this technology to all types of food products (both liquid and solid) makes it a good candidate as an alternative to classical pasteurization and sterilization treatments. Guacamole, deli meat, juices, salads and oysters are some of the examples of pressurized food products commercially produced using HHP process in the United States; also jams, fellies, sauces, fish, meat products, sliced ham, salad dressing, rice cakes, and yogurt are marketed in Japan; and fruit juices are produced in France and

Portugal (Balasubramaniam et al. 2016).. High pressure processing can prolong the shelf life of meat products in addition to chilling but the pressurelabile nature of protein systems limits the commercial range of applications (Buckow et al. 2013).

High pressure treatment on meat and meat products has been a popular subject in the last decade for researchers. The effect of this technology on foodborne pathogenic bacteria has been widely studied (Campus 2010; Norton & Sun 2008) and reported that most vegetative pathogens are susceptible to pressure, and pressure treatments achieve significant reductions (Rendueles et al. 2011). Inactivation of *Escherichia coli* O15:H7 (EHEC) in ground beef at steady pressure (Podolak et al. 2006) and under different cyclic HHP operating conditions were investigated and up to 4.96 log CFU/g *E. coli* population reduction was reported when three 5-min cycles at 400Mpa was applied (Morales, P., Calzada, J., Avila, M., and Nunez et al. 2008).

Apart from the numerous advantages that HHP technology presents, there are some drawbacks of high pressure on fresh meat products such as increased lipid oxidation and discoloration through protein denaturation (Campus 2010; Buckow et al. 2013; Aymerich et al. 2008; Bajovic et al. 2012; Cheftel & Culioli 1997; Simonin et al. 2012)[8, 15, 30-33]. Mostly commercially available pressurized meat products are ready to eat foods except fresh minced beef now (Bajovic et al. 2012).

Color change is one of the drawbacks in HHP treatments. As the pressure applied increases, the redness and total color difference becomes significant. Pressure higher than 300 MPa induces modifications of meat color parameters such a decrease of the total color difference while the redness of the 520 MPa samples decreases gradually, in relation to the increase of metmyoglobin

(Jung et al. 2003). In order to overcome discoloration problem, various ingredients were added such as sodium chloride (NaCl), sodium tripolyphosphate (STPP) and beta-glucan (BG). However, additives showed little improvement on color characteristics but anti-oxidative property of beta-glucan in meat system was revealed (Omana et al. 2011). Induced lipid oxidation of beef is another problem in HHP treatment. Aiming to increase the meat quality after HHP treatment, many researchers have tried to add functional ingredients such as phosvitin (Jung et al. 2013) or vegetable oil (Jung et al. 2012). Addition of phosvitin was reported to synergistically reduce microbial growth and delayed the lipid oxidation when applied with HHP. However, it had no effect on the color changes of raw ground beef attributable to HHP (Jung et al. 2013). On the contrary, addition of vegetable oil followed by the application of HHP has been proven to inhibit the bacterial growth and lipid oxidation (Jung et al. 2012).

HHP can cause partial denaturation of protein and increase the potential for protein breakdown with tenderization (Buckow et al. 2013). It is a fact that HHP treatment easies the tenderization process of beef muscle by fractioning the myofibrillar and sarcoplasmic proteins, followed by formation of a strengthened myofibrillar structure that is more brittle so is tenderer (Sikes & Tume 2014).

An increase in TBARS values was observed at the higher pressure levels (300, 400 MPa) (McArdle et al. 2010). But still, TBARS values were found to be lower in pressurized samples compared to cooked samples. The reported results of McArdle and his co-workers showed that HHP alters meat quality to a lesser extent than conventional cooking, thereby minimizing the processing impact (McArdle et al. 2013).

Thus, recent researches have been focused on applying HHP in hurdle technologies to reduce the quality changes as providing the enough microbial inactivation (Chen et al. 2012). A synergic effect on bacterial inactivation has been observed when HHP combined with some antimicrobials, such as bacteriocins, nisin and lysozyme (Hauben et al. 1996; Ananou et al. 2010; Jofré, Aymerich, et al. 2008; de Alba et al. 2013; Jofré, Garriga, et al. 2008; Marcos et al. 2008; Abriouel et al. 2014; Pérez Pulido et al. 2012; Vercammen et al. 2011). Also, there was a synergy between high pressure and salt or nitrite has been reported. Because high pressure can limit the addition of salt and nitrite while ensuring extended shelf life, it is promising for the manufacture of meat products (Duranton et al. 2012).

Essential oils (EO) are volatile natural mixtures extracted from different plant parts (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots), and are composed of terpenoid structures with broad activities (Seow et al. 2014). EOs are known to work as potential antimicrobial agents having the ability to control foodborne pathogenic bacteria. Use of plant based EOs in food and beverage industries has been exploited for decades. They have been shown to work as potent antibacterial agents because of the presence of bioactive volatile components (Bajpai & Baek 2016). Besides, concerns on reducing chemical preservatives in food industry have been raised due to the adversary effects of chemical preservatives, resulting in the release of toxic materials inside the packed food products. Use of EOs can be an effective application as food preservatives naturally due to their potent antimicrobial nature (Bajpai et al. 2012). The use of EOs as biopreservatives is a matter of great interest for the food industry since consumers prefer natural additives instead of synthetic ones. That is why many studies have been performed on this subject in the last few years (Lang & Buchbauer 2012). Undesirable organoleptic effects can be limited by

careful selection of EO according to the type of food. Synergism and antagonism between components of EOs and food constituents require more study before these substances can be reliably used in commercial applications. If the active substances are to be added to foods in greater concentrations than is currently normal practice for flavourings, further safety studies may be necessary. A significant number of essential oils and phytochemicals are bioactive against foodborne pathogens in vitro, and to a smaller degree, in foods. To prevent off flavor or other undesirable sensory qualities, essential oils have to be carefully selected according to the specific food type. Synergism between different phytochemicals or other chemical compounds has to be investigated further before they can be applied commercially. A compound should be checked for its safety limits if its antimicrobial activity depends on a concentration greater than the usual for flavorings. Despite having disadvantages, essential oils prove beneficial with their broad antimicrobial activities and lack of apparent resistance development (Seow et al. 2014; Burt 2004).

It is apparent that minimal processing technologies such as HHP that will prevent contamination in meat based products needs to be developed to provide a strong alternative to severe heat treatment for sanitary purposes. A negative aspect such as induced lipid oxidation and discoloration needs to be reduced to improve the methodology. It has been suggested before high pressure should be combined with other preservation methods to improve the final product quality and investigated combination of HHP and antimicrobials. However, consumers are getting sensitive to purchase added synthetic preservatives and looking for more natural and safe products. That is why natural antimicrobials and antioxidants are getting popularity in food industry recent years.

Thus, in this study, it is proposed to optimize the synergistic effects of high hydrostatic pressure and natural antimicrobials on meat quality.

MATERIAL AND METHODS

Test strain and cultures. The test strain used in this study was *Escherichia coli* ATCC 25922 (Culti-loop™ Remel Inc., Lenexa, KS, USA). The stock culture was prepared by sub-culturing the strain on tryptic soy agar (TSA) at 37±1 °C for 24 h and stored at 4°C at most for one month. Fresh cultures used in inoculation studies were sub-cultured from stock cultures on TBA plates, which further sub-cultured in tryptic soy broth (TSB). Test inoculum was prepared by transferring 1 ml of 24-hour old culture to 10 ml of 0.85% saline. The saline suspension was adjusted to an optical density of 0.1, which corresponds to 0.5 McFarland standard (1x 10^8 cfu/ml).

Essential oils. The essential oils (EOs) of cumin seed (*Cuminum cyminum*) and oregano (*Origanum vulgare*) were purchased from Eden Botanical (Petaluma, CA). In previous lab work, these two essential oils were shown to have the antimicrobial activity against *E. coli* ATCC 25922, and were proven to have synergistic effect on *E. coli* ATCC 25922 when applied with HHP (400MPa for 10 min).

Stock nano-emulsions of EOs were prepared as 50000 ppm oil-in-water emulsions using Tween-80 (Sigma) as the emulsifying agent using ultrasonication as described Ghosh et. al. (Ghosh et al. 2012). Briefly, oil and Tween-80 were mixed at a ratio of 1:1 in an aqueous phase, to give a final oil concentration of 50000 ppm. The coarse emulsion was initially mixed with a magnetic stirrer for two minutes under room temperature and sonicated using 20 kHz sonicator with

maximum power output of 500 kW (Qsonica Sonicator Q500, Fisher Scientific, USA). Emulsions were sonicated at ambient temperature for 15 min having %60 amplitude; and each cycle consisted of 30 s pulses on and 1 s pulses off. In order to control temperature rise in the emulsions, during the sonication, emulsions were hold in ice bucket. Those stock emulsions stored in amber glass at 4^{o}C for at most 3 weeks and before each use they were sonicated for one minute. Stock solutions were diluted in water to give the desired concentrations.

Meat sample preparation. Two batches of whole sirloin samples (≅6 kg/batches) were obtained from Virginia Tech Meat Center (Blacksburg, VA, USA) 4 days after slaughtering. Vacuum packed samples stored at 4 °C for 3 days before experiments start.

Meat samples were cut into cubes of approximate 2 cm thickness using sterile knife. Randomly selected 5 pieces of samples were tested for presence of initial microbial load of untreated meat. It was confirmed that bacterial contaminants were absent.

Beef cuts were inoculated with *E. coli* ATCC 25922 so as to have a final bacterial concentration of $1x10^7$ cfu/g. Three batches of 4kg beef cuts were clustered in different containers and 400ml *E. coli* ATCC 25922 suspension (overnight cultures, which had been incubated in TSB, were diluted in saline solution to adjust the absorbency at 600nm to 0.5 McFarland's standard (10^8 CFU/ml)) was added and mixed well for 5 min. All steps were performed aseptically.

Inoculated meat samples were kept in sterile zip-lock bags (double bags) and kept in the fridge for two hours. The initial microbial load of samples was determined using random sampling and the enumeration technique as described in microbial analysis and enumeration method section.

The final concentration on each batch was found to be 7.8±0.09 log CFU/g meat. Beef cuts were divided into 40±5 g sub-samples and placed into pre-sterilized (boiling in water for 15 min), labeled vacuum pouches. The amount of EOs that needs to be added in each vacuum bag was calculated carefully to have same amount of liquid in each bag.

High Pressure Processing. A high-pressure system (Avure's Food Press QFP 35L-600 with a 7XS-6000 Intensifier Pump, Middletown, OH, USA) having a 35 L capacity was used to treat the samples. All runs were performed at 4 °C and water ice mixture was used as transmitter medium. Based on the experimental design decided according to surface response methodology, nine runs were done one after another at the same day and the sample loaded on the pressure vessel for each run was adjusted to 30 bags. The highest adiabatic temperature rise was 13 °C in the vessel. After pressurization the samples were placed in ice filled container and immediately transferred and stored in refrigerator at 4 °C.

Color. CIElab color parameters lightness (L: 1000white, 00black), redness (a*±red–green) and yellowness (b*±yellow–blue) of the samples were made using a Minolta chromometer (Model CR-300, Minolta Camera, Ltd., Osaka, Japan). Tests were replicated five times for each sample. Chroma (ΔC), total color difference (ΣE), whiteness index (WI), hue angle (H), and discoloration (a*/b*) were calculated from L* a* b* values in order to describe the color change as compared to the control sample. These values are calculated using measured L*, a* and b* values as follows and used to where subscript “o” indicates the color reading of control sample used as the reference and a larger ΔE indicates greater color change from the reference sample (Hunt et al. 2012).

$$\Delta C = (a^2 + b^2)/2 \quad (7)$$

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2} \quad (8)$$

$$\Delta H = arctangent\left(\frac{b}{a}\right) \quad (9)$$

Lipid oxidation. Lipid peroxidation of samples was determined by thiobarbituric acid reactive substance (TBARS; Oxford Biomedical Research,) kit, which quantifies the malondialdehyde (MDA) content, following the manufacturer's procedure. TBARS was expressed as μg/L MDA. Blanks were prepared for each sample.

First to obtain standard curve, 7 standards were prepared between 0 and 3 mg/L MDA concentration and read at 532 nm. Blank for each sample was prepared separately. For sample preparation, 5.0 g of the sample was added to 5.0 mL of deionized water in a 15 mL centrifuge tube and homogenized the sample to a smooth suspension. Then enough water added to bring the volume to exactly 10 mL, so dilution factor became 2 and solids concentration was 500 g/L. 1.0 mL of the Indicator Solution was added to 1.0 mL of the emulsion and vigorously agitated the sample for one minute with a vortex mixer. Then the sample was allowed to react for 60 min at room temperature. Tubes containing samples were centrifuged at 15,000 g for 5 min. carefully transferred to a cuvette and measured the absorbance at 532 nm.

Tenderness Measurement. After HHP treatment, the steaks were cooked according to American Meat Science Association (AMSA) guidelines to 71°C or medium doneness (Belk et al. 2015). They were placed in a single layer with no overlapping and cooled to 4°C overnight before

testing to enable ease of coring. Three 1.27 cm diameter cores, parallel to the orientation of the muscle fibers were sampled from cooked steaks from each treatment group using a manual coring device. WBS testing were carried out on a Texture Analyzer TA-XT2 (Texture Technologies Corp., Scarsdale, NY). Each core was sheared once through its center using a TA-7 USDA Warner-Bratzler shear blade, perpendicular to the longitudinal orientation of the muscle fibers which is standard for this method. Crosshead speed was set at 200 mm/min. The maximum peak force recorded was taken as the shear force.

Experimental design and HHP. The experimental design matrix was determined using Response surface methodology (RSM) using a statistical software (Design Expert version 8.0.7. , Stat-Ease Inc., Minneapolis, MN, USA). This empirical modelling approach is used to understand the quantitative relationship between multiple input variables of HP process parameters (pressure and time) and EOs concentrations and the output response of microbial log reduction to optimize the conditions to get 5 log reduction on meat samples. Basically, RSM includes mathematical and statistical procedures to design experimental matrix, model selection to fit results for best description and optimization on the fitted model (Chen & Chen 2008). The three-level, four-factorial Box–Behnken experimental design was applied to investigate and validate HP process parameters and EOs concentrations affecting the inactivation of *E. coli* ATCC 25922 in raw beef. The design consists of replicated 5 center points and the set of midpoints of each independent variable boundary values. Error assessment was determined from repeated data of center points. Four experimental factors were pressure (P, MPa), time (t, min), cumin concentration (CC, ppm) and oregano concentration (CO, ppm). The actual factor levels corresponding to coded factor levels are shown in Table 11.

Bacterial inactivation was assessed in terms of logarithmic reductions as the difference between counts after the treatments and the initial inoculum, i.e. Log (N/N_0). Mean values for the responses were fitted to a quadratic model to generate the regression equations. Analysis of variance (ANOVA) was performed to check the adequacy and accuracy of the fitted models and response surface plots were generated using the same software. The adequacy of the regression model was tested by normal probability plots of the residuals, the predicted versus actual plots, lack of fit test, and the coefficients of determination (R^2) values. Three-dimensional surface plots were obtained for the various responses by keeping two independent variables at zero level while the other two were varied.

RESULTS AND DISCUSSION

Analysis of variance of data for the five responses, discoloration, total color change, whiteness index, hue angle and chroma, showed the model for each response was highly significant ($p < 0.001$) with R^2 values of 0.9555, 0.9139, 0.8939, 0.8362 and 0.8128, respectively. These indicated that the models adequately explained the responses observed (Table 14). Quadratic model fits for discoloration, total color change and hue angle and linear model fits for whiteness index and chroma. Discoloration value (a*/b*) of samples decreased by increasing pressure and holding time (Table 12). For a*/b*, lower ratios are indicative of more discoloration, so the color of the beef samples was changed by pressure and treatment time. Statistically cumin and oregano EOs were found ineffective on the color change parameters as pressure and holding time were responsible the changes on color. Also it can be seen from the captured images of samples after

HHP treatment (Table 13). Total color change, chroma and hue angle differences and whiteness index significantly increase by the intensity of the high pressure conditions.

The surface color of beef is the most important factor among consumers at first sight when buying fresh beef retail cuts. Above 150 MPa there were similar color changes to those in cooked meat observed for fresh meat (Cheftel & Culioli 1997). Neither cumin nor oregano essential oils helped to improve the color quality of beef in the limits of their dosage used. On the other hand, using EOs as antimicrobials may decrease the intensity of the pressure needed for aimed log reduction and can help to improve color quality indirectly. For example, the optimum process conditions for minimize total color change and maximize discoloration value while targeting five log reduction of *E.coli* (used the data from the previous chapter) were calculated as P=200 MPa, t=15 min, C=312.5 ppm and O=156 ppm giving 2.5 log reduction but with this process parameters (P, t), without essential oils, only 1.25 log reduction can be achieved. In Figure 4. the reddish area includes more close a*/b* values to fresh meat and this area is bigger on Figure 4.c which is oregano added sample and Figure 4.d is oregano-cumin combinations added sample. Although it is not statistically significant, oregano has more positive effect on preventing discoloration of pressure treated beef.

It is a widely held view that lipid oxidation is the primary causes of quality deterioration in meat products (Love & Pearson 1971; Cheftel & Culioli 1997). At sufficiently high pressure, meat becomes more susceptible to lipid oxidation (Bajovic et al. 2012). Oxidation was not increased immediately after the pressure treatment, but pressure induced lipid oxidation during subsequent storage of the meat. TBARS indices reported here obtained from the long term storage samples

(Table 15). According to Tume et al (2010), high-pressure processing accelerates the oxidation of lipid peroxides, effectively reducing their concentration while increasing later breakdown products (detected as TBARS). In contrast to earlier findings, however, pressure and holding time found statistically not effective on lipid oxidation in terms of TBARS value. On the other hand, oregano essential oil's concentration and its interaction with cumin are found statistically important ($P<0.01$, Table 14) in this study. As shown in Figure 9, presence of EOs in the sample, especially oregano EO, helped to decrease the level of oxidation. Although cumin showed antioxidant activity, not as much as oregano, contrary to expectations the combination of them showed less antioxidant effect then their single use. These results would seem to suggest that the combination of essential oils exerted an antagonistic effect on lipid oxidation.

For untreated samples, oregano and cumin-oregano interaction were found effective on lipid oxidation ($p<0.01$, Table 16) while cumin found less effective ($p<0.1$). In contrast to the conduct of oregano-cumin EOs combination after pressure treatment, their combinations showed a synergy against lipid oxidation without high pressure. There was no research related to antioxidant property of oregano and cumin EOs combinations with or without high pressure in the literature. There would therefore seem to be a definite need for studying their combinations' effect on lipid oxidation with HHP.

Because of high concentrations of phenolic compounds such as thymol and carvacrol oregano shows antioxidant effect (Shahidi 2016). There are many food application of oregano as an antioxidant agent in the literature. Oussalah et al. (2004) applied an oregona containing milk protein-based edible films containing on beef muscle slices, resulting in stabilized lipid oxidation.

The addition of oregano spice increased the oxidative stability of tuna salad although addition of spices had an antioxidative effect, the taste introduced to the product by the addition might be unappreciated in this type of products (Sørensen et al., 2010). Oregano essential oil significantly reduced ($p < 0.05$) lipid and protein oxidation, and improved color stability of raw and cooked meat (Al-Hijazeen et al. 2016). The stability of cold-smoked sardine muscle was improved by high hydrostatic pressure processing and a coating the muscle with functional gelatin- based edible films enriched with an oregano or a rosemary extract were evaluated separately and in combination by Gomez-Estaca et al. (2007), and they reported all treatments were able to slow lipid oxidation. Also cumin EO's antioxidant activity reported by several researchers. However, there are no food application of cumin as an antioxidant in the literature to the best of our knowledge.

WBSF is the most common used indicator of tenderness of meat. From Table 16 it can be seen that quadratic model fitted to WBSF data set and actual and predicted values demonstrated on Table 15. The effect of pressure and EO's were found ineffective while only time significantly effective ($P<0.01$). As it can be seen from Figure 11, essential oils increased tenderness. However, the maximum value of WBSF is 3.5 kgf which is still reasonable for beef meat.

As the estimated regression equations fitted sufficiently well to actual data for various quality attributes ($R^2 > 0.8$), the developed models can be adequately used for predictive modeling for the use of hurdle concept of HHP and EOs combination for production of microbial safe and high quality fresh beef product. The criteria selected for optimization of process parameters were based on minimum changes in quality and maximum reduction in spoilage causing factors, which are as follows: minimization of total color change and TBARS; maximization of discoloration; and

targeting of 5 log reduction of *E. coli* inactivation (Table 18). Based on the above criteria, the optimized process conditions were: high pressure level of 200 MPa, hold-time of 14.9 min, 312.3 ppm cumin and 156.2 ppm oregano EOs with a desirability of 0.552. In Appendix II, a hundred different optimum conditions listed and thre of them reported on Table 19. For designing the process, optimization by RSM can be used as an easy and useful method. However, the validation of optimum conditions is needed to be sure that it works well.

RSM effectively analyzed combination treatments involving hydrostatic pressure, and EOs concentration to control undesirable effects of pressure on beef. Although there were no effect of EOs on color parameters of beef, especially oregano EO showed a promising antioxidant activity. Thus it can be concluded that EOs at least minimize lipid oxidation during the storage and help to extend shelf life of beef.

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FIGURES

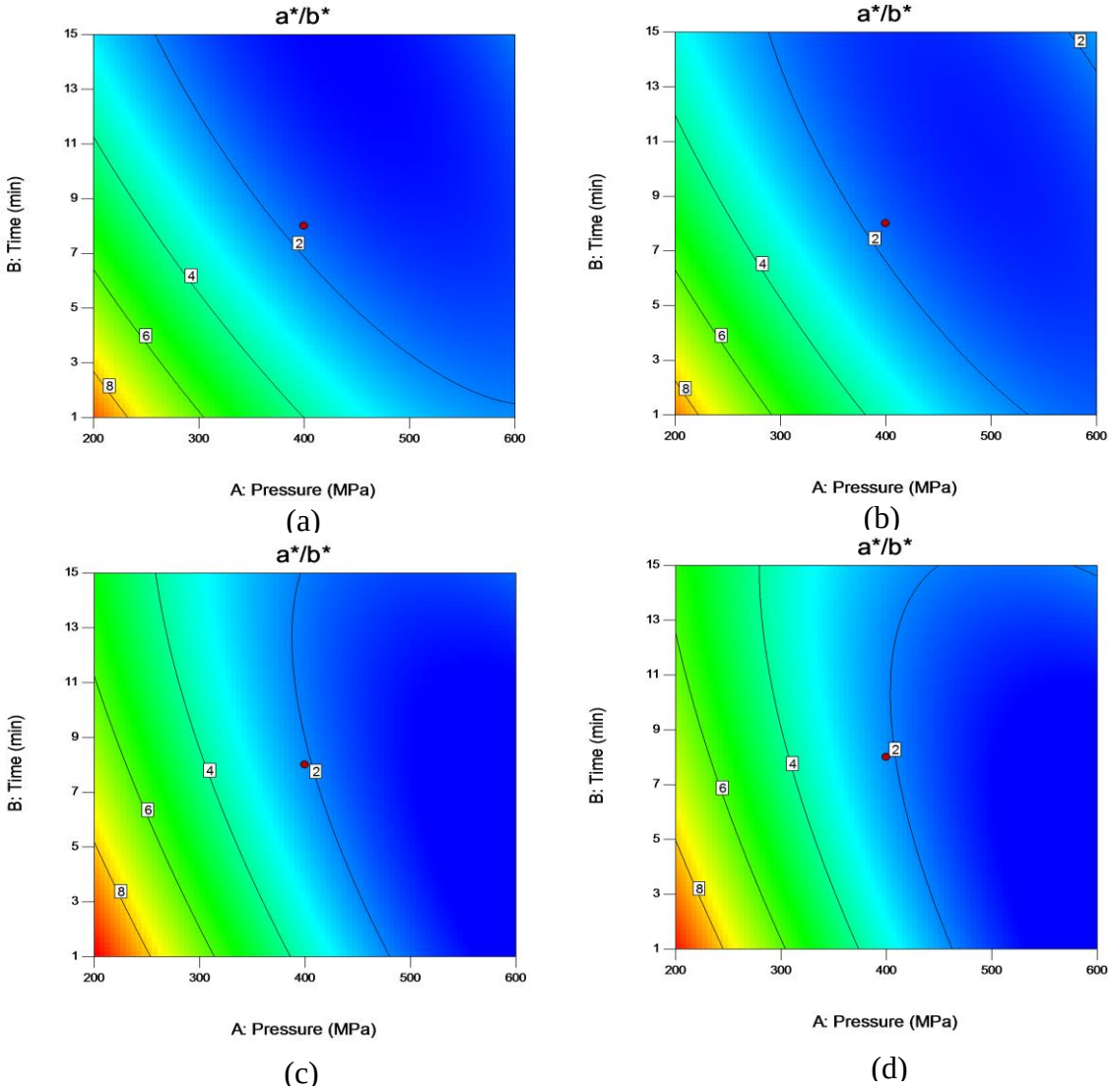


Figure 4 Response surface plots describing the effect of HHP (pressure, holding time) on discoloration of beef samples. (a) No EO added; (b) only cumin EO added at highest level; (c) only oregano added at highest level; (d) both cumin and oregano EOs added at highest level

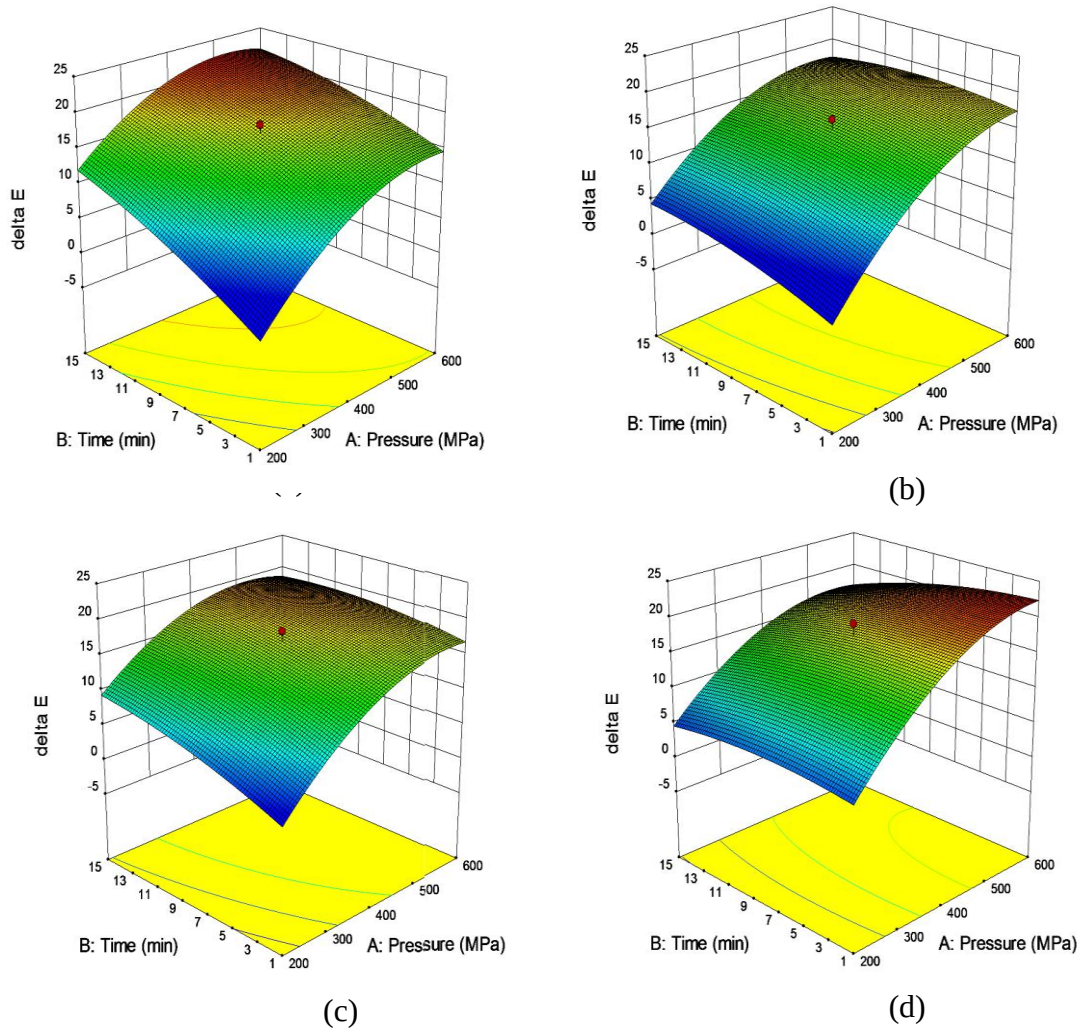


Figure 5 Response surface plots describing the effect of HHP variables (pressure, holding time) on the total color change of beef samples. (a) No EO added; (b) only cumin EO added at highest level; (c) only oregano added at highest level; (d) both cumin and oregano EOs added at highest level

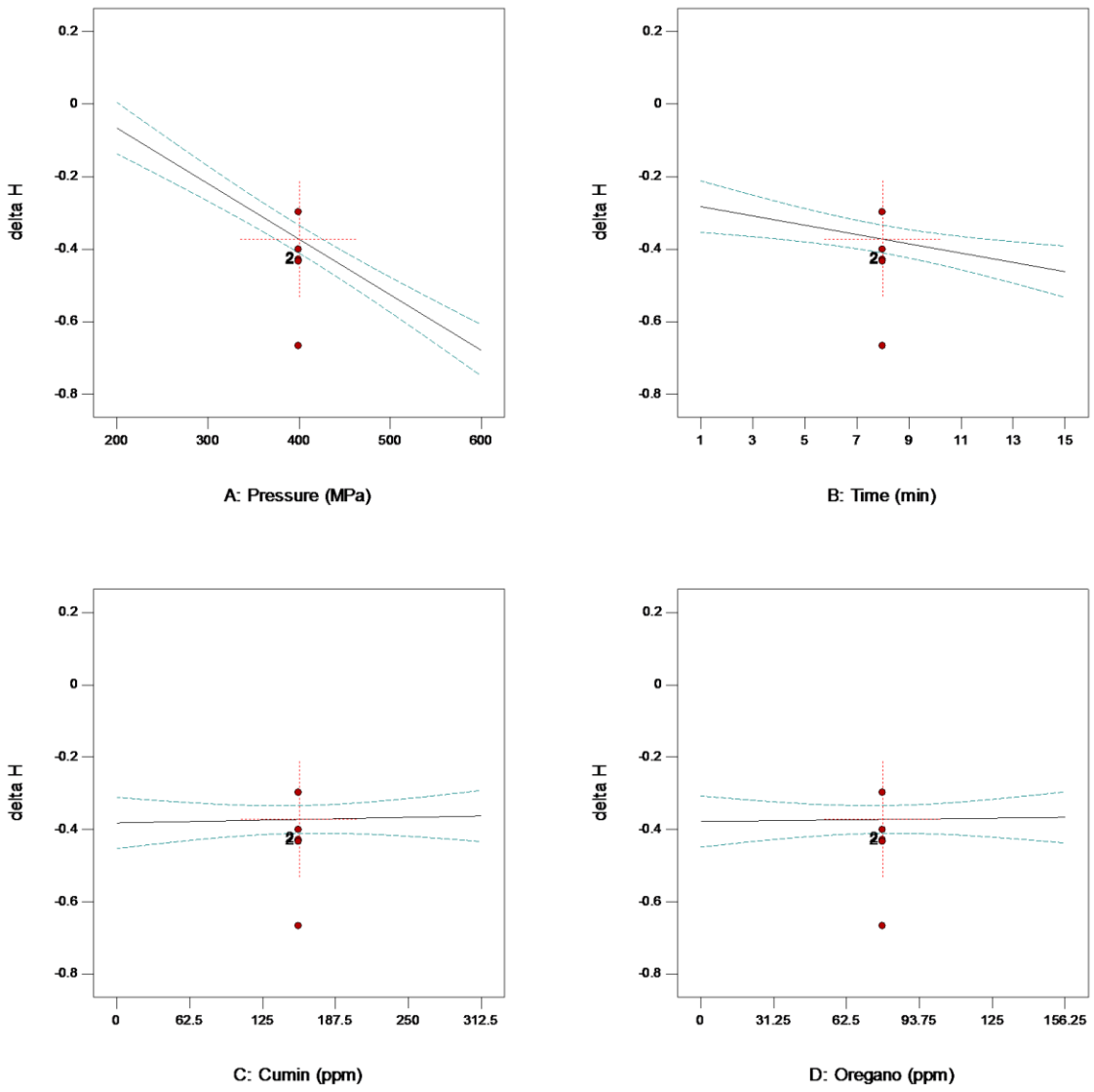


Figure 6 Effects of all factors on hue angle value of samples

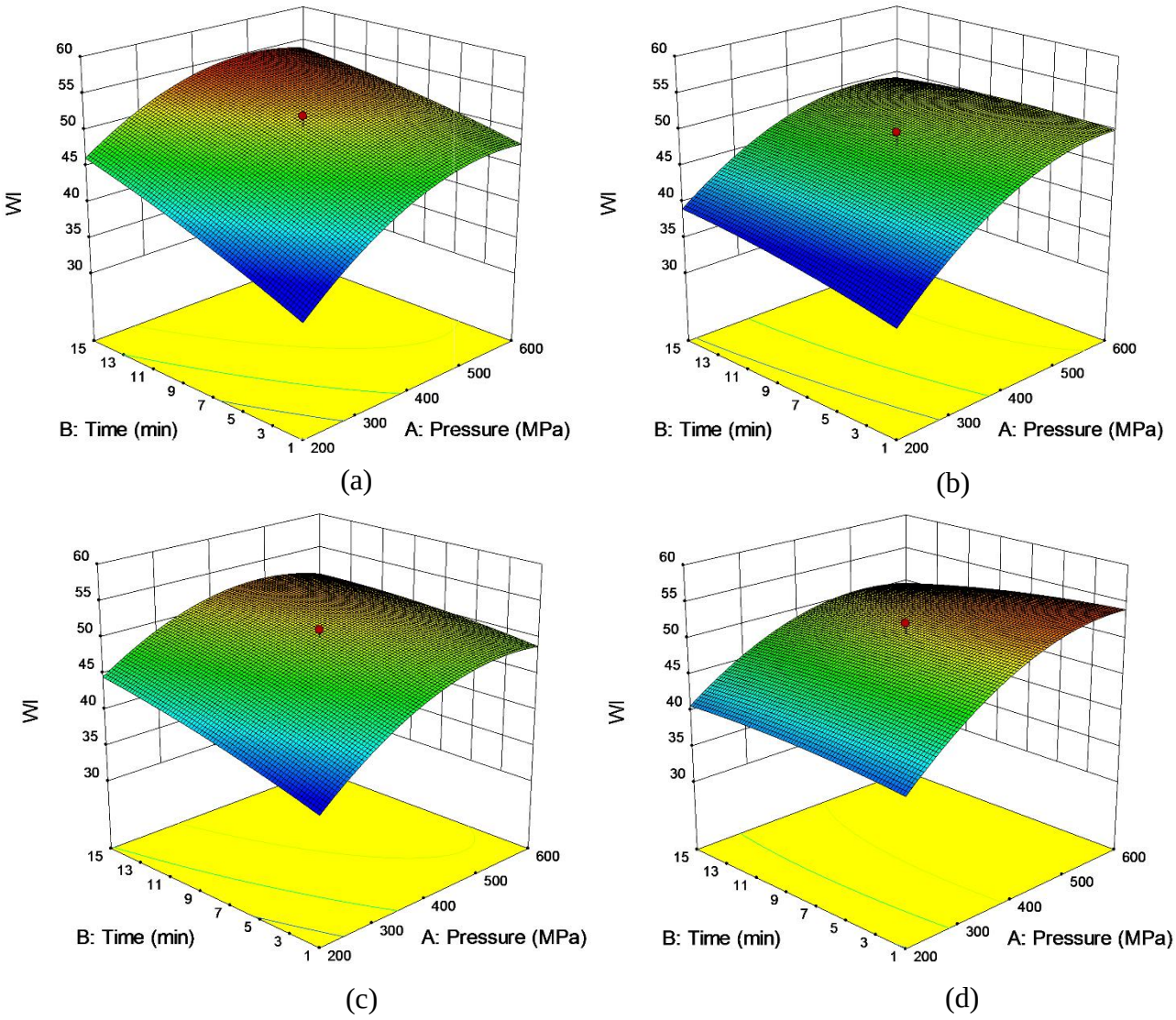


Figure 7 Response surface plots describing the effect of HHP variables (pressure, holding time) on the total whiteness index of beef samples. (a) No EO added; (b) only cumin EO added at highest level; (c) only oregano added at highest level; (d) both cumin and oregano EOs added at highest level

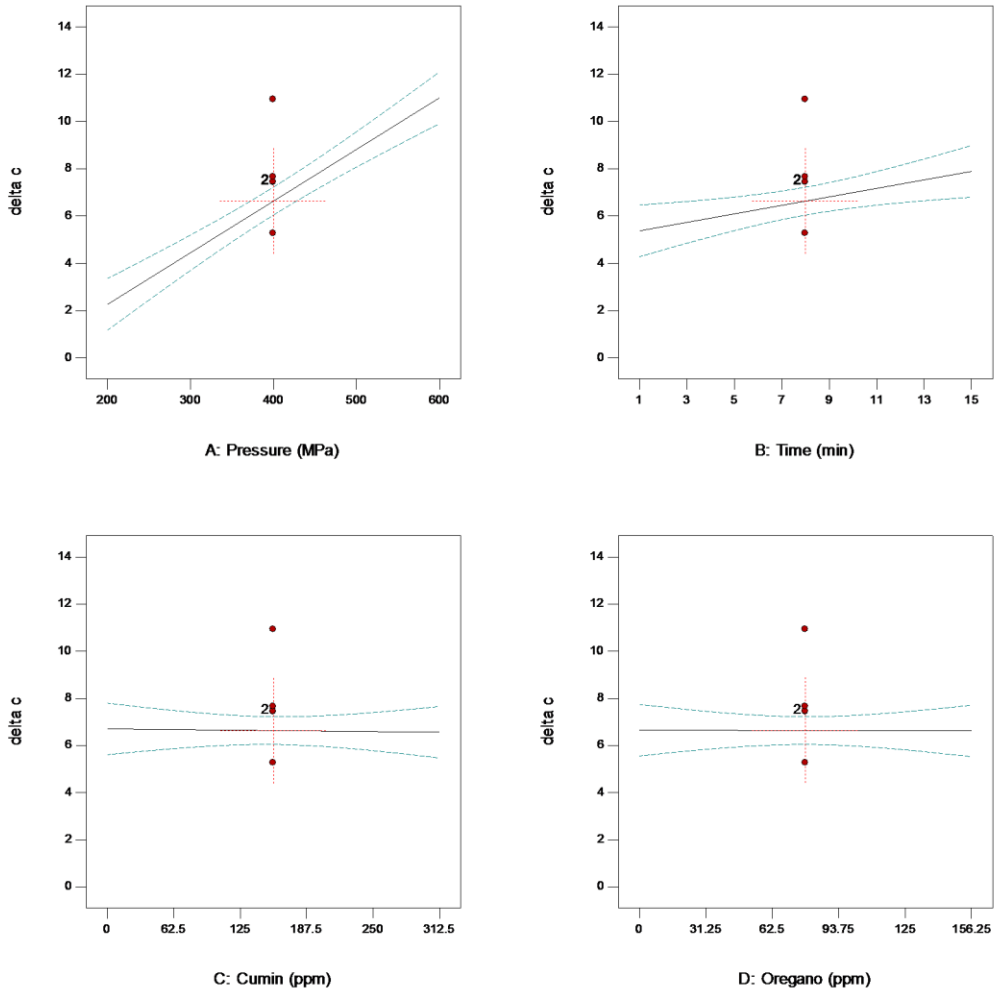


Figure 8 Effects of all factors on chroma of samples

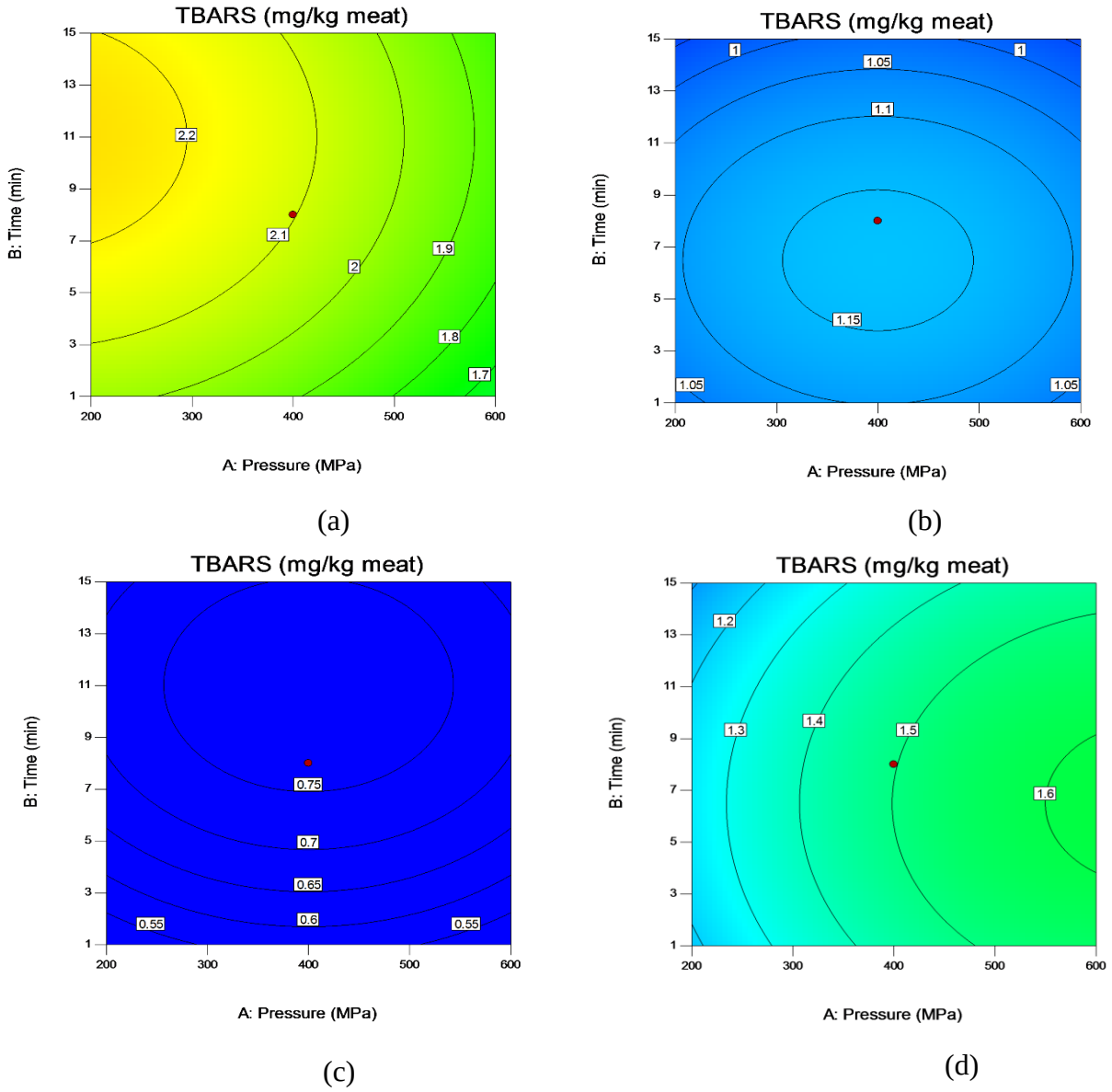
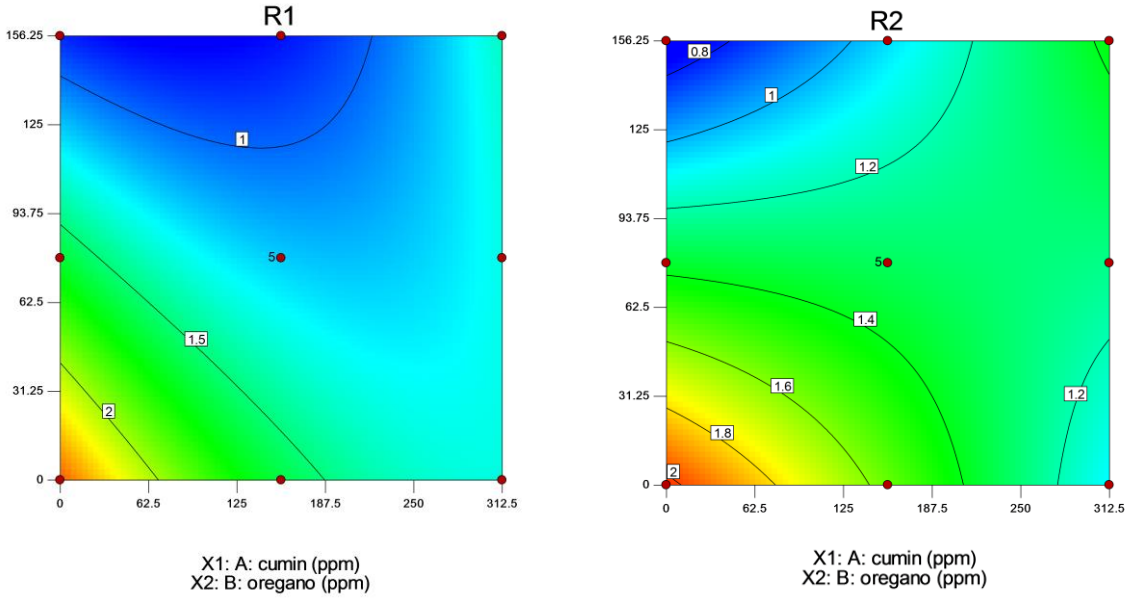


Figure 9 Response surface plots describing the effect of HHP variables (pressure, holding time) on TBARS of beef samples. (a) No EO added; (b) only cumin EO added at highest level; (c) only oregano EO added at highest level; (d) both cumin and oregano EOs added at highest level



(a) (b)

Figure 10 Response surface plots describing the effect of Cumin and Oregano EOs on TBARS: (a) untreated samples, (b) treated at 400 MPa for 8 min

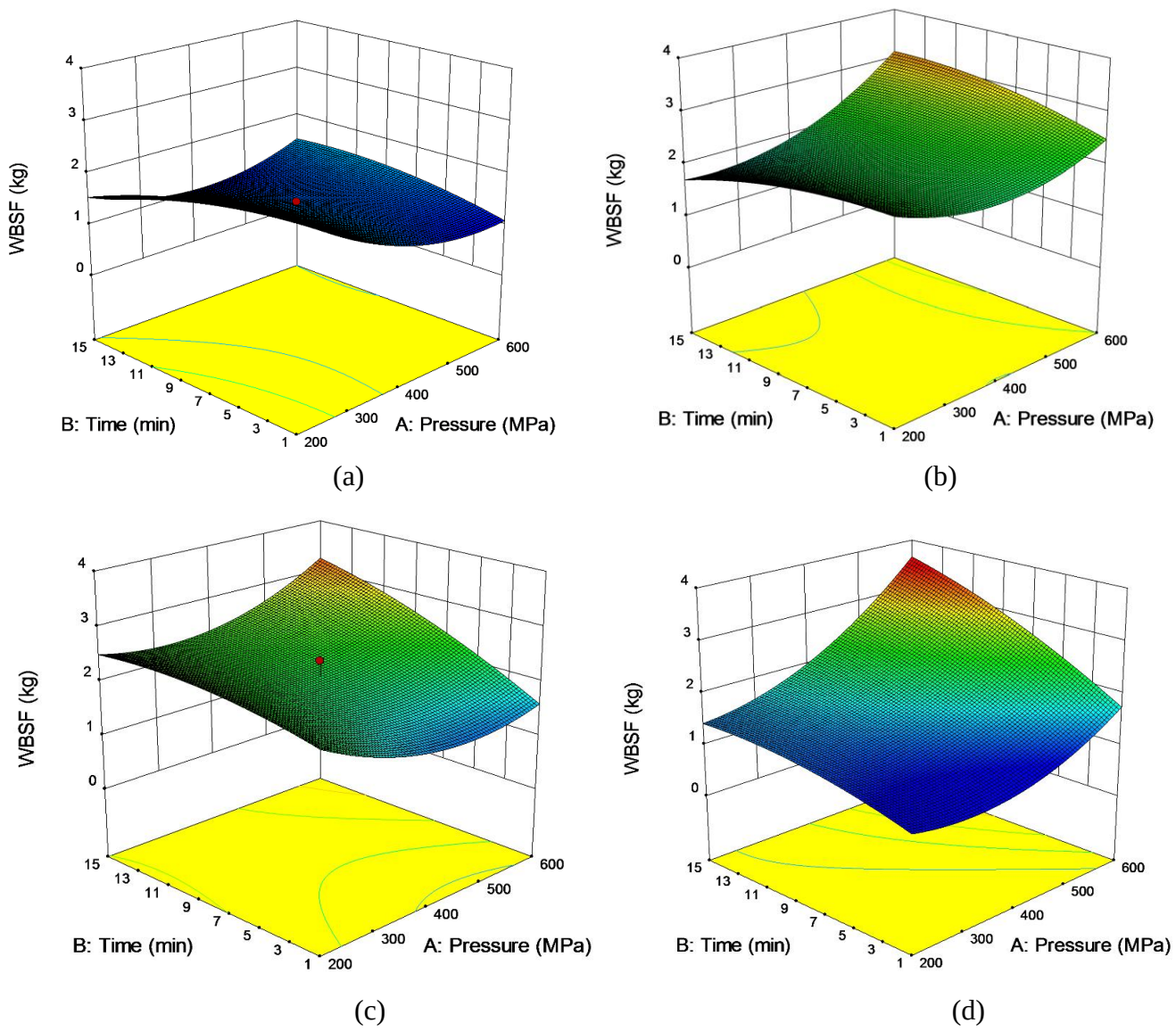


Figure 11 Response surface plots describing the effect of HHP variables (pressure, holding time) on WBSF of beef samples. (a) No EO added; (b) only cumin EO added at highest level; (c) only oregano added at highest level; (d) both cumin and oregano EOs added at highest level

TABLES

Table 12 Levels of factors chosen for the experimental design

			Actual levels of coded factor		
Factors	Symbols	Units	-1	0	+1
Pressure	P	MPa	200	400	600
Time	t	minute	1	8	15
Concentration of cumin EO	C_C	ppm	0	156.25	312.5
Concentration of oregano EO	C_O	ppm	0	78.125	156.25

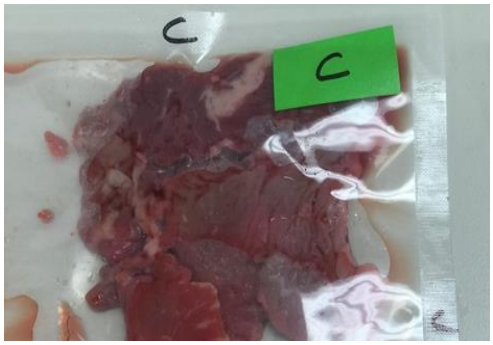
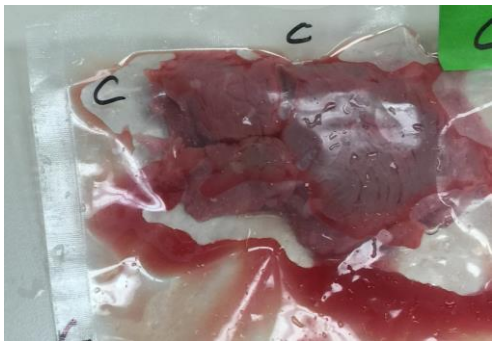
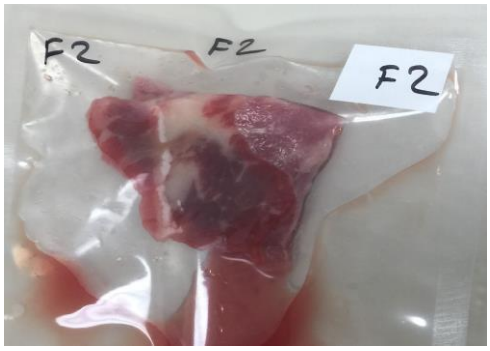
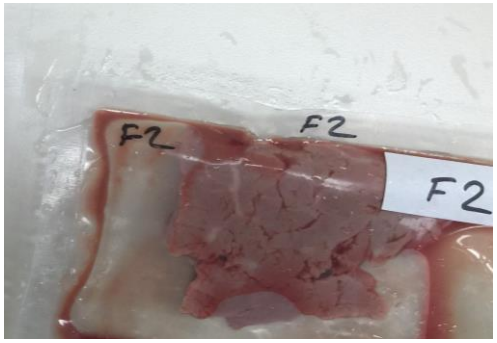
Table 13 Observed and predicted values for a*/b*, ΣE, ΔH, WI, and ΔC

Variables				a*/b		ΣE		ΔH		WI		ΔC	
P (MPa)	t (min)	C (ppm)	O (ppm)	observed	predicted	observed	predicted	observed	predicted	observed	predicted	observed	predicted
200	1	156.25	78.125	9.96	9.25	4.208	2.57	0.045	0.024	39.193	37.425	1.833	1.005
200	8	0	78.125	6.48	6.29	5.27	6.99	-0.076	-0.076	39.729	41.575	2.039	2.334
200	8	156.25	0	4.11	4.99	6.109	5.91	-0.193	-0.072	39.901	40.179	3.911	2.279
200	8	156.25	156.25	6.79	6.67	6.979	7.32	-0.076	-0.060	41.907	41.954	2.21	2.247
200	8	312.5	78.125	6.53	6.34	3.28	4.52	0.01	-0.056	38.287	39.268	0.731	2.192
200	15	156.25	78.125	3.87	4.19	10.034	8.58	-0.123	-0.156	44.745	43.357	3.304	3.521
400	1	0	78.125	3.93	4.02	12.305	12.55	-0.164	-0.292	46.379	46.515	3.143	5.448
400	1	156.25	0	3.43	3.52	11.892	12.88	-0.141	-0.288	45.912	46.794	2.734	5.392
400	1	156.25	156.25	2.68	3.20	15.889	16.65	-0.278	-0.277	49.003	49.720	5.511	5.361
400	1	312.5	78.125	3.66	3.68	14.288	15.27	-0.178	-0.273	47.309	48.709	3.98	5.305
400	8	0	0	1.89	1.73	18.366	17.26	-0.391	-0.387	52.043	50.664	6.95	6.722
400	8	0	156.25	1.7	2.10	18.453	17.03	-0.4322	-0.376	51.203	50.220	7.571	6.690
400	8	156.25	78.125	2.38	1.81	17.706	17.60	-0.43	-0.372	50.822	50.538	7.426	6.635
400	8	156.25	78.125	1.04	1.81	14.262	17.60	-0.435	-0.372	47.283	50.538	7.442	6.635
400	8	156.25	78.125	1.8	1.81	15.817	17.60	-0.299	-0.372	49.891	50.538	5.259	6.635
400	8	156.25	78.125	1.83	1.81	17.568	17.60	-0.402	-0.372	49.524	50.538	7.647	6.635
400	8	156.25	78.125	2.02	1.81	22.612	17.60	-0.668	-0.372	55.174	50.538	10.926	6.635
400	8	312.5	0	1.83	1.73	16.327	14.96	-0.405	-0.368	49.818	48.040	7.015	6.579
400	8	312.5	156.25	1.67	2.14	19.129	17.45	-0.449	-0.357	52.219	50.837	7.88	6.548

Table 12 Observed and predicted values for a*/b*, ΔE, ΔH, WI, and ΔC (Continued)

Variables				a*/b		ΔE		ΔH		WI		ΔC	
P (MPa)	t (min)	C (ppm)	O (ppm)	observed	predicted	observed	predicted	observed	predicted	observed	predicted	observed	predicted
400	15	0	78.125	1.6	1.61	20.307	19.66	-0.464	-0.472	53.723	53.018	7.966	7.964
400	15	156.25	0	1.63	0.76	17.272	18.97	-0.454	-0.468	50.506	51.848	7.768	7.909
400	15	156.25	156.25	2.29	1.85	15.99	17.46	-0.32	-0.456	50.099	51.276	5.698	7.877
400	15	312.5	78.125	2.03	1.98	14.971	15.06	-0.383	-0.452	48.256	48.816	6.974	7.822
600	1	156.25	78.125	1.01	0.99	20.102	18.77	-0.685	-0.589	52.298	50.925	11.146	9.748
600	8	0	78.125	1.23	1.07	17.888	19.11	-0.589	-0.688	50.067	51.145	9.789	11.078
600	8	156.25	0	0.87	1.03	19.822	19.83	-0.762	-0.684	51.002	51.651	12.239	11.022
600	8	156.25	156.25	0.96	0.12	20.136	20.68	-0.723	-0.673	51.812	52.229	11.558	10.991
600	8	312.5	78.125	1.22	1.06	18.954	19.69	-0.596	-0.669	51.232	51.445	10.022	10.935
600	15	0	78.125	1.6	1.61	20.307	19.66	-0.464	-0.472	53.723	53.018	7.966	7.964

Table 14 The visual comparison of the color of untreated (on the left) and treated (on the right) samples (a) 200 MPa, 15 min, 156.25 ppm cumin and 78.125 ppm oregano, (b) 400 MPa, 15 min, 156.25 ppm cumin and 78.125 ppm oregano, and (c) 600 MPa, 15 min, 156.25 ppm cumin and 78.125 ppm oregano

Untreated	Treated
 L*=37.63 a*=17.54 b*=1.714	 P=200 MPa, t=15 min, Cumin=156.25ppm, Oregano=78.12ppm L*=47.49 a*/b*=3.87 a*=14.89 ΣE=10.034 b*=3.848 ΔH=-0.123
 L*=37.63 a*=17.54 b*=1.714	 P=400 MPa, t=15 min, Cumin=312.5ppm, Oregano=78.12ppm L*=50.33 a*/b*=2.03 a*=13.905 ΣE=14.971 b*=6.842 ΔH=-0.383

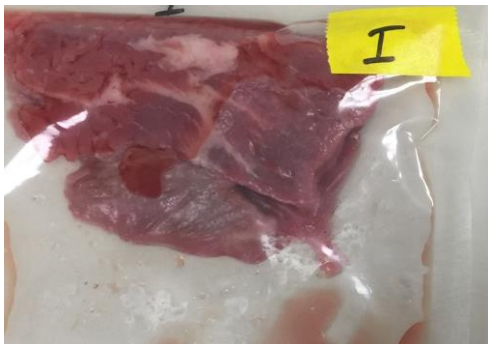
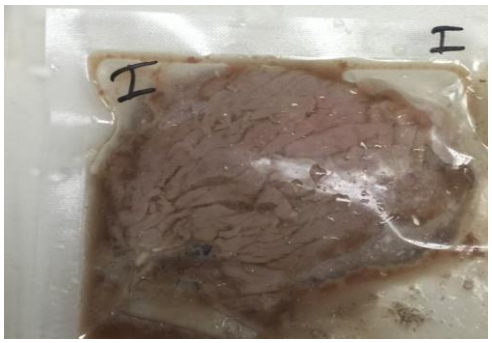
 L*=37.63 a*=17.54 b*=1.714	 P=600 MPa, t=15 min, Cumin=156.25ppm, Oregano=78.12ppm L*=54.75 a*/b*=0.93 a*=9.42 ΣE=11.735 b*=10.12 ΔH=0.781

Table 15 Regression coefficients and ANOVA of the models fitted for color response variables (for actual factors)

	a*/b	ΔE	ΔH	WI	ΔC
Constant	1.810E+01	-2.154E+01	3.277E-01	1.569E+01	-3.460E+00
Pressure	-4.992E-02[a]	1.209E-01[a]	-1.532E-03[a]	1.141E-01[a]	2.186E-02[a]
Time	-8.825E-01[a]	1.411E+00[d]	-1.282E-02[c]	1.178E+00[c]	1.797E-01c
Cumin	-4.940E-03	7.607E-03	6.144E-05	2.909E-03	-4.565E-04
Oregano	1.863E-02	2.228E-02	7.232E-05	1.700E-02	-2.016E-04
Pressure * Time	1.073E-03	-9.127E-04	-	-9.382E-04	-
Pressure * Cumin	-4.800E-07	2.445E-05	-	2.086E-05	-
Pressure * Oregano	-4.144E-05	-8.896E-06	-	-1.914E-05	-
Time * Cumin	1.600E-04	-1.673E-03	-	-1.462E-03	-
Time * Oregano	6.446E-04	-2.413E-03	-	-1.599E-03	-
Cumin * Oregano	6.144E-07	5.560E-05	-	6.638E-05	-
Pressure^2	3.939E-05[a]	-1.032E-04[b]	-	-1.015E-04[b]	-
Time^2	1.445E-02	-2.184E-02	-	-1.331E-02	-
Cumin^2	1.236E-05	-3.633E-05	-	-2.545E-05	-
Oregano^2	-3.084E-05	-5.543E-06	-	3.894E-06	-
R^2	0.9555	0.9139	0.8362	0.8939	0.8128
F value	21.49	10.62	30.64	8.42	26.06
P value (Prob>F)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Statistical significance	Significant[a]	Significant[a]	Significant[a]	Significant[a]	Significant[a]
Lack of fit	Not significant	Not significant	Not significant	Not significant	Not significant

[a] <0.0001; [b] <0.001; [c] <0.01; [d] <0.1

Table 16 Observed and predicted values for TBARS, and WBSF

Variables				TBARS(mg MDH/kg meat)		WBSF (kgf)	
P (MPa)	t (min)	C (ppm)	O (ppm)	observed	predicted	observed	predicted
200	1	156.25	78.125	1.242	1.051	1.991	2.013
200	8	0	78.125	1.312	1.543	2.465	2.513
200	8	156.25	0	1.441	1.435	1.905	2.095
200	8	156.25	156.25	0.818	0.750	1.635	1.803
200	8	312.5	78.125	1.072	1.252	2.528	1.937
200	15	156.25	78.125	1.242	1.097	1.651	1.813
400	1	0	78.125	1.040	1.326	1.423	1.526
400	1	156.25	0	1.072	1.286	1.802	1.528
400	1	156.25	156.25	0.818	0.785	1.066	1.011
400	1	312.5	78.125	1.441	1.356	1.256	1.564
400	8	0	0	2.636	2.103	1.463	1.374
400	8	0	156.25	0.992	0.767	2.395	2.108
400	8	156.25	78.125	1.242	1.250	1.887	1.830
400	8	156.25	78.125	1.316	1.250	1.199	1.830
400	8	156.25	78.125	1.200	1.250	2.385	1.830
400	8	156.25	78.125	1.340	1.250	1.887	1.830
400	8	156.25	78.125	1.150	1.250	1.792	1.830
400	8	312.5	0	1.272	1.161	1.812	2.157
400	8	312.5	156.25	1.300	1.497	1.496	1.643

Table 15 Observed and predicted values for TBARS, and WBSF (Continued)

Variables				TBARS(mg MDH/kg meat)		WBSF (kgf)	
P (MPa)	t (min)	C (ppm)	O (ppm)	observed	predicted	observed	predicted
400	15	0	78.125	1.312	1.507	1.898	1.883
400	15	156.25	0	1.072	1.331	1.675	1.379
400	15	156.25	156.25	0.818	0.831	2.192	2.116
400	15	312.5	78.125	1.441	1.265	1.974	2.163
600	1	156.25	78.125	1.242	1.051	1.848	1.744
600	8	0	78.125	1.312	1.358	1.946	2.187
600	8	156.25	0	1.072	1.250	1.979	2.103
600	8	156.25	156.25	0.818	0.934	2.512	2.615
600	8	312.5	78.125	1.441	1.437	3.480	3.081
600	15	156.25	78.125	1.242	1.097	2.865	2.900

Table 17 Regression coefficients and ANOVA of the models fitted for TBARS for treated and untreated samples and WBSF (for actual factors)

	TBARS (HHP)	TBARS (no HHP)	WBSF
Constant	1.948E+00	2.45E+00	4.664E+00
Pressure	4.952E-04	-	-1.309E-02[e]
Time	4.720E-02	-	-5.777E-02[d]
Cumin	-6.498E-03	-7.29E-03[e]	-4.346E-03
Oregano	-8.698E-03[c]	-1.12E-02[c]	-1.392E-03
Pressure * Time	-5.833E-19	-	2.423E-04[e]
Pressure * Cumin	2.952E-06	-	1.177E-05[e]
Pressure * Oregano	5.904E-06	-	1.286E-05
Time * Cumin	-6.217E-05	-	5.542E-05
Time * Oregano	-5.041E-19	-	5.732E-04
Cumin * Oregano	3.424E-05[c]	3.37E-05[c]	-2.556E-05
Pressure^2	-1.772E-06	-	1.167E-05[c]
Time^2	-2.140E-03	-	-3.652E-03
Cumin^2	8.960E-06[c]	1.19E-05[e]	5.451E-06
Oregano^2	-1.417E-05	6.95E-06	-2.330E-05
R^2	0.6962	0.9037	0.7372
F value	2.29	13.13	2.80
P value (Prob>F)	0.0663	0.0019	0.0317
Statistical significance	Significant[e]	Significant	Significant[d]
Lack of fit	Not significant	Significan	Not significant

[a] <0.0001; [b] <0.001; [c] <0.01; [d] <0.01; [e]<0.1

Table 18 Constrains for the optimization

Name	Goal	Lower Limit	Upper Limit	Importance
A:Pressure	is in range	200	600	3
B:Time	is in range	1	15	3
C:Cumin	is in range	0	312.5	3
D:Oregano	is in range	0	156.25	3
log reduction	is target = 5	1.6	5.8	5
delta H	none	-0.762	0.045	3
delta E	minimize	3.28	22.612	4
delta c	none	0.731	12.239	3
WI	none	38.287	55.174	3
a*/b*	maximize	0.87	9.96	4
TBARS	minimize	0.818	2.636	4
WBSF	none	1.0656	3.4804	3

Table 19 Optimum Conditions

Pressure (MPa)	Time (min)	Cumin (ppm)	Oregano (ppm)	log reduction	delta H	delta E	delta c	WI	a*/b*	TBARS	WBSF	Desirability
200.0	14.9	312.3	156.2	2.50	-0,139	4.701	3.412	40.849	5.719	1.095	1.433	0.552
336.2	15.0	312.5	156.2	3.09	-0,349	12.568	6.412	47.526	3.076	1.28	1.77	0.45
594.5	1.0	0.4	0.0	4.95	-0,596	14.68	9.715	48.276	2.11	1.659	1.088	0.437

CHAPTER VI

CONCLUSION

Ready-to-eat meat products treated by high pressure have been on the market shelves for years. However, it has some undesired effects on the quality of fresh red meat; such as discoloration and induced lipid oxidation. Therefore researchers have started to combine HHP with other preservation methods in the hurdle concept. EOs are consisting of secondary metabolites which involves the self defense mechanism of plants against microorganisms or insects. Thus, they have good antimicrobial properties naturally. Because of the rising health conscious among consumers, recently, researchers have shown an increased interest in developing minimally processed and more natural products. Although both HHP and EOs have a good potential with their effectiveness against spoilage and pathogen microorganisms, they have limitations due to their negative effects on organoleptic properties of food. In this study, it is aimed to decrease intensity of pressure and shorten the process time by the help of EOs to achieve the sufficient microbial inactivation. Thus, the potential of a hurdle technology of HHP and combination for fresh likely beef product was evaluated.

This research was divided into three parts. In the first part, the aim was to assess antimicrobial effect of different essential oils alone or in a combination against *E. coli* ATCC 25922 with or without HHP treatment. Cumin, oregano and thyme were found the most effective three EOs against *E. coli* ATCC 25922. These EOs were also evaluated after HHP treatment (400 MPa for 10

min at 20°C). Their minimum inhibitory concentrations decreased half of their untreated MIC values. None of the combinations of those three EOs has not shown synergy without HHP treatment. However with HHP, all combinations showed synergy. These findings suggest that HHP and EOs (alone or in combination) enhance each other's antimicrobial activation. Cumin and oregano EOs combination was described the best combination because of their lowest fractional inhibitory concentration index (FICI). Thus, it was selected for the further studies.

Secondly, the effect of selected antimicrobials with HHP on the inactivation of *E. coli* ATCC 25922 in beef describes. Response surface methodology, using Box–Behnken design and regression analysis, was used to understand the contribution of four variables, pressure, process time and concentration of cumin and oregano EOs on inactivation of *E. coli* ATCC 25922. A predictive model was established, and the adequacy of the predictive model was validated. Second-order polynomial models and response surface plots were generated. Log reduction after HHP was significantly affected by all variables except oregano. It can be said that cumin's effectiveness increased under pressure. The theoretical implications of these findings are unclear and should be taken seriously for further research. In addition, samples were stored for short term (3 days) and long term (120 days) and not a significant post growth observed in contrast to earlier findings indicating complete recovery of *E. coli* ATCC 25922 after 120 h of incubation at 25°C after HHP. It can be conclude that *E.coli* recovery after pressure application can be impeded when cumin and oregano EOs were added. Also our results suggest that there is an additive effect of pressure application on activity of cumin EO.

In the last study, the combination of HHP and EOs on beef quality was evaluated and optimum process conditions and antimicrobial concentrations for effective reduction of the bacteria

and maintenance or improvement of beef quality were identified by RSM. Color indices were significantly affected by pressure, time and their interactions. Above 400 MPa the discoloration was similar to cooked beef and EO addition did not help color improvement. Oxidation was not increased immediately after the pressure treatment, but pressure induced lipid oxidation during long term storage of the meat. Oregano essential oil's concentration and its interaction with cumin are found statistically important ($P<0.01$) on controlling lipid oxidation. Thus it can be concluded that EOs at least minimize lipid oxidation during the storage and help to extend shelf life of beef. Contrary to expectations the combination of them showed less antioxidant effect then their single use. These results would seem to suggest that the combination of essential oils exerted an antagonistic effect on lipid oxidation. WBSF is the most common used indicator of tenderness of meat. EOs increased tenderness. However, the maximum value of WBSF is 3.5 kgf which is still reasonable for beef meat. The optimum process conditions were decided as pressure=200 MPa, time= 14.9 min, cumin concentrations=312.3 ppm, and oregano concentration= 156.2 with 0.552 desirability. RSM was found effective to analyze combination treatments involving hydrostatic pressure, and EOs concentration to control undesirable effects of pressure on beef. The development of a response surface model to describe the optimization of HHP-EOs combinations' conditions will be beneficial for the development of an *E. coli* safe beef product with desired quality parameters

Overall, this study strengthens the idea that applying HHP and EOs in the hurdle concept have a great potential to produce microbiologically safe and better quality beef product. Moreover RSM effectively analyzed combination treatments involving hydrostatic pressure, and EOs

concentration to control undesirable effects of pressure on beef. Besides having better inhibition of pathogens, the limitations of their usage can be eliminated.

CHAPTER VII

FUTURE RECOMMENDATIONS

Further research in the area of sensory testing is needed to understand the level of EOs that can be added to various formulations and still maintain the expected sensory characteristics of the product. The scope of this study was limited in terms of the variety of EOs. Therefore a future study investigating the synergistic effect of other essential oils combination with HHP would be very interesting. It is suggested that this hurdle concept should be evaluated against other food pathogens. More detailed research is needed to better understand how the antimicrobial mechanism of EOs and the HHP combination works together. This would provide a knowledge to make selection of the EOs and HHP combinations more consciously. A further study could assess the long-term effects of EOs-HHP combination on the microbial, physical, and organoleptic quality of meat products.

APPENDICES

Appendix A: Experimental Designs and Data Sets

Table 20 Data sheet for Chapter IV (Factors and responses)

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3
Std	Run	A:Pressure	B:Time	C:Cumin	D:Oregano	log reduction	log(N/N0), t=3days	log(N/N0), t=120
		MPa	min	ppm	ppm			
1	28	200	1	156,25	78,125	1,6	1,94	0,14
11	8	200	8	0	78,125	1,95	1,9	0,2
9	11	200	8	156,25	156,25	2,06	1,77	0,17
19	16	200	8	312,5	78,125	2,07	2,31	0,91
17	25	200	8	156,25	0	1,7	1,36	0,06
3	5	200	15	156,25	78,125	1,9	1,87	0,27
21	4	400	1	312,5	78,125	2,32	1,82	0,82
15	15	400	1	0	78,125	2,1	1,48	0,68
23	27	400	1	156,25	0	2,54	2,54	1,34
13	29	400	1	156,25	156,25	2,24	2,07	1,4
26	3	400	8	156,25	78,125	3,32	3,04	0,54
6	7	400	8	312,5	0	3,54	3,55	2,55
7	9	400	8	156,25	78,125	3,5	3,2	2,33
8	17	400	8	312,5	156,25	3,89	4,18	3,18
5	19	400	8	0	156,25	2,95	3,67	2,57
25	22	400	8	0	0	2,5	2,5	1,7
29	23	400	8	156,25	78,125	3,26	3,38	2,48
27	24	400	8	156,25	78,125	3,2	2,96	2,26
28	26	400	8	156,25	78,125	3,56	3,8	3
24	10	400	15	156,25	156,25	3,38	3,47	2,47
16	12	400	15	156,25	0	3,38	3,38	2,08
14	18	400	15	0	78,125	2,95	3,72	3,02
22	20	400	15	312,5	78,125	3,6	3,6	2,8
2	13	600	1	156,25	78,125	7,8	7,8	----
20	1	600	8	312,5	78,125	7,8	7,8	----
12	2	600	8	0	78,125	7,8	7,8	----
18	6	600	8	156,25	0	7,8	7,8	----
10	21	600	8	156,25	156,25	7,8	7,8	----
4	14	600	15	156,25	78,125	7,8	7,8	----

Table 21 Data sheet for Chapter V (Factors and responses)

Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Response 7	Response 8
A:Pressure	B:Time	C:Cumin	D:Oregano	log reduction	delta H	delta E	delta c	WI	a*/b*	TBARS	WBSF
MPa	min	ppm	ppm							mg/kg meat	kg
400	15	156,25	156,25	3,38	-0,32	15,99	5,698	50,099	2,29	0,818	2,1922
400	8	156,25	78,125	3,32	-0,43	17,706	7,426	50,822	2,38	1,242	1,887
400	8	312,5	0	3,54	-0,405	16,327	7,015	49,818	1,83	1,272	1,81215
400	8	156,25	78,125	3,5	-0,435	14,262	7,442	47,283	1,04	1,316	1,1985
600	8	312,5	78,125	5,8	-0,596	18,954	10,022	51,232	1,22	1,441	3,4804
200	8	0	78,125	1,95	-0,076	5,27	2,039	39,729	6,48	1,312	2,4645
200	15	156,25	78,125	1,9	-0,123	10,034	3,304	44,745	3,87	1,242	1,6508
200	8	156,25	156,25	2,06	-0,076	6,979	2,21	41,907	6,79	0,818	1,63465
400	8	312,5	156,25	3,89	-0,449	19,129	7,88	52,219	1,67	1,3	1,4963
600	15	156,25	78,125	5,8	-0,734	20,817	11,735	52,596	0,93	1,242	2,8645
400	8	0	156,25	2,95	-0,4322	18,453	7,571	51,203	1,7	0,992	2,3952
600	8	0	78,125	5,8	-0,589	17,888	9,789	50,067	1,23	1,312	1,94645
400	8	0	0	2,5	-0,391	18,366	6,95	52,043	1,89	2,636	1,4628
400	15	156,25	0	3,38	-0,454	17,272	7,768	50,506	1,63	1,072	1,6752
200	8	312,5	78,125	2,07	0,01	3,28	0,731	38,287	6,53	1,072	2,5277
600	1	156,25	78,125	5,8	-0,685	20,102	11,146	52,298	1,01	1,242	1,84755
600	8	156,25	0	5,8	-0,762	19,822	12,239	51,002	0,87	1,072	1,97865
200	8	156,25	0	1,7	-0,193	6,109	3,911	39,901	4,11	1,441	1,9053
400	8	156,25	78,125	3,26	-0,299	15,817	5,259	49,891	1,8	1,2	2,3845
400	1	312,5	78,125	2,32	-0,178	14,288	3,98	47,309	3,66	1,441	1,25585
400	15	0	78,125	2,95	-0,464	20,307	7,966	53,723	1,6	1,312	1,8977
400	8	156,25	78,125	3,2	-0,402	17,568	7,647	49,524	1,83	1,34	1,887
400	1	0	78,125	2,1	-0,164	12,305	3,143	46,379	3,93	1,04	1,4225

400	15	312,5	78,125	3,6	-0,383	14,971	6,974	48,256	2,03	1,441	1,9735
400	1	156,25	0	2,54	-0,141	11,892	2,734	45,912	3,43	1,072	1,8024
600	8	156,25	156,25	5,8	-0,723	20,136	11,558	51,812	0,96	0,818	2,512
400	1	156,25	156,25	2,24	-0,278	15,889	5,511	49,003	2,68	0,818	1,0656
200	1	156,25	78,125	1,6	0,045	4,208	1,833	39,193	9,96	1,242	1,9908
400	8	156,25	78,125	3,56	-0,668	22,612	10,926	55,174	2,02	1,15	1,7915

Appendix B: Optimization

Table 22 Optimum conditions for minimizing total color change and TBARS, maximizing discoloration and targeting 5 log reduction of *E. coli*

Number	Pressure	Time	Cumin	Oregano	log reduction	delta H	delta E	delta c	WI	a*/b*	TBARS	WBSF	Desirability
1	200,000	14,986	312,490	156,248	2,501	-0,140	4,661	3,432	40,832	5,710	1,092	1,432	0,552
2	200,000	14,767	312,500	156,249	2,504	-0,137	4,731	3,392	40,857	5,730	1,099	1,434	0,552
3	200,000	14,826	312,162	156,249	2,503	-0,138	4,721	3,403	40,857	5,723	1,096	1,434	0,552
4	200,044	14,939	312,498	156,250	2,502	-0,140	4,679	3,424	40,840	5,713	1,093	1,432	0,552
5	200,000	14,726	310,956	156,250	2,504	-0,137	4,784	3,386	40,892	5,725	1,095	1,437	0,552
6	200,000	14,986	310,792	156,249	2,500	-0,140	4,706	3,432	40,867	5,702	1,085	1,435	0,552
7	200,000	14,510	311,773	156,248	2,507	-0,134	4,829	3,346	40,898	5,750	1,105	1,438	0,552
8	200,000	14,390	312,494	156,250	2,509	-0,133	4,847	3,324	40,896	5,766	1,112	1,437	0,551
9	200,000	14,851	309,421	156,248	2,502	-0,139	4,785	3,409	40,909	5,706	1,084	1,439	0,551
10	200,000	14,335	312,492	156,249	2,509	-0,132	4,864	3,315	40,902	5,771	1,114	1,438	0,551
11	200,000	14,137	312,498	156,249	2,511	-0,129	4,922	3,279	40,921	5,792	1,121	1,439	0,551
12	200,000	14,805	312,422	155,123	2,504	-0,138	4,721	3,399	40,841	5,714	1,099	1,440	0,551
13	200,000	14,156	309,115	156,250	2,509	-0,130	5,001	3,284	40,983	5,773	1,106	1,445	0,551
14	200,002	14,998	305,890	156,250	2,497	-0,141	4,831	3,437	40,964	5,676	1,064	1,443	0,551
15	200,001	13,958	312,500	156,240	2,511	-0,127	4,973	3,247	40,937	5,812	1,127	1,440	0,551
16	200,000	14,003	309,461	156,248	2,510	-0,128	5,035	3,256	40,990	5,792	1,113	1,445	0,551
17	200,000	14,828	302,759	156,250	2,499	-0,139	4,964	3,408	41,044	5,675	1,058	1,451	0,551
18	200,000	14,302	303,339	156,039	2,506	-0,132	5,103	3,313	41,078	5,727	1,079	1,456	0,551
19	200,001	14,289	301,879	156,249	2,505	-0,132	5,143	3,311	41,109	5,724	1,073	1,458	0,551
20	200,000	15,000	312,500	154,110	2,500	-0,141	4,656	3,434	40,807	5,687	1,094	1,443	0,551

21	200,451	14,291	308,543	156,249	2,509	-0,132	5,009	3,318	41,011	5,745	1,100	1,446	0,551
22	200,001	14,838	306,054	155,148	2,500	-0,139	4,877	3,408	40,966	5,679	1,072	1,451	0,551
23	200,000	13,724	312,491	156,249	2,511	-0,124	5,037	3,205	40,957	5,840	1,134	1,441	0,551
24	200,001	13,658	310,713	156,250	2,511	-0,123	5,099	3,194	40,995	5,839	1,129	1,444	0,551
25	200,000	14,649	312,489	153,189	2,506	-0,136	4,766	3,372	40,834	5,708	1,107	1,452	0,550
26	200,000	13,693	305,197	156,249	2,509	-0,124	5,222	3,202	41,093	5,808	1,105	1,454	0,550
27	201,133	14,676	309,547	156,249	2,507	-0,138	4,918	3,402	40,997	5,695	1,092	1,441	0,550
28	200,000	15,000	295,061	156,070	2,491	-0,141	5,109	3,442	41,176	5,621	1,022	1,464	0,550
29	200,000	13,311	301,903	156,249	2,506	-0,119	5,393	3,135	41,175	5,841	1,103	1,461	0,550
30	200,000	14,796	291,479	156,249	2,492	-0,139	5,258	3,407	41,264	5,624	1,016	1,473	0,550
31	200,000	13,085	312,500	156,250	2,508	-0,116	5,201	3,090	41,004	5,922	1,153	1,441	0,549
32	200,762	13,868	312,500	152,993	2,513	-0,127	5,045	3,248	40,951	5,771	1,135	1,461	0,549
33	200,000	14,742	312,500	149,632	2,504	-0,138	4,735	3,389	40,783	5,662	1,108	1,471	0,549
34	200,005	14,139	283,879	156,249	2,495	-0,131	5,612	3,292	41,447	5,658	1,009	1,495	0,549
35	200,000	12,920	303,362	156,249	2,503	-0,114	5,449	3,064	41,170	5,903	1,120	1,458	0,549
36	200,000	14,072	312,499	148,687	2,511	-0,129	4,923	3,269	40,831	5,722	1,132	1,484	0,549
37	202,332	14,325	301,466	156,151	2,509	-0,136	5,312	3,369	41,260	5,658	1,074	1,460	0,549
38	203,486	14,762	312,494	156,249	2,512	-0,143	4,983	3,468	41,076	5,644	1,105	1,437	0,548
39	200,000	12,278	290,667	156,249	2,487	-0,107	5,848	2,955	41,393	5,946	1,085	1,481	0,547
40	200,000	14,985	273,613	156,250	2,474	-0,143	5,640	3,449	41,585	5,528	0,945	1,508	0,546
41	200,000	12,489	270,711	156,247	2,479	-0,111	6,212	3,002	41,692	5,832	1,006	1,527	0,546
42	200,038	13,728	312,430	141,961	2,511	-0,125	4,996	3,209	40,773	5,692	1,149	1,525	0,546
43	200,000	15,000	312,499	142,097	2,497	-0,141	4,656	3,437	40,673	5,554	1,106	1,504	0,546
44	200,000	14,542	264,255	156,243	2,474	-0,138	5,964	3,374	41,773	5,533	0,929	1,534	0,545
45	200,001	12,111	264,878	156,249	2,468	-0,106	6,372	2,937	41,766	5,875	0,995	1,539	0,545
46	207,498	14,719	312,495	156,250	2,521	-0,148	5,283	3,547	41,330	5,549	1,113	1,441	0,545
47	200,000	14,234	312,497	138,169	2,506	-0,132	4,859	3,300	40,689	5,590	1,136	1,538	0,544
48	200,000	12,347	251,638	156,250	2,462	-0,110	6,584	2,985	41,955	5,789	0,946	1,573	0,544
49	200,003	12,357	247,911	156,243	2,458	-0,110	6,649	2,988	42,005	5,775	0,934	1,583	0,543

50	207,584	13,648	307,381	156,250	2,526	-0,135	5,731	3,359	41,537	5,628	1,127	1,455	0,543
51	200,001	14,302	312,497	135,105	2,504	-0,133	4,836	3,313	40,647	5,547	1,135	1,552	0,543
52	200,000	15,000	312,498	133,960	2,492	-0,142	4,654	3,439	40,584	5,459	1,112	1,542	0,542
53	200,000	11,826	236,970	156,249	2,437	-0,104	6,875	2,898	42,108	5,838	0,911	1,609	0,541
54	200,001	12,430	233,914	156,248	2,445	-0,112	6,881	3,008	42,190	5,721	0,890	1,620	0,540
55	211,565	14,299	309,374	156,249	2,534	-0,149	5,780	3,562	41,687	5,470	1,121	1,453	0,540
56	200,000	10,972	234,869	156,250	2,410	-0,094	6,942	2,746	42,054	6,003	0,919	1,606	0,538
57	200,001	11,098	230,606	156,247	2,410	-0,095	7,000	2,770	42,112	5,965	0,904	1,620	0,538
58	200,026	10,812	212,324	156,249	2,380	-0,093	7,243	2,728	42,258	5,984	0,858	1,671	0,534
59	200,228	10,214	261,444	140,771	2,403	-0,084	6,456	2,605	41,475	6,118	1,058	1,624	0,533
60	200,000	12,753	312,500	113,856	2,485	-0,115	5,042	3,039	40,406	5,529	1,190	1,686	0,533
61	200,000	14,999	312,498	114,766	2,471	-0,143	4,649	3,442	40,375	5,218	1,118	1,619	0,530
62	200,000	11,153	312,497	110,491	2,446	-0,094	5,155	2,752	40,296	5,811	1,227	1,727	0,529
63	200,000	11,696	291,030	105,225	2,448	-0,103	5,575	2,860	40,635	5,539	1,164	1,763	0,525
64	200,000	12,547	191,803	149,755	2,388	-0,117	7,519	3,049	42,646	5,544	0,818	1,770	0,525
65	200,000	14,998	312,499	103,220	2,452	-0,144	4,644	3,444	40,250	5,063	1,117	1,657	0,523
66	200,001	8,777	195,132	156,250	2,255	-0,068	7,245	2,369	42,011	6,468	0,831	1,685	0,520
67	221,546	12,603	231,739	156,249	2,500	-0,148	8,413	3,511	43,551	5,117	0,910	1,618	0,520
68	200,000	11,857	173,693	146,264	2,348	-0,109	7,733	2,934	42,696	5,616	0,818	1,840	0,517
69	237,344	15,000	290,916	156,250	2,587	-0,199	7,744	4,260	43,452	4,737	1,063	1,512	0,517
70	200,001	9,271	312,497	95,307	2,337	-0,071	4,950	2,417	39,835	6,130	1,253	1,829	0,515
71	200,000	13,762	312,494	84,475	2,435	-0,130	4,788	3,226	40,032	4,975	1,149	1,766	0,513
72	200,001	9,504	312,497	73,636	2,314	-0,076	4,662	2,463	39,446	5,815	1,233	1,942	0,506
73	200,000	10,439	246,579	89,963	2,355	-0,091	6,277	2,658	40,961	5,514	1,132	1,889	0,504
74	200,001	15,000	312,500	69,052	2,369	-0,147	4,618	3,452	39,888	4,554	1,092	1,733	0,494
75	200,060	15,000	312,472	64,829	2,356	-0,147	4,620	3,454	39,849	4,485	1,087	1,739	0,490
76	200,000	14,947	312,500	53,947	2,321	-0,147	4,607	3,445	39,728	4,314	1,072	1,753	0,479
77	200,003	8,171	312,498	36,527	2,131	-0,062	3,822	2,231	38,416	5,775	1,186	2,135	0,472
78	200,022	11,118	40,522	156,250	1,999	-0,107	8,382	2,861	43,200	5,924	0,680	2,360	0,431

79	436,056	1,000	312,500	0,001	2,940	-0,334	14,161	6,109	47,717	3,068	1,099	2,013	0,431
80	434,261	1,000	312,274	0,000	2,920	-0,331	14,094	6,070	47,662	3,094	1,099	2,010	0,431
81	430,839	1,000	309,919	0,000	2,882	-0,326	13,978	5,996	47,566	3,138	1,099	1,997	0,431
82	432,432	1,045	312,423	0,000	2,906	-0,329	14,044	6,038	47,616	3,106	1,100	2,012	0,431
83	445,143	1,000	312,494	0,470	3,049	-0,348	14,509	6,308	48,006	2,939	1,100	2,022	0,430
84	426,541	1,103	312,498	0,000	2,847	-0,321	13,841	5,920	47,442	3,175	1,102	2,010	0,430
85	442,234	1,000	304,132	0,001	3,020	-0,344	14,436	6,248	47,948	2,957	1,096	1,984	0,430
86	446,286	1,080	312,497	0,001	3,075	-0,350	14,569	6,347	48,048	2,896	1,099	2,029	0,430
87	423,343	1,179	312,498	0,000	2,823	-0,317	13,748	5,863	47,357	3,198	1,104	2,012	0,430
88	444,725	1,111	312,500	0,000	3,061	-0,348	14,527	6,319	48,011	2,908	1,100	2,029	0,430
89	465,134	1,008	312,498	0,000	3,296	-0,378	15,169	6,746	48,541	2,673	1,094	2,053	0,428
90	420,593	1,512	312,500	0,000	2,845	-0,317	13,785	5,863	47,361	3,127	1,112	2,027	0,428
91	593,010	1,000	0,155	46,304	4,975	-0,590	15,343	9,673	48,480	1,803	1,392	1,351	0,424
92	361,148	3,385	312,498	10,410	2,542	-0,249	12,165	4,898	45,848	3,535	1,184	2,046	0,422
93	430,242	1,000	221,307	0,004	2,899	-0,330	14,207	6,024	47,821	3,007	1,146	1,672	0,420
94	449,632	1,000	0,002	108,318	2,877	-0,366	14,564	6,527	48,006	2,938	1,036	1,448	0,418
95	449,510	1,000	0,000	109,013	2,874	-0,366	14,571	6,524	48,009	2,936	1,030	1,448	0,418
96	461,094	1,000	0,000	91,368	3,029	-0,385	14,602	6,781	48,096	2,839	1,180	1,431	0,417
97	451,440	1,000	202,911	0,000	3,146	-0,364	14,906	6,495	48,420	2,701	1,163	1,611	0,417
98	471,044	1,000	0,000	85,268	3,156	-0,400	14,740	6,999	48,220	2,716	1,225	1,416	0,416
99	465,406	1,000	0,001	84,009	3,085	-0,392	14,595	6,876	48,118	2,810	1,240	1,419	0,416
100	451,799	1,000	9,021	92,985	2,938	-0,370	14,591	6,573	48,085	2,943	1,154	1,430	0,416

Appendix C: Certificate of Analysis of Essential oils

CERTIFICATE OF ANALYSIS

Common Name	:	Basil, Sweet ct. Linalool - ORGANIC
Latin Name	:	*Ocimum basilicum*
Country of Origin	:	Egypt
Lot Index #	:	*5
Cultivation Method	:	Cultivated - Certified Organic (NOP, EU, JAP)
Type	:	Essential Oil
Extraction Method	:	Steam Distilled
Plant Part	:	Leaves and Tops
Use	:	Aromatherapy, Natural Perfumery
Manufactured	:	August 2014

*Located on the bottom right hand corner of each label. The Lot Index # is the last dash number after the SKU and Size Reference Code. Example: 445-2-3 refers to SKU-Size Reference Code-Lot Index #.

Specific Gravity @20°C:	0.901
Refractive Index @20°C:	1.473
Optical Rotation @20°C:	*n/a*

Physical appearance	:	Pale yellow liquid
Odor	:	Aromatic-herbal, clove, citrus, minty and penetrating
Solubility	:	Soluble in alcohol and fixed oils
Primary Constituents	:	Linalool, 1,8-Cineole, Eugenol, Methyl Chavicol

Comments : Odor quality is excellent.

Chromatographic Values of the Volatile Components of the Oil (in %):

Components	%		Components	%
1 – Camphene	0.47		16 – Bornyl Acetate	0.95
2 – Sabinene	0.08		17 – Eugenol	5.68
3 – β-Pinene	1.04		18 – β-Elemene	1.28
4 – Myrcene	1.24		19 – α-Bergamotene(E)	5.72
5 – Cineole-1,8	8.51		20 – α-Humulene	0.75
6 – Ocimene (E)	0.67		21 – Germacrene D	3.26

7 – γ -Terpinene	0.11		22 – α-Selinene	2.70
8 – Terpinolene	0.07		23 – δ-Cadinene	2.57
9 – Linalool	52.52		24 – α-Bulnesene	0.52
10 – Camphor	0.57		25 – γ-Cadinene	0.10
11 – Borneol	0.28		26 – Muurolol	0.42
12 – Terpinen 1-ol-4	0.25		27 – Cadinol	2.39
13 – α-Terpineol	0.77			
14 – Estragol	0.98			
15 – Geraniol	0.11			

This Essential Oil is an authentic natural product and does not contain any artificial ingredients or adulteration of any kind to the best of my knowledge. The analysis and statements herein constitute the most complete information available to Eden Botanicals. This product is guaranteed by Eden Botanicals to be of excellent quality.

Eden Botanicals
www.edenbotanicals.com
info@edenbotanicals.com
Tel: 1-707-509-0041 / Fax: 1-707-949-2526
Document updated: 12.09.14

1114-4219-95

CERTIFICATE OF ANALYSIS

Common Name	:	Black Cumin Seed CO2 - ORGANIC
Latin Name	:	*Nigella sativa*
Country of Origin	:	India
Lot Index #	:	*8
Cultivation Method	:	Cultivated
Type	:	CO2 Total Extract
Extraction Method	:	Super Critical Extraction
Plant Part	:	Seeds
Use	:	Body/Skin Care
Manufactured	:	August 2014
Best By	:	August 2019

Specific Gravity @20°C:	0.918
Refractive Index @20°C:	1.4747
Optical Rotation @20°C:	*na*

Physical appearance	:	Clear to pale yellow transparent liquid
Odor	:	Spicy, warm
Solubility	:	Soluble in fixed oils, Insoluble in alcohol
Primary Constituents	:	Linoeic Acid, Eicosadienoic Acid, Thymoquinone, Cymene, Thujene, Carvacrol, Rosmarinus officinalis Leaf Extract
Comments	:	Odor quality is excellent.

Chromatographic Values of the Volatile Components of the Oil (in %):

Fatty Acid Composition	**%**		**Volatile Components**	**%**
1 – Palmitic acid	12.9		Total Content of Essential Oil	2.2
2 – Palmitoleic acid	0.28			
3 – Stearic acid	2.5		1 - Alpha Thujene	8.4
4 – Oleic acid	21.9		2 - Alpha Pinene	1.7
5 – Vaccenic acid	0.79		3 – Sabinene	0.77
6 – alpha Linoleic acid	0.20		4 - Beta Pinene	1.6
7 – Arachidic acid	0.13		5 - Cymene	30.3
8 – Eicosenoic acid	0.28		6 - Limonene	1.2

9 – Eicosadienoic acid	1.9		7 - Gamma Terpinene	1.4
			8 – Thymoquinone	39.3
			9 – Carvacrol	2.5
			10 – Longifolene	2.8
			11 - Thymohydroquinone	2.2

This CO2 Extract is an authentic natural product and does not contain any artificial ingredients or adulteration of any kind to the best of our knowledge. The analysis and statements herein constitute the most complete information available to Eden Botanicals. This product is guaranteed by Eden Botanicals to be of excellent quality.

Eden Botanicals
www.edenbotanicals.com
info@edenbotanicals.com
Tel: 1-707-509-0041 / Fax: 1-707-949-2526
Document updated: 06.03.15

0415-83863-134

CERTIFICATE OF ANALYSIS

Common Name	:	Pepper, Black
Latin Name	:	*Piper nigrum*
Country of Origin	:	Madagascar
Lot Index #	:	*5
Cultivation Method	:	Cultivated
Type	:	Essential Oil
Extraction Method	:	Steam Distilled
Plant Part	:	Fruit
Use	:	Aromatherapy, Natural Perfumery

*Located on the bottom right hand corner of each label. The Lot Index # is the last dash number after the SKU and Size Reference Code. Example: 445-2-3 refers to SKU-Size Reference Code-Lot Index #.

Specific Gravity @25°C:	0.8632
Refractive Index @20°C:	1.4755
Optical Rotation @20°C:	*na*

Physical appearance	:	Straw colored to greenish oil
Odor	:	Sharp, stimulating aroma
Solubility	:	Insoluble in water, soluble in alcohols and oils
Primary Constituents	:	beta-Caryophyllene, alpha-Guaiene, Carvacrol methyl ether

Comments	:	Odor quality is excellent.

This Essential Oil is an authentic natural product and does not contain any artificial ingredients or adulteration of any kind to the best of my knowledge. The analysis and statements herein constitute the most complete information available to Eden Botanicals. This product is guaranteed by Eden Botanicals to be of excellent quality.

Eden Botanicals
www.edenbotanicals.com
info@edenbotanicals.com
Tel: 1-707-509-0041 / Fax: 1-707-949-2526
Document updated: 02.19.15

1014-7246-780

CERTIFICATE OF ANALYSIS

Common Name	:	Cilantro - Organic
Latin Name	:	*Coriandrum sativum*
Country of Origin	:	Egypt
Lot Index #	:	*3
Cultivation Method	:	Cultivated
Type	:	Essential Oil
Extraction Method	:	Steam Distilled
Plant Part	:	Leaves
Use	:	Aromatherapy, Natural Perfumery
Manufacture Date	:	March 2015

*Located on the bottom right hand corner of each label. The Lot Index # is the last dash number after the SKU and Size Reference Code. Example: 445-2-3 refers to SKU-Size Reference Code-Lot Index #.

Specific Gravity @20°C:	0.853
Refractive Index@20°C:	1.453
Optical Rotation@20°C:	na

Physical appearance	:	Pale yellow liquid
Odor	:	Powerful, aldehydic-orange twist, fresh, herbaceous, cilantro-like
Solubility	:	Insoluble in water, Soluble in alcohol and fixed oils
Primary Constituents	:	NA
Comments	:	Odor quality is excellent.

Chromatographic Values of the Volatile Components of the Oil (in %):

Components	%		Components	%
1- Nonane	1.79		12- Camphor	0.39
2- α Pinene	1.76		13- Borneol	n.d
3- Sabinene	0.19		14- Decanal	26.60
4- β Pinene	0.18		15- 2 Decenal (E)	29.44
5- Octanal	1.09		16-1 Decanol	1.30
6- Myrcene	n.d		17- Undecenal	1.99
7- p Cymene	1.03		18- 2 Decenal (Z)	2.93
8- Limonene	n.d		19- Deodecanal	1.31
9- y Terpinene	n.d		20-2 Dodecenal	5.67

CERTIFICATE OF ANALYSIS

Common Name	:	Cumin Seed - ORGANIC
Latin Name	:	*Cuminum cyminum*
Country of Origin	:	Egypt
Lot Index #	:	*2
Cultivation Method	:	Cultivated, Certified Organic (NOP)
Type	:	Essential Oil
Extraction Method	:	Steam Distilled
Plant Part	:	Seeds
Use	:	Aromatherapy, Natural Perfumery
Manufactured	:	July 2013

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Specific Gravity @20°C:	0.900 - 0.949
Refractive Index @20°C:	1.490 - 1.515
Optical Rotation @20°C:	+1° to +9°

Physical appearance	:	Initially colorless, becoming yellow or greenish brown
Odor	:	Spicy "curry like" odor, oily and bitter
Solubility	:	Soluble in alcohol and fixed oils
Primary Constituents	:	Cuminic aldehyde, γ-Terpinene, ρ-Mentha-1,3-en-al-7, ρ-Mentha-1,4-en-al-7, β-Pinene, ρ-Cymene
Comments	:	Odor quality is excellent.

Chromatographic Values of the Volatile Components of the Oil (in %):

Components	%
1- CUMINIC ALDEHYDE	15.0 – 46.0
2 – *Y*-TERPINENE	10.0 – 32.0
3 – P-CYMENTHA-1,3-EN-AL-7	2.8 – 22.0
4 – P-CYMENTHA-1,4-EN-AL-7	0.3 – 20.0
5 – BETA-PINENE	7.0 – 20.0
6 – P-CYMENE	3.5 – 12.0

*n.d. = not detected

CERTIFICATE OF ANALYSIS

Common Name	:	Fenugreek CO2
Latin Name	:	*Trigonella foenumgraecum*
Country of Origin	:	India
Lot Index #	:	*2
Cultivation Method	:	Cultivated
Type	:	CO2 Select Extract
Extraction Method	:	Super Critical Extraction
Plant Part	:	Dried Seeds
Use	:	Aromatherapy, Natural Perfumery
Manufacturing Date	:	May 2014
Best By	:	April 2019

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Specific Gravity @25°C:	0.93
Refractive Index@20°C:	1.481
Optical Rotation@20°C:	*na*

Physical appearance	:	Yellow to clear liquid
Odor	:	Fresh, sweet, calming aroma, characteristic of Fenugreek
Solubility	:	Soluble in alcohol and fixed oils
Primary Constituents	:	E-anethole, Fenchone, Methyl chavicol

Comments	:	Odor quality is excellent.

This Essential Oil is an authentic natural product and does not contain any artificial ingredients or adulteration of any kind to the best of my knowledge. The analysis and statements herein constitute the most complete information available to Eden Botanicals. This product is guaranteed by Eden Botanicals to be of excellent quality.

Mollie Jensen
Eden Botanicals
www.edenbotanicals.com
info@edenbotanicals.com

CERTIFICATE OF ANALYSIS

Common Name	:	Ginger CO2 - ORGANIC
Latin Name	:	*Zingiber officinalis*
Country of Origin	:	Vietnam
Lot Index #	:	*7
Cultivation Method	:	Cultivated, Certified Organic (NOP)
Type	:	CO2 Total Extract
Extraction Method	:	Super Critical Extraction
Plant Part	:	Rhizome
Use	:	Aromatherapy, Natural Perfumery
Production Date	:	June 2014
Best By	:	June 2019

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Specific Gravity @20°C:	0.9705
Refractive Index@20°C:	1.5080
Optical Rotation@20°C:	*na*

Physical appearance	:	Brown clear liquid
Odor	:	Spicy, warm aroma, deeply complex
Solubility	:	Soluble in alcohol and fixed oils

Comments	:	Odor quality is excellent.

Chromatographic Values of Components of the Oil (in %):

Pungent Compounds	**%**		**Volatile Compounds**	**%**
1 – Zingerone	0.03		1 – Neral/Geranial	1.7/2.9
2 – 6-Gingerol	17.9		2 – ar-Curcumene	7.9
3 – 8-Gingerol	3.7		3 – α Zingiberene	31.5
4 – 6-Shogaol	1.4		4 – α Farnesene	6.5
5 – 10-Gingerol	4.4		5 – β Bisabolene	9.2
6 – 8-Shogaol	0.18		6 - Sesquiphellandrene	13.3
7 – 10-Shogaol	0.09			
Sum of Gingerols	26.0		Content of water	0.73
Sum of Shogaols	1.6		Content of Essential Oil	41.7

CERTIFICATE OF ANALYSIS

Common Name	:	Oregano - Wild
Latin Name	:	*Origanum vulgare*
Country of Origin	:	Turkey
Lot Index #	:	*7
Cultivation Method	:	Cultivated
Type	:	Essential Oil
Extraction Method	:	Steam Distilled
Plant Part	:	Leaves / Tops
Use	:	Aromatherapy

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Specific Gravity @15°C:	0.918
Refractive Index @20°C:	1.4580 to 1.4730
Optical Rotation @20°C:	-1°40'

Physical appearance	:	Pale yellow transparent liquid
Odor	:	Sharp, pungent, herbaceous, typical of Oregano
Solubility	:	Soluble in alcohol and fixed oils
Primary Constituents	:	Carvacrol, γ-Terpinene, p-Cymene, β-Caryophyllene, Thymol
Comments	:	Odor quality is excellent.

Chromatographic Values of the Volatile Components of the Oil (in %):

Components	%		Components	%
1- Carvacrol	64.95		6- Mycene	0.74
2- Thymol	16.67		7- Linalool	0.70
3- p-Cymene	7.78		8- Alpha Humulene	0.64
4- gamma Terpinene	2.10		9- 1,8-Cineole	0.62
5- trans-beta Caryophyllene	1.48		10- alpha Terpinene	0.59

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CERTIFICATE OF ANALYSIS

Common Name	:	Rosemary Antioxidant CO2 - ORGANIC
Latin Name	:	*Rosmarinus officinalis*
Country of Origin	:	Spain
Lot Index #	:	*11
Cultivation Method	:	Wild Grown, Certified Organic
Type	:	CO2 Extract
Extraction Method	:	Super Critical Extraction
Plant Part	:	Leaves
Use	:	Antioxidant for Body/Skin Care
Manufactured	:	March 2015
Best By	:	March 2020

*Located on the bottom right hand corner of each label. The Lot Index # is the last dash number after the SKU and Size Reference Code. Example: 445-2-3 refers to SKU-Size Reference Code-Lot Index #.

Specific Gravity @25°C:	NA
Refractive Index @20°C:	NA
Optical Rotation @20°C:	NA

Physical appearance	:	Thick dark brownish green paste
Odor	:	Pleasant, mildly stimulating, low camphor
Solubility	:	Insoluble in water and alcohol, soluble in fixed oils
Primary Constituents	:	Phenolic diterpenes, Carnosic acid, 12-methyl-carnosic acid, Carnosol

Comments : Odor quality is excellent.

Chromatographic Values of the Components of the Oil (in %):

Components	%		Components	%
1- Rosmanol	0.05		7 – Reference AO Compounds Carnosol + Carnosic Acid (Calc. as Carnosic Acid)	8.9
2 – 7-Methyl-Rosmanol	0.11		8 – Ursolic Acid	0.49
3 - Carnosol	1.5		9 – Oleanolic Acid	0.39
4 – Carnosic Acid	11.9		Content of essential oil	1.9

CERTIFICATE OF ANALYSIS

Common Name	:	Turmeric - Organic
Latin Name	:	*Curcuma longa*
Country of Origin	:	Madagascar
Lot Index #	:	*4
Cultivation Method	:	Cultivated
Type	:	Essential Oil
Extraction Method	:	Steam Distilled
Plant Part	:	Rhizomes
Use	:	Aromatherapy, Natural Perfumery

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Specific Gravity @23°C:	0.900 to 0.936
Refractive Index @23°C:	1.49 to 1.50
Brix @23°C:	76.5 to 86.1%

Physical appearance	:	Light yellow liquid
Odor	:	Warm, spicy, slightly woody aroma
Solubility	:	Insoluble in water, soluble in alcohol and fixed oils
Primary Constituents	:	ar-tumerone, α + β-tumerones, α-phellandrene, 1.8-cineole, α-zingiberene, β-sesquiphellandrene
Comments	:	Odor quality is excellent.

Chromatographic Values of the Volatile Components of the Oil (in %):

Components	%		Components	%
1 – ALPHA-PINENE	0.48		10 – AR-CURCUMENE	1.95
2 – BETA-MYRCENE	0.43		11 – ALPHA-ZINGIBERENE	2.45
3 – ALPHA-PHELLANDRENE	10.37		12 – BETA-BISABOLENE	0.57
4 – P-CYMENE	1.64		13 – BETA-SESQUIPHELLANDRENE	2.40
5 – 1,8-CINEOLE	3.46		14 – AR-TUMERONE	23.76
6 – GAMMA-TERPINENE	0.59		15 – BETA-TUMERONE	22.37
7 – TERPINOLENE	0.78		16 – ALPHA-TUMERONE	13.40
8 – BETA-	0.72		17 – (E)-ALPHA-ALIANTONE	1.56

CARYOPHYLLENE				
9 – ALPHA-HUMULENE	0.77			

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Eden Botanicals
www.edenbotanicals.com
info@edenbotanicals.com
Tel: 1-707-509-0041 / Fax: 1-707-949-2526
Document updated: 03.17.15

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CERTIFICATE OF ANALYSIS

Common Name	:	Thyme ct. Linalool - ORGANIC
Latin Name	:	*Thymus vulgaris ct. linalool*
Country of Origin	:	France
Lot Index #	:	*6
Cultivation Method	:	Cultivated
Type	:	Essential Oil
Extraction Method	:	Steam Distilled
Plant Part	:	Leaves and Tops
Use	:	Aromatherapy
Manufactured	:	October 2014
Best By	:	October 2019

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Specific Gravity @25°C:	0.868
Refractive Index@20°C:	1.466
Optical Rotation@20°C:	-5.94

Physical appearance	:	Transparent, clear liquid
Odor	:	Sweet, strongly herbal, light, fresh, cleansing
Solubility	:	Insoluble in water, soluble in alcohols and oils
Primary Constituents	:	Linalool, Terpinene-4-ol, Gamma-Terpinene, 3-Octanone

Comments	:	Odor quality is excellent.

Chromatographic Values of the Volatile Components of the Oil (in %):

Components	%		Components	%
1 – MW 136	0.188		28 – CAMPHRE	0.936
2 – TRICYCLENE	0.041		29 – BORNEOL	1.185
3 – ALPHA-THUJENE	2.093		30 – TERPINEN-4-OL	5.004
4 – ALPHA-PINENE	3.296		31 – ALPHA-TERPINEOL	1.148
5 – CAMPHENE	0.943		32 – CIS-DIHYDROCARVONE	0.110
6 – SABINENE	0.911		33 – TRANS-DIHYDROCARVONE	0.091

7 – BETA-PINENE	0.359		34 – VERBENONE	0.486
8 – OCTEN-3-OL	0.079		35 – FORMATE DE BORNYLE	0.036
9 – 3-OCTANONE	0.047		36 – NEROL	0.052
10 – MYRCENE	4.547		37 – CARVACROL METHYL-ETHER	0.042
11 – ALPHA-PHELLANDRENE	0.242		38 – ACETATE DE LINALYLE	0.860
12 – ALPHA-TERPINENE	2.502		39 – ACETATE DE BORNYLE	0.103
13 – PARA-CYMENE	1.694		40 – THYMOL	0.434
14 – LIMONENE	2.054		41 – CARVACROL	0.064
15 – BETA-PHELLANDRENE	0.382		42 – ACETATE D'ALPHA-TERPENYLE	0.078
16 – 1,8-CINEOLE (EUCALYPTOL)	1.150		43 – ACETATE DE GERANYLE	0.227
17 – (Z)- BETA-OCIMENE	0.081		44 – BETA-BOURBONENE	0.020
18 – (E)-BETA-OCIMENE	0.274		45 – BETA-CARYOPHYLLENE	0.542
19 – GAMMA-TERPINENE	4.499		46 – ALPHA-HUMULENE	0.017
20 – CIS-HYDRATE DE SABINENE	2.208		47 – GERMACRENE D	0.045
21 – TERPINOLENE	0.867		48 – BICYCLOGERMACRENE	0.117
22 – TRANS-OXYDE DE LINALOL	0.295		49 – GAMME-CADINENE	0.016
23 – LINALOL	57.474		50 – DELTA-CADINENE	0.040
24 – HOTRIENOL	0.303		51 – BUTANOATE DE GERANYLE	0.018
25 – ACETATE D'OCTENE-3-YLE	0.119		52 – GERMACRENE-D-4-OL	0.022
26 – CIS-PARA-MENTH-2-EN-1-OL	0.250		53 – OXYDE DE CARYOPHYLLENE	0.044
27 – ISOBUTYRATE D'HEXYLE	0.253			

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Eden Botanicals
www.edenbotanicals.com
info@edenbotanicals.com
Tel: 1-707-509-0041 / Fax: 1-707-949-2526
Document updated: 05.08.15

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